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Design, synthesis and pharmacological evaluation of new heterocyclic ligands of Formyl Peptide Receptors, potentially useful as analgesic and anti-inflammatory agents

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DECLARATION:

This dissertation is submitted in partial fulfilment of the requirements for the Certificate of Postgraduate Studies. It describes work carried out in the Pharmaceutical Sciences Department, University of Florence, between January 2008 and December 2010 under the supervision of Prof. Vittorio Dal Piaz. During the PhD course, a 7-months period was spent at the Chemistry Department, University of Cambridge, under the supervision of Dr. David R. Spring. Unless otherwise indicated, the research described is my own and not the product of collaboration.

Signed…….. Date……

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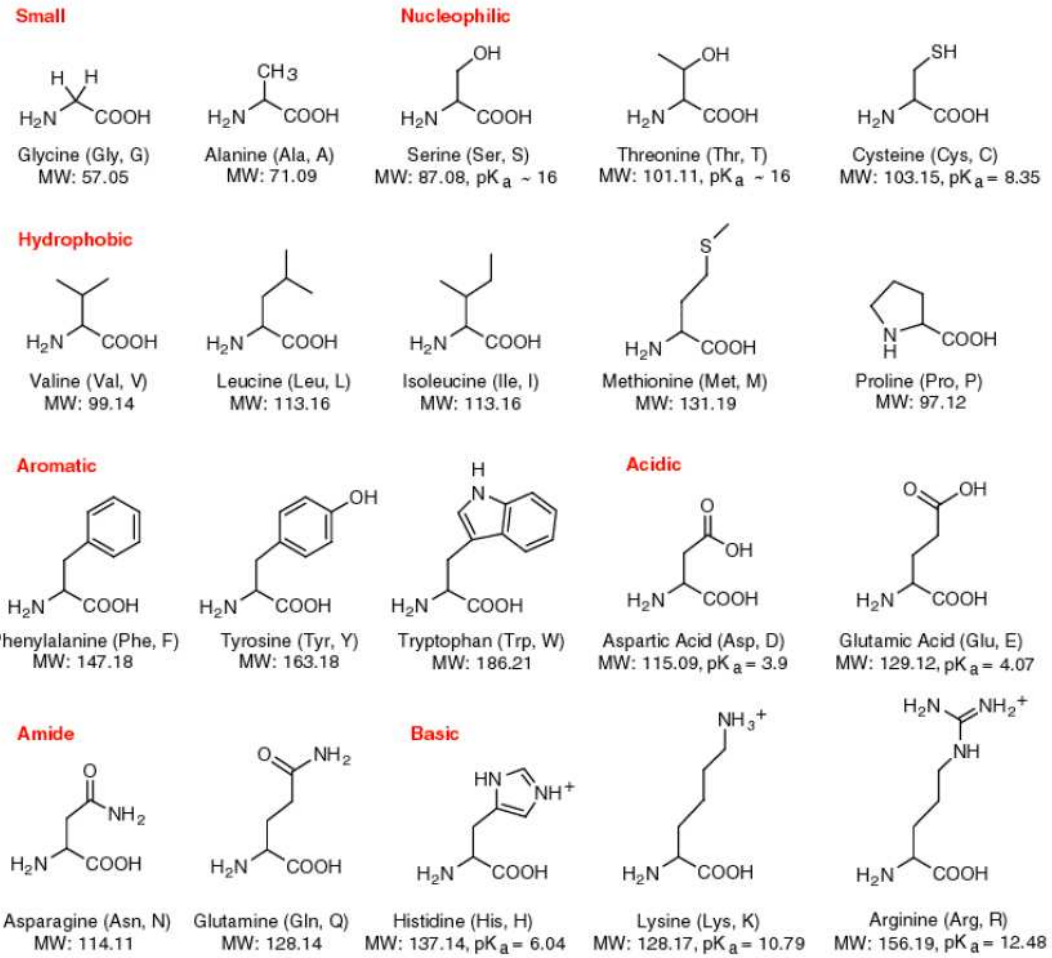
ABBREVIATIONS

$[\alpha]^{20}_{D}$ = optical specific rotation
$\Delta\Delta G^*$ = quantity of the transition state stabilization
δ = chemical shifts
& = cyclic peptides
λ = wavelength
λ_{em} = emission wavelength
λ_{ex} = excitation wavelength
λ_{max} = maximum wavelength
7TM = seven-trans membrane
Aβ = β-amyloid peptide
AβAc = Alzheimer's β-amyloidogenic conformer
aa = amino acid
Ab = antibody
Ab.TS = antibody-bound transition state
Ac = annexin-1-derived peptide(s)
AcOH = acetic acid
Agr = agr = accessory gene regulator(s)
AIP(s) = autoinducing (oligo)peptide(s)
Alloc = allyloxycarbonyl group
ALX = lipoxin A4 receptor
an. = anhydrous
ANXA1 = annexin A1
aq. = aqueous
Ar = aromatic ring
Asu = aspartimide (aminosuccinimide, 3-amino-pyrrolidine-2,5-dione)
Bn = benzyl
Boc = tert-butoxycarbonyl group
c = concentration
C5a = complement component 5a
calcd. = calculated
cAMP = cyclic adenosine monophosphate
CAN = ceric ammonium nitrate
CD = circular dichroism
CD4+ = T helper cells
CD8+ = cytotoxic T cells
CDCA = chenodeoxycholic acid
cDNA = complementary deoxyribonucleic acid
CHIPS = chemotaxis inhibitory protein of S. aureus
CHX = cyclohexane
CI = chemical ionisation
CM = complex mixture
CSP(s) = chiral stationary phase(s)
CsH = cyclosporin H
c-suPAR = cleaved soluble uPAR
CTC = chlorotrityl chloride polystyrene resin
d = day(s)
DCA = deoxycholic acid
DCC = dicyclohexylcarbodiimide
DCP = diethyl cyanophosphonate
DC(s) = dendritic cell(s)
DIC = diisopropylcarbodiimide
DIPEA = DIEA = *N,N*-Diisopropylethylamine or Hünig's base
DKP = diketopiperazine
DMAP = 4-dimethylaminopyridine
DMF = dimethylformamide
DMSO = dimethyl sulfoxide
EC_{50} = half maximal effective concentration
E. coli = escherichia coli
EDC = 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide hydrochloride
EDTA = ethylenediaminetetraacetic acid
ee = enantiomeric excess
EI = electron ionisation
equiv = equivalent(s)
EMRSA-16 = Epidemic methicillin-resistant Staphylococcus aureus 16
ERK = extracellular regulated kinase
ESI = electrospray ionisation
EtOAc = ethyl acetate
F2L = peptide derived from heme-binding protein
FAB (LSIMIS) = fast atom bombardment (liquid secondary ion mass spectrometry)
FBS= fetal bovine serum
$Fe(acac)_3$ = iron(III) acetylacetonate
FLIPR = fluorometric imaging plate reader
FLIPr = FPRL1 inhibitory protein
fMLF = *N*-formyl-methionine-leucine-phenylalanine
FMLPR(s) = formil-metinine-leucine-phenilalanine receptor(s)
Fmoc = Fluorenylmethyloxycarbonyl group
FPR(s) = formyl peptide receptor(s)
FPRH1= formyl peptide receptor-homolog 1
FPRH2= formyl peptide receptor-homolog 2
FPRL1= formyl peptide receptor like1
FPRL2= formyl peptide receptor-like 2
FT-IR = Fourier Transform Infrared Spectroscopy
G-CSF = granulocyte macrophage-colony-stimulating factor
gp41 = envelope glycoprotein 41 of HIV-1

GPCR(s) = G-protein-coupled receptor(s)
GTPase(s) = enzymes that bind GTP (guanine triphosphate)
h = hour(s)
HATU = 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluoro-phosphate 3-oxide
HBA= hydrogen bond acceptor
HBD= hydrogen bond donor
HBSS = Hanks' Balanced Salt Solution
HEPES = 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
Hex = hexane
HIV = Human immunodeficiency virus
HIV-1 = Human immunodeficiency virus type 1
HK= histidine kinase
HL-60= Human promyelocytic leukemia 60 cell line
HM63 = FPR2 antibody
^{1}H NMR = proton nuclear magnetic resonance
HOAt = 7-aza-1-hydroxybenzotriazole
HOBt = 1-hydroxybenzotriazole
HPLC = high-performance liquid chromatography
HSV-2= herpes simplex virus type 2
IC_{50} = half maximal inhibitory concentration
I.D. = internal diameter
IFN-γ = interferon gamma
IgE = immunoglobulin E
IL = interleukin
IPA = isopropanol
IR = Infrared spectroscopy
IUPAC = International Union of Pure and Applied Chemistry
J = coupling constants
Kd = dissociation constant
kDa = kilodalton
Ki = inhibition constant
KTS = transition state (TS) dissociation constant
LC-MS = LCMS = liquid chromatography-mass spectrometry
LL-37 = a Cathelicidin-derived peptide
LPS = lipopolysaccharide
LTB4 = leucotriene B4
LXA4, LXB4 = lipoxin A4, lipoxin B4
LXA4R= lipoxine A4 receptor
MALDI = matrix-assisted laser desorption /ionisation
MAPK = mitogen-activated protein kinase
TBHP = tert-butyl hydroperoxide
m-CPBA = meta-chloroperbenzoic acid
mdeg = millidegrees (for the angle of polarization in CD spactra)
MHC= major histocompatibility complex
min = minute(s)
mp = melting point
MRSA = Methicillin-resistant strains of Staphylococcus aureus
m/z = mass to charge ratio
MS = mass spectrometry
N.A.= no activity
NADPH = nicotinamide adenine dinucleotide phosphate-oxidase
NBS = *N*-bromosuccinimide
N.D. = non determined
NF-*k*B = eukaryotic transcription factor
NK= natural killer cells
NMR = nuclear magnetic resonance spectroscopy
NO = nitric oxide
o/n = overnight
P = product
PAF = platelet-activating factor
PGE2 = prostaglandin E2
pKa = -log(acidity constant)
PKC(s) = protein kinase C(s)
PMN(s) = polymorphonuclear leukocyte(s)
ppm = part(s) per milion
PPA = Polyphosphoric Acid
PrP = prion protein
PyAOP = (7-azabenzotriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluoro-phosphate
QS = quorum sensing
QQ = quorum quenching
Q-ToF = Quadrupole-Time of Flight
RBL-2H3 = Rat basophilic leukemia 2H3 cell line
RPMI 1640 = a medium used for the culture of human normal and neoplastic leukocytes
rt = room temperature
S = substrate
SAA = serum amyloid A
SAR(s) = structure-activity relationship(s)
S. aureus = Staphylococcus aureus
S.D. = standard deviation
SM = starting material
SN_2 = bimolecular nucleophilic substitution
SPPS = solid-phase peptide synthesis
^{t}Bu = *t*-Bu = *tert*-butyl group

TFA = trifluoroacetic acid
THF = tetrahydrofuran
TLC = thin layer chromatography
TLR(s) = toll-like receptor(s)
TNF-α = tumor necrosis factor alpha
t_R = retention time (in min)
TS = Transition-State
uPA= urokinase-type plasminogen activator
uPAR = urokinase-type plasminogen activator receptor
UV = ultraviolet spectroscopy
UV-VIS = ultraviolet-visible spectroscopy
v/v = volume/volume
V3 = vasopressin receptor
WBC(s) = white blood cell(s)
WT = wild-type cells
w/v = weight/volume

Table of the amino acids and their abbreviations.



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1. INTRODUCTION

1.1 Inflammation and infection: an overview

Inflammation is the first response of the immune system to infection or irritation. Through this process the white blood cells and chemicals released from them protect human organism against exogenous substances and pathogenic microorganisms such as bacteria and viruses.[1] Nevertheless, if the injurious agent continues or the control of cellular recruitment breaks down, both acute and chronic inflammatory disorders will ensue.[2]

Innate immunity is a very important mechanism in defending humans against infectious microbes but in some diseases the immune system inappropriately triggers an inflammatory response when there are no foreign substances to fight off.[3] In these cases, the decompensations are called "autoimmune diseases" and the body responds as if normal tissues are infected or somehow abnormal: as result of this response, the normal protective immune system of the body causes damage to its own tissues. Thus, immune dysregulation exposes patients to life-threatening risks. As known, infection and inappropriate inflammatory processes play a central role in many diseases such as asthma, rheumatoid arthritis and multiple sclerosis. Moreover, as consequence of the increasing aging of the population, in more developed countries it was observed that older people exhibit a natural immune function dysregulation, which may be exacerbated in chronic stress conditions.[4] In addition, the ongoing emergence of resistant bacterial and viral strains to multiple classes of chemotherapeutics increases morbidity, mortality, and costs associated with nosocomial infections.[5]

Nowadays there is a large number of treatments for inflammatory diseases and many anti-inflammatory therapeutic agents have been developed. Unluckily, the most part of currently used anti-inflammatory drugs has some limitations due to their interference with pro-inflammatory mediators, whereas less is understood about the biochemical processes that resolve inflammation. Principally, selective activation of such a pathway might lead to an alternative treatment for inflammation.[6] Moreover, identification of immunomodulatory agents enhancing innate immune responses represents a promising strategy for combating inflammation and infectious diseases and development of bioactive molecules that selectively stimulate the innate immune response is an important challenge both for biologists and chemists.[7]

1.2 White blood cells or leukocytes

White blood cells (WBCs), or leukocytes, are immune system cells delegated to defend the body against both infectious disease and foreign materials.[8] There are several different types of white blood cells and they all derive from a multipotent cell in the bone marrow, known as a hematopoietic stem cell. Leukocytes are found throughout the body, including the blood and lymphatic system (**figure 1.1**).[9] A major distinguishing feature of some leukocytes is the presence of granules, so that white blood cells are often characterized as granulocytes or agranulocytes. Granulocytes (neutrophils, basophils, eosinophils)

are polymorphonuclear leukocytes and they are characterised by the presence in their cytoplasm of differently staining granules visible under light microscopy. These granules are membrane-bound enzymes which primarily act in the digestion of endocytosed particles through the action of lysosomes.

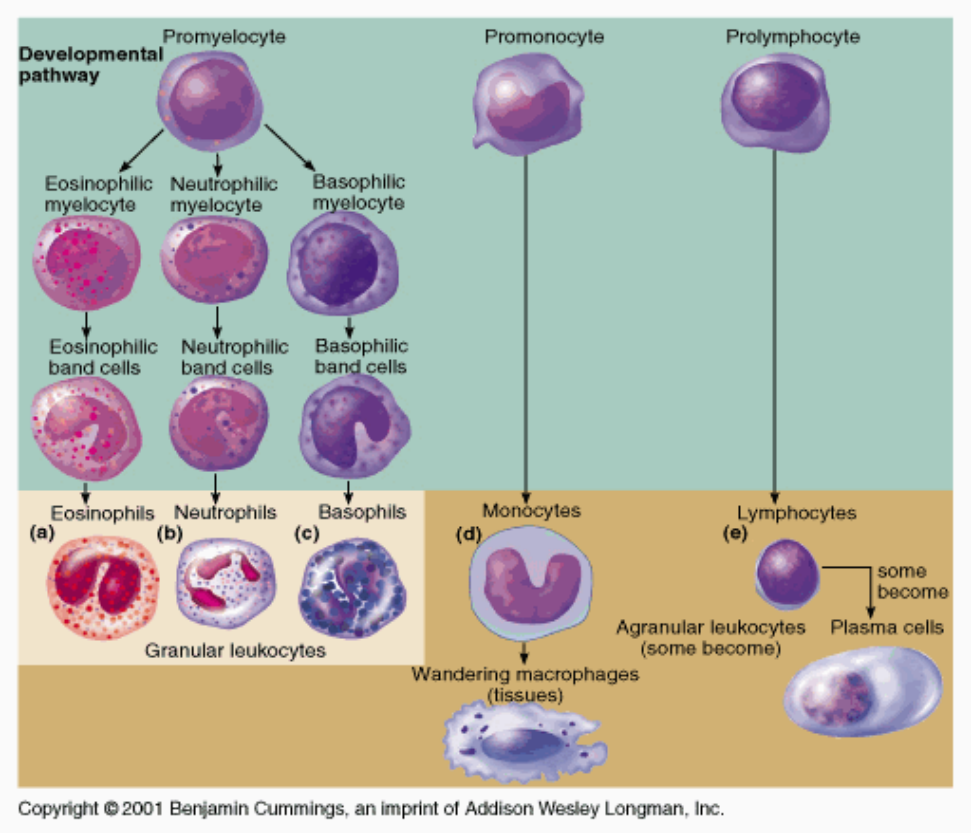


Figure 1.1. The different types of white blood cells and hematopoietic cells from which they derive.

Agranulocytes (lymphocytes, monocytes, macrophages) are mononuclear leucocytes and they are characterized by the apparent absence of granules in their cytoplasm. Although the name implies a lack of granules, these cells contain lysosomes.[10] Some leukocytes migrate into the tissues of the body to take up a permanent residence at that location rather than remaining in the blood. Often these cells have specific names depending on the tissue in which they settle in but, generically, they are well-known as “fixed macrophages” and dendritic cells.

1.2.1 Neutrophils

Neutrophils are the most abundant type of white blood cells in mammals and form an essential part of the innate immune system. They belong to polymorphonuclear leukocytes (PMNs) together with basophils and eosinophils (in fact, technically, PMN refers to all granulocytes). Normally found in the blood, neutrophils are usually the first responders to microbial infection, defending against bacteria, fungi and other inflammatory process.[11] Being highly motile, they quickly congregate at the focus of inflammation.

Thus, neutrophils are the most common cells recruited in acute phase of inflammation, coming in and destroying foreign organisms or substances.[12,13,14] Considering their ability to migrate toward the site of inflammation, through the blood vessels and the interstitial tissue, following "chemical signals" in the process of chemotaxis, neutrophils are recruited in the site of injury within few minutes from trauma and they should be considered as the hallmark of acute inflammation.[15] Cell surface receptors allow neutrophils to detect chemical gradients of molecules such as interleukin-8 (IL-8), interferon gamma (IFN-gamma) and complement component 5a (C5a,) which these cells use to direct the path of their migration. They are very active in phagocytosing bacteria and are able to release soluble anti-microbial proteins.[16,17] As a consequence of their activity and death usually they determine pus formation. Moreover, neutrophils express and release cytokines,[18] which, in turn, amplify inflammatory reactions through several other cell types.

1.2.2 Basophils

Basophils appear in many specific kinds of inflammatory reactions, particularly those that cause allergic symptoms. They can be found in unusually high numbers at sites of infection and play a crucial role in both parasitic infections and allergies.[19] Basophils are usually chiefly responsible for allergic and antigenic response by releasing histamine, proteoglycans (e.g. heparin and chondroitin), proteolytic enzymes (e.g. elastase and lysophospholipase), causing inflammation and contributing in this manner to the severity of the allergic response. When activated, they also secrete lipid mediators like leukotrienes and several cytokines. Histamine and proteoglycans are pre-stored in the cell's granules while the other secreted substances are newly generated. Each of these substances contributes to inflammation.[20] A specific receptor on basophilic cell surface is involved in the IgE binding and the interaction with this immunoglobulin involved in parasite defense and allergy confers to the basophils a selective response to environmental substances, such as pollen proteins or helminth antigens. Recent studies in mice suggest that basophils may also regulate the behavior of T cells and mediate the magnitude of the secondary immune response.[21]

1.2.3 Eosinophils

Among the immune system components, eosinophils are white blood cells responsible for combating multicellular parasites and certain infections.[22] Usually they fight viral infections and helminth colonization, being slightly elevated in the presence of certain parasites. Eosinophils are also important mediators involved in the control of allergy and asthma and are closely associated with the severity of disease. In addition, the are frequently involved in many other biological processes, including allograft rejection and neoplasia.[23] After maturation in bone marrow, eosinophils circulate in blood and migrate to

inflamed tissues in response to chemokines and certain leukotrienes (e.g. leukotriene B4).[24] Following activation by an immune stimulus, eosinophils degranulate and release an array of cytotoxic granule cationic proteins that are capable to induce tissue damage and dysfunction.[25,26] Their effector functions include also production of reactive oxygen species (such as superoxide, peroxide, and hypobromite), lipid mediators like the eicosanoids (leukotrienes and prostaglandins),[27] growth factors,[28,29] cytokines and TNF-α.[23]

1.2.4 Lymphocytes

Also lymphocytes are white blood cells of the vertebrate immune system and they are much more common in the lymphatic system. All lymphocytes originate during haematopoiesis process and, after maturation, enter in the circulation and peripheral lymphoid organs (e.g. the spleen and lymph nodes) where they survey for invading pathogens and/or tumor cells. The three major types of lymphocyte are natural killer (NK) cells, T cells and B cells.[30]

NK cells are a part of the innate immune system and play a fundamental role in defending the host from both tumors and viral infected cells. They distinguish infected cells and tumors from normal and uninfected cells by recognizing changes of the level of a surface molecule called MHC (major histocompatibility complex) class I. NK cells are activated in response to a family of cytokines called interferons and they release cytotoxic granules which are able to destroy the altered cells.[20]

T and B cells are the major cellular components of the adaptive immune response. T cells are involved in cell-mediated immunity whereas B cells are primarily responsible for humoral immunity (relating to antibodies). B cells mature into B lymphocytes at the level of the bone marrow, while T cells migrate to and mature in a distinct organ, called thymus.[31] The function of T cells and B cells is to recognize specific “non-self” antigens, during a process known as “antigen presentation”. Once these cells have identified an invader, they generate specific responses that are tailored to maximally eliminate specific pathogens or pathogen infected cells. B cells respond to pathogens by producing large quantities of antibodies which neutralize foreign organism like bacteria and viruses. Always in response to pathogens, some T cells, called T helper cells (CD4+), produce cytokines that direct the immune response,[32] while other T cells, called cytotoxic T cells (CD8+), produce toxic granules which induce the death of pathogen infected and tumor cells. Following activation, B cells and T cells leave a lasting legacy of the antigens they have encountered, in the form of “memory cells”. During all the host life, these memory cells will “remember” each specific pathogen encountered, and will be able to determine a strong response if the pathogen will be detected again.[31]

1.2.5 Monocytes

Monocytes are white blood cells that develop in the bone marrow and then go into blood, where they circulate for few days to finally migrate into tissues. In response to inflammation signals, they can move quickly to the sites of infection and differentiate into resident macrophages and dendritic cells to elicit an immune response.[33] Monocytes are responsible for phagocytosis of foreign substances in the body and this action is performed by using intermediary proteins such as antibodies or complement factors. In addition, they are able to directly bind the microbes via pattern-recognition receptors which recognize pathogens. Finally, monocytes are also capable to kill infected host cells through antibody recognition in a process called "antibody-mediated cellular cytotoxicity".[34,35]

1.2.6 Macrophages

Macrophages are mononuclear leucocytes responsible to protect tissues from foreign substances. When a leukocyte enters injured tissues attracted by a range of various stimuli in the process of chemotaxis, it undergoes a series of changes to become a macrophage. The majority of macrophages migrate into the tissues of the body to take up a permanent residence at that location ("fixed macrophages"), rather than remain in the blood. Each type of macrophage has a specific name determined by its location. Some examples of this differentiation are the Kupffer cells (in the liver), the histiocytes (in the connective tissue), the microglia (in the neural tissue), the mesangial cells (in the kidney), sinusoidal lining cells (in the spleen), the osteoclasts (in the bone) and the dust cells (in the lung).[20] As a consequence of their different fixed location, macrophages are versatile cells that play many roles. They act as phagocytes in both non-specific defense (innate immunity) and specific adaptive immunity. Their main roles consist to phagocytize necrotic tissues and pathogens, either as stationary or as mobile cells, and to stimulate lymphocytes and other immune cells to respond to the pathogen.

1.2.7 Dendritic cells

Dendritic cells (DCs) are immune cells that, as well as macrophages do, migrate into the tissues of the body to take up a permanent residence at that location. As mentioned above, these cells always arise from monocytes that depending on the specific signal, can became either dendritic cells or macrophages respectively. Dendritic cells can also be found as immature form in the blood,[36] but they are usually found in lung, stomach, intestine and, in small amount, in tissues that are in contact with the external environment, such as the skin. Langerhans cells are indeed a specialized kind of dendritic cells. Their main function is to process antigen material and to present it on the surface to other cells of the immune system.[37] They are the most potent of all the "antigen-presenting cells" and, once activated, they migrate

to the lymphoid tissues where they interact with T cells and B cells to initiate and shape the adaptive immune response.[38]

1.3 Leukocytes: involvement in inflammation and infection

The host defense response of humans is complex and multileveled, involving many cell types with distinct but overlapping roles. Leukocyte infiltration is an important feature in host response and defense to invading potentially pathogenic organisms and to fight a variety of inflammation processes. Phagocytic leukocytes are one of the earliest cell types responding to an inflammatory stimulus and they are key participants in innate immune response.[39] Leukocytes accumulate at sites of inflammation and microbial infection as response to locally produced "chemical signals".[40] In fact their recruitment is strictly dependent on the presence of a gradient of chemotactic factors.[41] In response to inflammatory challenges, phagocytes such as neutrophils, monocytes and macrophages migrate to the site of infection in a process of adhesion and transmigration through blood-vessel walls, where they engulf and destroy bacteria or other damage stimuli.[42] They are usually activated by a wide variety of inflammatory stimuli, including cytokines and other endogenous chemical messengers, pathogen-associated molecular structures and oligopeptides derived from various pathogens or endogenously produced.[43,44] After phagocytosis of microorganisms or other particulate substances, leukocytes secrete a variety of mediators that possess potent proinflammatory and antimicrobial activities. These mediators include antibiotic peptides and proteases which are sometimes stored in granules and are released during the process of degranulation.[45] Leukocytes perform as well a variety of other complex microbicidal functions, including chemotaxis (migration to site of inflammation), margination (rolling and adhesion to vessel walls), diapedesis (transmigration across the endothelial barrier),[46-47] phagocytosis of foreign particles, destruction of targeted organisms and reactive free radicals production.[16]

In this scenario, more detailed signalling studies in leukocytes may help to identify new targets for potential therapeutic interventions. In addition, future combinatorial therapies with higher selectivity for certain leukocyte subsets promise improved approaches for treating acute and chronic inflammatory disorders.

1.4 Formyl Peptide Receptors (FPRs) role in inflammation and infection

Formyl peptide receptors (FPRs) are a small family of chemoattractant receptors and they play an essential role in host defense mechanisms against pathogen infection and trauma. In addition, they are involved at different levels in the regulation of inflammatory reactions and sensing cellular dysfunction.[48] These receptors belong to the seven transmembrane domain G-protein-coupled receptor (GPCR) family which are expressed in the majority of white blood cells and are known to be important

in host defense and inflammation.[49] All major neutrophil functions stimulated towards FPRs can be inhibited by treatment of the cells with pertussis toxin,[42,50] indicating that the G proteins coupled to them belong to the Gi family of heterotrimeric proteins.[51] Several studies conducted throughout the 1980s led to the identification of the formyl peptide receptors as highly promiscuous receptors that can be activated by a wide range of structurally unrelated non-peptide and peptide agonists, including synthetic, or both host-derived and pathogen-derived agents.[42,48,50,52,53] Further investigations resulted in the identification of different chemotactic factors derived from bacteria as low-molecular weight peptides having a blocked amino terminus group, which were able to bond and activate FPRs. Because prokaryotes initiate protein synthesis with *N*-formyl methionine, short peptides starting with *N*-formyl methionine (CHOMet) were chemically synthesized and tested.[54] It was found a potent chemotactic activity for neutrophils in many of the synthetic *N*-formyl peptides tested, especially peptides containing *N*-formyl-methionyl-leucine and *N*-formyl-methionyl-phenylalanine.[55] The above findings led Freer et al.[56] to propose that the *N*-formyl group is essential for the bioactivity of these chemotactic peptides through interaction with FPRs. These studies resulted in identification of *N*-formyl-methionine-leucine-phenylalanine (fMet-Leu-Phe, or fMLF) as the most potent agonist among 24 synthetic peptides tested in neutrophil chemotaxis assays.[57]

Owing to its ability to bind and activate the G protein-coupled formyl peptide receptors (FPRs), the tripeptide fMLF became a prototype of formylated chemotactic peptides for neutrophils able to act through FPRs activation. At subnanomolar to nanomolar concentrations, this binding event translates into directional movement of neutrophils, while at higher concentrations (> 100 nM), the same peptide also stimulates bactericidal functions including lysosomal enzyme release,[56] degranulation and production of superoxide.[40,58,59] Because these peptides are derived from bacterial or mitochondrial proteins,[1,55] it has been proposed that a primary FPR function is to promote trafficking of phagocytic myeloid cells to sites of infection and tissue damage, where they exert antibacterial effector functions and clear cell debris. In support of this hypothesis, mice lacking a known murine FPR variant were more susceptible to bacterial infections.[60] Intriguingly, the bactericidal activities triggered by these chemotactic peptides contribute to tissue damage when neutrophils are activated in certain different pathological conditions. Therefore, an understanding of the pharmacological basis of FPR binding and signaling has the potential to enhance anti infective activity as well as to reduce unwanted neutrophil activation and the resulting tissue damage.[61]

1.5 Formyl Peptide Receptors (FPRs) localization and classification

Three FPR subtypes have been identified till now in humans (FPR1, FPR2/ALX , FPR3), whereas eight FPR-related receptors have been discovered in mice (**figure 1.2**).[48] Activation of FPRs induces a variety

of responses, which depend on the agonist, cell type, receptor subtype and species involved. The receptors of the FPR gene family are primarily found in myeloid cells, but the distribution changes within myeloid cell subsets. Indeed, the three different FPRs display a quite different expression profile on phagocytic leukocytes (**figure 1.2**). For example, neutrophils express functional FPR1 and FPR2/ALX, while monocytes express all the three receptors at their surface. Differently, monocyte-derived DCs express FPR1 and FPR3 when immature and only retain FPR3 after maturation.[62,63] Many studies also indicate the presence of formyl peptide receptors in non-myeloid cells. In this contest, there is evidence that FPRs might play different key roles in the activation of the immune system cells and functions in different body districts.

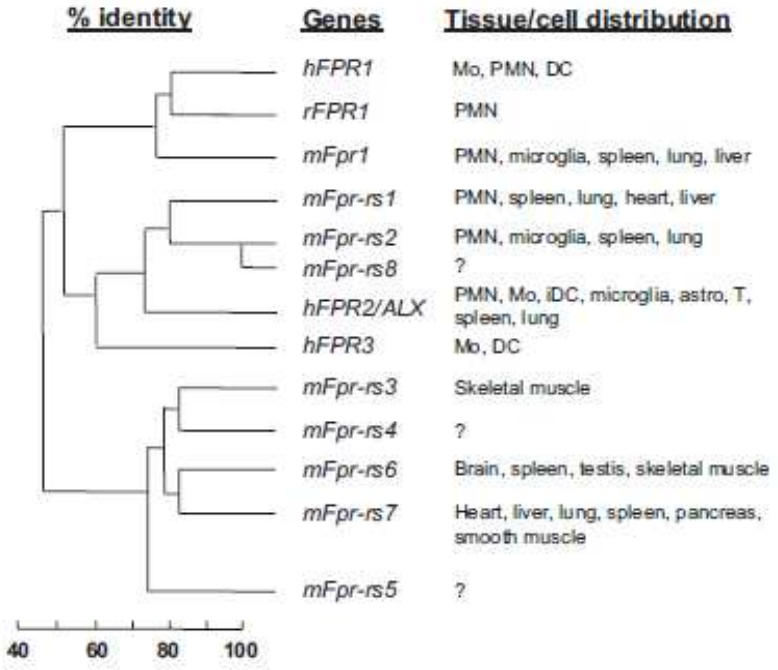


Figure 1.2. Sequence homology between the FPR family members and their tissue distribution. The predicted protein sequences of the three human (h) FPR genes, the eight mouse (m) Fpr genes, and the rabbit (r) FPR1 gene were compared. Based on sequence homology, the hFPR1, mFpr1, and rFPR1 are in the same cluster. Note that some of these genes are not expressed in neutrophils and monocytes. The tissue expression profiles for mFpr-rs4, mFpr-rs5, and mFpr-rs8 have not been determined. Mo, monocytes; PMN, polymorphnuclear leukocytes; iDC, immature dendritic cells; astro, astrocytes; T, T lymphocytes. Adapted from Ye et al. (2009).[49]

1.5.1 Formyl Peptide Receptor 1 (FPR1)

Human FPR1 is a relatively abundant chemoattractant receptor on phagocytic cells and it was first defined biochemically in 1976,[40] as a high affinity binding site on the surface of neutrophils for the prototypic peptide fMLF. Other names used in the literature to define FPR1 include FPR ("classic FPR") and FMLPR (formil-metinine-leucine-phenilalanine receptor).[49] Biochemical studies indicated that the receptor is a glycoprotein of 55-70 kDa that functionally couples to G proteins for transmembrane signalling.[64] It was then cloned in 1990 by Boulay *et al.*[65,66] from a differentiated HL-60 myeloid

leukemia-cell cDNA library. Cloning of the cDNA for FPR1 identified the receptor to be a single polypeptide of 350 amino acids with a typical hydropathy plot pattern for a seven transmembrane domain structure.[65-68] In transfected cell lines, FPR1 binds fMLF with high affinity (Kd < 1 nM) and it is activated by picomolar to low nanomolar concentrations of fMLF in chemotaxis assays.[40] Besides being expressed on phagocytes (e.g. neutrophils and monocytes) and a small number of non-phagocytic cells (e.g. hepatocytes, immature dendritic cells, astrocytes, microglial cells),[69] FPR1 has been identified also in the tunica media of coronary arteries.[40] Using an antibody recognizing the carboxyl terminal 11 amino acids of FPR1, Becker et al.[70] found immunoreactivity for this subtype of receptor in multiple organs and tissues, including epithelial cells in organs with secretary functions, endocrine cells (including follicular cells of the thyroid and cortical cells of the adrenal gland), liver hepatocytes and Kupffer cells, smooth muscle cells and endothelial cells, brain, spinal cord and both motor and sensory neurons.[49]

1.5.2 Formyl Peptide Receptor 2 (FPR2 or FPR2/ALX)

In 1990s several laboratories reported the identification of a cDNA and a gene,[71-74] coding for a putative seven transmambrane receptor that shares significant sequence homology to human FPR1. Different names were given to the gene product, including FPR2/ALX for its low-affinity binding of fMLF,[73] FPRL1 (formyl peptide receptor like1),[71] FPRH1 (formyl peptide receptor-homolog 1),[74] and "receptor related to formyl peptide receptor" based on its sequence homology to the human FPR1.[72] Other names used in the literature include HM63,[75] and FMLF-related receptor II.[49] Pharmacological characterization has led to the identification of the eicosanoid lipoxin A4 (LXA4),[76,77] of aspirin-triggered lipoxins,[78,79] and of a variety of peptides,[80] as ligands for this receptor.[40,48] Therefore, in addition to FPRL1, which frequently appears in the literature, the name ALX (or LXA4R, "Lipoxine A4 receptor") has been introduced to convey the ability of the receptor to interact with LXA4 and aspirin-triggered lipoxins.[81] FPR2/ALX is a 7TM receptor with 351 amino acids and shares 69% of amino acidic identity with human FPR1. Despite the relatively high level of sequence homology with FPR1, FPR2/ALX is a low-affinity receptor for fMLF, with a *K*d of 430 nM .[67,71,73] It has been reported that mitochondria-derived formyl peptides are more potent agonists for FPR2/ALX than fMLF,[82] suggesting that its primary function may be to recognize host-driven mitochondrial peptides or possibly other bacterially derived formyl peptides. Human FPR2/ALX has a tissue distribution similar to that of FPR1, but FPR2/ALX is expressed also in other cell types, including phagocytic leukocytes, hepatocytes, epithelial cells, T lymphocytes, neuroblastoma cells, astrocytoma cells and microvascular endothelial cells.[69] These patterns of tissue expression suggest that FPR1/FPR2/ALX may also participate in a number of functions other than host defense. In addition to formyl peptides and LXA4, FPR2/ALX is also able to interact with non formylated peptides. Compared with FPR1, FPR2/ALX exhibits a high level of ligand promiscuity and it

is activated by numerous and chemically unrelated ligands, including synthetic peptides, pathogen derived peptides, host-derived peptides, lipids[49] and small synthetic compounds.

1.5.3 Formyl Peptide Receptor 3 (FPR3)

A second gene with significant sequence homology to human FPR1 was identified using a similar cloning strategy used to discover FPR2/ALX. This gene encodes for FPR3,[49] a putative 7TM receptor of 352 amino acids initially named FPRL2,[71] (formyl peptide receptor-like 2) and FPRH2 (formyl peptide receptor-homolog 2),[74] taking into account the sequence homology to FPR1. FPR3 shares 56% and 83% sequence identity with human FPR1 and FPR2/ALX respectiely. FPR3 does not bind N-formyl peptides,[83] such as fMLF, but it responds to some non formylated chemotactic peptides identified for FPR2/ALX.[84,85] Migeotte et al.[86] recently reported that a naturally occurring endogenous acylated peptide, derived from the N-terminal sequence of heme-binding protein, is a potent agonist for FPR3. Unlike FPR1 and FPR2/ALX, FPR3 had been not found in neutrophils.[71] This receptor is characterized by its specific expression on monocytes and DCs.[86] As already seen, monocytes express the three receptors at their surface, whereas monocyte-derived DCs express FPR1 and FPR3 when immature and only retain FPR3 after maturation.[62,63] In particular, in the process of monocyte differentiation into immature dendritic cells (DCs), the cellular expression of FPR2/ALX progressively declines,[63] whereas FPR2/ALX expression remains unchanged during monocyte differentiation into macrophages. There is also a progressive loss of FPR1 during differentiation of immature DC to mature DC, such that FPR3 becomes the predominant human formyl peptide receptor in mature DC.[62,86] The biological significance of differential expression of formyl peptide receptors in monocytes, macrophages and DCs has not yet been clearly defined, but it seems that the three receptors might play key role in the differential migration pattern of these antigen presenting cells.[86]

1.6 FPRs activation and cell functions

The stimulation of FPRs is regulated at the levels of receptor by G protein activation, transduction and amplification of signals through various effectors, including kinases and small GTPases (**figure 1.3**), and integration of effector signals leading to phagocyte functions such as chemotaxis, degranulation, and superoxide generation. Regulation of FPRs at the receptor level concern mainly three different processes: desensitization, phosphorilation and interaction with β-arrestins, which in turn involve the following uncoupling of G proteins and internalization of the receptors (**figure 1.4**). It is noteworthy that, although there are many similarities between FPR1 and FPR2/ALX (and, perhaps, also FPR3),[88] major differences exist between these receptors in signalling.

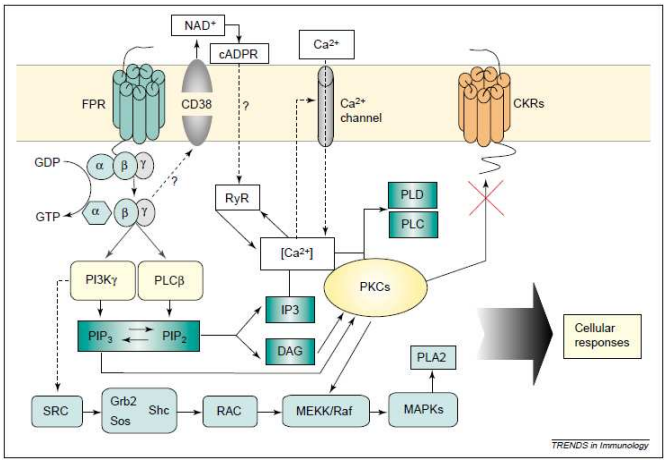


Figure 1.3. Signaling pathways of an activated FPR. On agonist binding, trimeric Gi-proteins are uncoupled and a series of signal transduction events ensue that result in cell activation and protein kinase C (PKC)-dependent desensitization of unrelated chemotactic receptors. CD38 induces the conversion of NAD^+ to cyclic ADP-ribose (cADPR), which acts at ryanodine receptors to release calcium ions (Ca^{2+}) from intracellular stores. This results in a sustained influx of extracellular Ca^{2+} required for fMLF-induced neutrophil migration. CKR, chemokine receptor; DAG, diacylglycerol; IP, inositol phosphate; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol diphosphate; PIP_3, phosphatidylinositol trisphosphate; PKC, protein kinase C; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D; RAC, Rac guanine triphosphatase family; SRC, Src-like tyrosine kinases. Adapted from Le et al. (2002).[40]

Evidence also suggests that the basic mechanism by which these receptors trigger a transient increase in intracellular Ca^{2+} may be different (**figure 1.4**).[89]

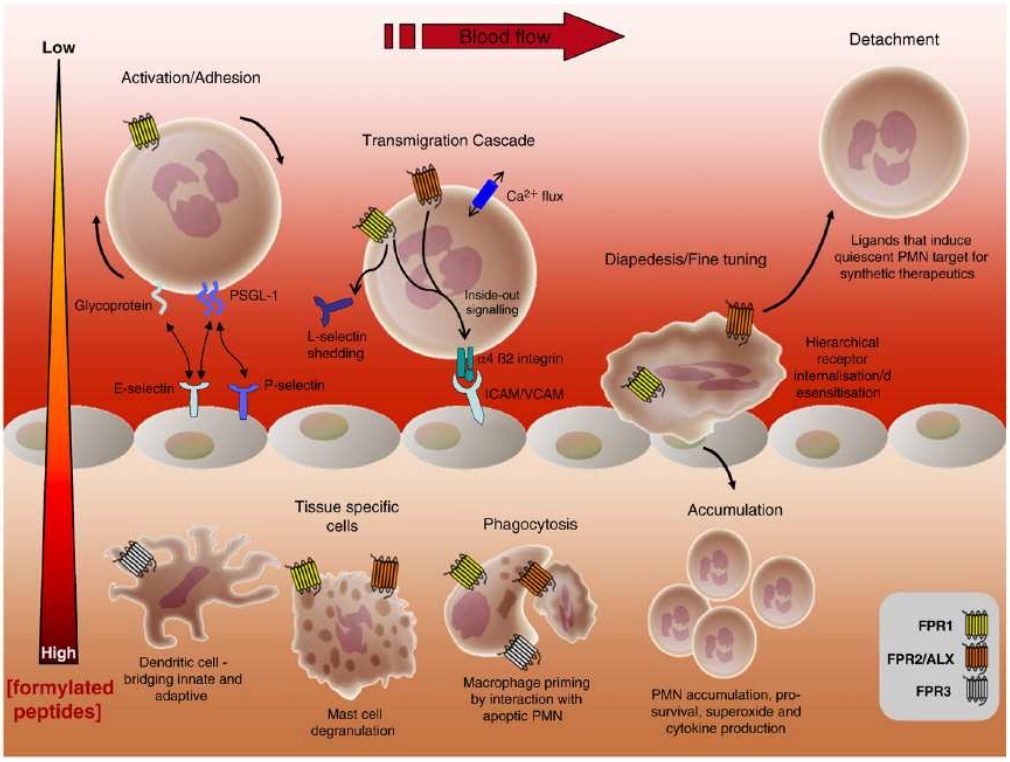


Figure 1.4. Leukocyte migration cascade via the FPRs family. FPR1 is activated at low concentration of formyl peptide (yellow). With increasing concentration of formyl peptide (orange) can also instigate FPR2 signalling. FPR activation of leukocytes, results in integrin expression and activation. Adherent leukocytes are an interesting therapeutic target for FPR2: interaction with endogenous ligands can lead to either leukocyte detachment (AnxA1/LXA4) or tissue accumulation (SAA). High concentration (red) or repeated stimulation by formyl peptide can lead to receptor desensitisation. FPRs play distinct role in myeloid cell priming and macrophage phagocytosis, mast cell membrane stabilisation. Adapted from Dufton and Perretti (2010). [87]

1.6.1 Chemotaxis

FPR1, along with the receptor for C5a, was the first identified phagocyte chemoattractant receptor.[90] Chemotaxis is a multistep process, induced by various chemoattractants such as PAF,[91] LTB4,[92] C5a anaphylotoxin,[93] and different chemokines such as the IL8.[94] In comparison with "classic chemoattractants", fMLF has predominant properties in cross-desensitization of other receptors,[95] and it is a potent agonist for stimulating bactericidal functions such as superoxide generation. Once established that its responses are induced by activation of specific G-protein-coupled receptors (FPRs) which are expressed on target cells, several N-formyl peptides have been extensively studied due to their abilities to induce directional migration of neutrophils.[96,97] Indeed, human neutrophils are able to detect a chemotactic gradient of subnanomolar concentrations of fMLF.[56,57] Therefore, exposure of neutrophils to a chemotactic agent creates an intracellular gradient of signalling molecules that defines the leading edge as well as the direction of cell migration. In addition, several exogenously and endogenously produced leukocyte chemoattractants that initiate leukocyte migration and activation have been identified. Migrating neutrophils in tissues are then exposed to multiple chemoattractants so that different and sometimes conflicting signals can sort out. In this scenario, that "navigation" of neutrophils is considered as a multistep process, and a migrating cell not only detects multiple chemoattractants but also integrates these different signals.[98-100]

A hierarchy in chemotactic signalling, determining the direction of migrating cells in opposing chemotactic gradients, have been identified and new insights into the molecular mechanisms that allow neutrophils to prioritize chemotactic signals have been provided.[101] It resulted evident that "end-target chemoattractants" such as fMLF, dominate over the "intermediary chemoattractants" as IL-8 and LTB4. The ability of neutrophils to distinguish between these chemoattractants is crucial for their optimal migration toward bacteria with minimal interference by the "intermediary chemoattractants" which are also present in the inflammatory site.[102] Considering all these evidences, it stands to reason to assume that net result of cell migration is determined by the type of orienting signals, the strength of the signals and the time sequence in which they appear.

1.6.2 Superoxide generation

Stimulation of neutrophils with fMLF, at concentrations higher than those required for chemotaxis, leads to generation of superoxide. In most published studies, fMLF concentrations of 50 to 100 nM are required for the induction of superoxide production by human neutrophils in suspension.[103,104] Superoxide production in neutrophils results from membrane assembly and activation of NADPH oxidase, which is a multicomponent enzyme complex for electron transfer, leading to one-electron reduction of molecular oxygen.[105] The different concentrations required for chemotaxis and superoxide generation, effectively

prevent oxidant-mediated tissue injury that may be caused by migrating neutrophils. It is not entirely clear why neutrophils require a 50- to 100-fold higher concentration of fMLF for superoxide production with respect to chemotaxis, but a high signalling strength is probably necessary for simultaneous activation of multiple pathways leading to NADPH oxidase activation in phagocytes. However, several studies reported in literature suggest that there are multiple mechanisms for the potentiation of the same enzyme.[49] It is noteworthy that, when used at low concentrations (e.g., 5 nM), fMLF is unable to stimulate neutrophil superoxide production but it can synergize with other neutrophils activators for a more robust response and.[106]

1.6.3 Degranulation

In addition to the induction of superoxide generation, at higher concentrations (usually 10-50 times higher than the optimal concentration for chemotaxis) formyl peptides stimulate the release of granule constituents from neutrophils.[57,107] The fMLF-induced mobilization of granules produces a variety of effects, including proteolytic cleavage of membrane-localized adhesion molecules such as L-selectin, cell surface expression of new adhesion molecules and release of proinflammatory proteins and enzymes that can cause tissue degradation and killing of bacteria. For instance, neutrophil myeloperoxidases, released from the azurophil granules, help to convert hydrogen peroxide to hypochlorous acid,[108] a metabolite important both for killing invading microbes and for the resolution of inflammation. Moreover, most of the membrane associated with NADPH oxidase components are localized on mobilizable granules which, if activated, become more available for the assembly of NADPH oxidase it self. Last but not the least, there are intracellular pools of FPR1 as well as of FPR2/ALX that are up-regulated to the cell surface when cells are stimulated with inflammatory mediators that mobilize the granules.[109,110] Degranulation in fMLF-stimulated neutrophils involves the second messenger diacylglycerol and PKCs which are activated by diacylglycerol and Ca^{2+}.[111] However, fMLF is still able to induce secretory granule release when extracellular and intracellular Ca^{2+} is chelated, suggesting the presence of Ca^{2+} independent pathways for degranulation.[112] Further investigations will be necessary to determine the cross-talk between FPRs and other components taking place in process and their roles in regulating vesicular fusion during degranulation.

1.6.4 Transcriptional regulation and anti-inflammatory functions

Although neutrophils are terminally differentiated myeloid cells with special bactericidal functions, these cells retain the ability to synthesize selected proteins, including certain cytokines.[113] fMLF has been found to stimulate neutrophil transcriptional regulation and cytokine production.[114] fMLF-induced IL-8 secretion is accompanied by the activation of NF-*k*B, a nuclear factor for transcription of a large number

of proinflammatory genes.[115] In addition NF-*k*B activation is also mediated by FPR2/ALX, in response to SAA (serum amyloid A) stimulation, which leads to IL-8 secretion.[116] These results are consistent with the ability of certain GPCRs to regulate transcriptional activation that contributes to the proinflammatory activities of the respective ligands.[117] In contrast, FPR2/ALX ligands such as LXA4 (lipoxin A4) and ANXA1 (annexin A1) exhibit anti-inflammatory activities.[76,79] ANXA1 has been shown to cause detachment of leukocytes and prevent transendothelial migration (diapedesis).[118] In comparison, the anti-inflammatory effect of LXA4 is shown to involve suppression of proinflammatory gene expression.[119] It is not entirely clear if this action of LXA4 is mediated through FPR2/ALX-dependent negative signalling or blockade of FPR2/ALX binding and activation by an endogenous, proinflammatory agonist for this receptor,[49] even if it is known that LXA4 can compete off the binding of FPR2/ALX agonists.[120] However, LXA4 stimulation does not lead to calcium mobilization in several types of transfected cells neither induce neutrophil degranulation and superoxide generation.[121-123] In contrast, LXA4 has been shown to activate monocytes.[124] The discrepancy suggests that signaling molecules essential for certain LXA4-induced functions might be missing in neutrophils and epithelial cells. It also reflects that LXA4 lacks full agonistic activities at FPR2/ALX.

1.6.5 Neutrophil apoptosis

Neutrophils released to blood circulation have a half-life of 8 to 10 h. If not activated, these cells are destined for apoptosis.[125] Stimulation of neutrophils with proinflammatory cytokines such as G-CSF (granulocyte macrophage-colony-stimulating factor) and IL-1β, but not with fMLF, C5a, or IL-8, prolongs the lifespan of neutrophils.[126] Other reports have shown that stimulation of neutrophils with fMLF can induce apoptosis and this process requires superoxide generation.[127] Neutrophil apoptosis and phagocytosis of apoptotic neutrophils are related to resolution of inflammation. Several ligands for the formyl peptide receptors are found to play different roles in neutrophil apoptosis. The underlying mechanism has not been fully identified, however these results suggest a potential role of FPRs, mainly of FPR2/ALX,[49] in the regulation of neutrophil apoptosis, but other receptors may be involved as well and in the future it will be important to determine how a single class of receptors mediates different functions in cell survival and apoptosis when stimulated with different ligands.

1.7 FPRs involvement in several diseases

Till now, a part from the clear involvement of FPRs in inflammation, host defense against bacterial infection and as well as in the clearance of damaged cells, additional and more complex physiological functions have been proposed for FPRs.

Receptor	Disease pathology	Mechanism
FPR1	Periodontitis	Functional polymorphisms
FPR2/ALX	Alzheimer's disease	Cellular infiltration and amyloidosis
	Asthma	Decreased expression in BALF samples and on peripheral blood granulocytes Increase FPR2/ALX expression
	Rheumatoid arthritis	Regulating extracellular matrix turnover Increased expression in synovial tissues
	Acute ischemic stroke	Increased expression in peripheral blood mononuclear cells

Table 1.1. FPR1 and FPR2/ALX receptor associations with human disease. Adapted from Dufton and Perretti (2010).[87]

Indeed these receptors have been found to interact with a menagerie of structurally different pro- and anti-inflammatory ligands associated with different diseases (**table 1.1** and **1.2**), including amyloidosis and Alzheimer's disease,[128] some kinds of cancers and related alopecia induced by most anticancer agents,[129,130] prion disease,[131] HIV,[132-135] and stomach ulcer.[136,137] In addition, it is has been demonstrated that exogenously administered fMLF as well as other FPRs agonists can peripherally and centrally inhibit the nociceptive transmission associated with inflammatory processes through a mechanism that involves formyl peptide receptors.[138]

Ligand	Disease pathology	Mechanism
AnxA1	Alzheimer's disease	Decreased expression following proteomic analysis of peripheral leukocytes.
	Coronary artery disease	Augmented levels in circulating neutrophils.
	Cystic fibrosis	Higher proteolysis in BAL Absence in epithelial cells (proteomic study)
	Rheumatoid arthritis	Higher expression in circulating T cells upon GC treatment
LXA_4	Severe asthma	Decreased levels in BALF samples
	Rheumatoid arthritis	Elevated levels in synovial fluid
SAA	Coronary artery disease	Increased systemic levels as a diagnostic marker of prevalents Promotes monocyte tissue factor and TNF induction from mononuclear cells
	Crohn's disease	Close correlation with IL-6 production
	Rheumatoid arthritis	Systemic disease progression and secondary amyloidosis Inducing IL-6 in synoviocytes

Table 1.2. FPR2/ALX ligand association with human disease. Adapted from Dufton and Perretti (2010).[87]

Moreover, these receptors have been proposed as prospective targets for therapeutic intervention against malignant gliomas.[139] How these receptors recognize so different ligands and how they contribute to disease pathogenesis and host defense, are basic questions currently under investigation.

1.8 FPRs ligands

Ligand diversity is a prominent and unusual feature of the FPRs (**table 1.3**). With the exception of the eicosanoid LXA4, all known FPR family ligands are peptides. More recently, small synthetic molecules have emerged from a number of compound library screens as ligands for the formyl peptide receptors.[40,48,80] Whereas many of the natural agonists and antagonists for FPRs are identified and purified from living organisms, a lot of peptides are synthesized based on the sequences of known

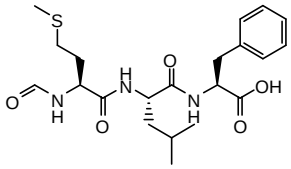
proteins of microbe and host origins (**table 1.3**). Whether these peptides are present in vivo and have physiological functions has yet to be determined for the most part of them.

Ligand	Origin/Description	Potency	Selectivity
N-formyl peptides			
fMLF and other bacterial formyl peptides*	Bacteria		FPR1 > FPR2/ALX
Mitochondrial formyl peptides*	Mitochondria		FPR1 ≈ FPR2/ALX
N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys	Synthetic	pK_d = 9.22	FPR1 ≫ FPR2/ALX
Microbe-derived nonformyl peptides			
T20 (DP178)	HIV-1 gp41 aa. 643–678	pEC_{50} = 8.30	FPR1
Hp (2–20)	*H. pylori*	pEC_{50} = 6.52	FPR2/ALX ≫ FPR3
T21 (DP107)	HIV-1 gp41 aa. 558–595	pEC_{50} = 6.30	FPR2/ALX
V3 peptide	HIV-1 gp120, V3 loop	pEC_{50} = 5.82	FPR2/ALX
N36 peptide	HIV-1 gp41 aa. 546–581	pEC_{50} = 5.00	FPR2/ALX
F peptide	HIV-1 gp120 aa. 414–434	pEC_{50} = 5.00	FPR2/ALX
Host-derived peptides			
CKβ8–1 (human CCL23)*	Chemokine	pEC_{50} = 9.00–7.82	FPR2/ALX ≈ CCR1
SHAAGtide*	CCL23 *N*-terminal 18 aa.	pEC_{50} = 7.72	FPR2/ALX > CCR1
Humanin*	Neuroprotective peptide	pEC_{50} = 8.46	FPR2/ALX
F2L*	Heme binding protein	pEC_{50} = 8.00	FPR3 ≫ FPR2/ALX
SAA*	Acute-phase protein	pEC_{50} = 7.35	FPR2/ALX, others
Annexin 1 / lipocortin 1*		pIC_{50} = 6.82	FPR1
Ac2–26*	Annexin 1	pEC_{50} = 6.05–5.77	FPR1, FPR2/ALX
Ac9–25	Annexin 1	pEC_{50} = 4.70	FPR1, FPR2/ALX
Aβ (1–42)*	Amyloid precursor	pEC_{50} = 7.00	FPR2/ALX
D2D3*	uPAR (88–274)	pEC_{50} = 7.08	FPR2/ALX
LL-37*	Cathelicidin	pEC_{50} = 6.00	FPR2/ALX
PrP (106–126)*	Prion protein	pEC_{50} = 4.60	FPR2/ALX
Temporin (from *Rana temporaria*)*	Anti-microbial peptide	pEC_{50} = 6.60	FPR2/ALX
	Pituitary adenylate cyclase activating polypeptide	pEC_{50} = 6.00	FPR2/ALX
Host-derived nonpeptide agonists			
Lipoxin A4 and aspirin-triggered lipoxins*	Eicosanoids	pK_d = 8.77	FPR2/ALX, AhR
Agonists from peptide library			
WKYMVm	Peptide library	pEC_{50} = 10.13	FPR2/ALX > FPR ≫ FPR3
WKYMVM	Peptide library	pEC_{50} = 8.70	FPR2/ALX ≫ FPR3
MMK-1	Peptide library	pEC_{50} = 8.70	FPR2/ALX
MMWLL, formyl-MMWLL	Peptide library	pEC_{50} = 8.96	FPR1
Agonists from nonpeptide library			
Quinazolinone derivative (Quin-C1)	Combinatorial library	pEC_{50} = 5.72	FPR2/ALX ≫ FPR1
Pyrazolone, 4-iodo-substituted, no. 43	Combinatorial library	pIC_{50} = 7.36	FPR2/ALX ≫ FPR1
AG-14	Drug-like molecule library	pEC_{50} = 7.38	FPR1

Table 1.3. Agonists for the human FPRs. The agonists are listed in the order of their potency within each group. The mitochondrial *N*-formylated peptides, listed in the first group, are also host-derived peptides and their potency is expressed in **table 1.4**, as well as the potency of other bacterial *N*-formylated peptides. Ligands that have been isolated from living organisms in the forms listed, and those generated by the actions of physiologically relevant enzymes, are indicated with an asterisk (*). aa., amino acid; pIC_{50}, negative logarithm of the IC_{50}; pEC_{50}, negative logarithm of the EC_{50}; p*K*d, negative logarithm of *K*d. Adapted from Ye et al. (2009).[49]

1.8.1 FPRs natural agonists

A) N-*Formyl Peptides.* The *E. coli*-derived tripeptide fMLF (**figure 1.5**, **table 1.4**) is the most widely used chemotactic peptide for several reasons. It was one of the first characterized synthetic chemotactic peptides and has been extensively studied because it is the smallest formyl peptide that displays full agonistic activities. Its potency and efficacy in activating major bactericidal functions of neutrophils equals that of C5a. Whereas fMLF is by far the most frequently used chemotactic peptide in studies of neutrophil functions, this prototypic formyl peptide should not be taken as the sole standard in judging the presence of functional formyl peptide receptors.[49]



fMLF

Figure 1.5. Chemical structures of fMLF, a FPR1 selective agonist.

Because bacterial protein synthesis starts with an *N*-formyl methionine, formyl peptides released from bacteria can be considered a type of microbe-associated molecular pattern, recognizable by specialized receptors in the innate immune cells of the host, such as the Toll-like receptors (TLRs). These non rearranging innate receptors have evolved to aid the host in detecting nonself such as bacterial products.[140] Ample evidence shows that the formyl peptide receptors can detect not only the *E. coli*-derived fMLF but also formyl peptides from other bacteria strains and from mitochondria of the host cells. **Table 1.4** lists selected bacterial and mitochondrial Formyl peptides that have been characterized for their bioactivities. Given the variety of formyl peptides from both bacteria and mitochondria (**table 1.4**), it is worthwhile to revisit some previous studies conducted with the use of fMLF and determine whether the receptors of interest are, at the present, more selective for formyl peptides of different sequences. For example, differently to FPR1, FPR2/ALX was first identified as a low affinity receptor for fMLF,[82] but it is always able to detect and respond to several different formyl peptides. Moreover FPR2/ALX is able to discriminate between N-formyl peptides of different sizes, hydrophobicities, and charges.[141] The biological relevance of this property of the receptor is not yet entirely understood but it is evident that several N-formyl peptides other than fMLF should demonstrate more selective for certain receptors (**table 1.4**). Indeed, although N-formyl peptides are a class of ligands representing a pattern recognized by the FPRs,[55] there are important differences in potency and receptor selectivity among the individual peptides.

Ligand	Origin	Assay	Potency
N-formyl-Met-Leu-Phe	*E. coli*	Chemotaxis	pEC_{50} = 10.15
		Lysozyme release	pEC_{50} = 7.49
		O_2^- production	pEC_{50} = 7.00
		Binding	pK_d = 9.28–7.61
			pK_d = 6.37
N-formyl-Met-Ile-Phe-Leu	*S. aureus*	Chemotaxis	pEC_{50} = 7.51
		Competitive binding	pIC_{50} = 8.01
		O_2^- production	pEC_{50} = 8.00
N-formyl-Met-Ile-Val-Ile-Leu	*L. monocytogenes*	Ca^{2+} flux	pEC_{50} = 8.66
			pEC_{50} = 6.80
		O_2^- production	pEC_{50} = 7.82
N-formyl-Met-Ile-Gly-Trp-Ile	*L. monocytogenes*	Ca^{2+} flux	pEC_{50} = 7.70
			pEC_{50} = 6.68
N-formyl-Met-Ile-Val-Thr-Leu-Phe	*L. monocytogenes*	Ca^{2+} flux	pEC_{50} = 8.57
			pEC_{50} = 6.70
N-formyl-Met-Ile-Gly-Trp-Ile-Ile	*L. monocytogenes*	Ca^{2+} flux	pEC_{50} = 7.40
N-formyl-Met-Phy-Glu-Asp-Ala-Val-Ala-Trp-Phy	*M. avium*	Chemotaxis	pEC_{50} = 6.00
N-formyl-Met-Met-Tyr-Ala-Leu-Phe	Mitochondria, ND6	Ca^{2+} flux	pEC_{50} = 7.92
			pEC_{50} = 7.82
N-formyl-Met-Leu-Lys-Leu-Ile-Val	Mitochondria, ND4	Ca^{2+} flux	pEC_{50} = 7.92
			pEC_{50} = 7.26
N-formyl-Met-Tyr-Phe-Ile-Asn-Ile-Leu-Thr-Leu	Mitochondria, ND1	Binding	pK_d = 9.00
N-formyl-Met-Phe-Ala-Asp-Arg-Trp	Cytochrome *c* oxidase subunit	Ca^{2+} flux	pEC_{50} = 6.80
			pEC_{50} = 6.68

Table 1.4. Binding affinity and potency of bacterial and mitochondrial formyl peptides. pEC_{50} is defined as the negative logarithm of the EC_{50} and pIC_{50} as the negative logarithm of the IC_{50}. HL-60 cells transfected to express FPR1 or FPR2/ALX, and Chinese hamster ovary cells transfected to express FPR1 were used in some studies. Adapted from Ye et al. (2009).[49]

Furthermore, there are several examples in which addition of an N-formyl group increases agonistic activity of the peptides.[142] Humanin is an endogenous peptide with neuroprotective activity that also binds to FPR2/ALX and FPR3.[143,144] For example, when humanin is N-formylated became a more potent agonist for these receptors,[145] in comparison to its non formilated form. In the latter example, although the primary sequences of FPR2/ALX and FPR3 differ considerably from those of FPR1, especially in the ligand binding domains,[146] these two receptors seem to retain the ability to preferentially interact with formylated peptides. Despite their evident importance in FPRs binding, it is not yet known whether these peptides are produced in vivo and whether they modulate inflammation.

B) Microbe-derived non-formyl peptides. Despite the absence of an *N*-formyl group, several different peptides have been found as FPRs full agonists (**table 1.3**).[85,137] In this context, it is well known that HIV-1 envelope proteins, such us several fragments derived from gp41, contain peptide sequences capable of interacting with either or both FPR1 and FPR2/ALX.[147-150] V3 peptide is another peptide derived from HIV-1 strain that showed to interact with FPRs.[151] The existence of these peptides in vivo and their biological significance are not known at present. In addition to HIV-1 proteins, other viral proteins contain sequences that can work as ligands for FPRs when tested in the form of synthetic peptides. gG-2p20, is a peptide present in Herpes simplex virus type 2 (HSV-2), activates neutrophils and monocytes via FPR1.[152] In addition, the same peptide activates phagocytes to release reactive oxygen species that inhibit NK cell cytotoxicity and accelerate apoptotic cell death. Recently, additional peptides from coronavirus have been identified as FPR1 ligands.[153]
It will be interesting to understand whether and how these peptides are generated during viral infection and as well the functional consequences of phagocyte response to these peptides.

C) Host-derived peptides. In addition to mitochondrial formyl peptides discussed above, a large number of endogenous peptides of various compositions (**table 1.3**), often without an *N*-formyl group, have been identified as agonists for the formyl peptide receptors, especially FPR2/ALX. Of particular interest are peptides associated with amyloidogenic diseases, peptides associated with inflammatory and antibacterial responses, and a peptide derived from heme-binding protein which acts as a potent endogenous FPR3 agonist.[49]
At least three amyloidogenic polypeptides, associated with chronic inflammation and amyloidosis, have been identified as FPR2/ALX agonists. Serum amyloid A (SAA) is an acute-phase protein whose serum concentration is increased by as much as 1000-fold in response to trauma, acute infection and other environmental stress causing acute-phase responses.[154] Studies with recombinant human SAA identified it as the first mammalian hostderived chemotactic peptide ligand for FPR2/ALX.[121] SAA, acting through

FPR2/ALX, is chemotactic for monocytes, neutrophils, mast cells, and T lymphocytes. In addition this protein stimulates production of metalloproteases and cytokines and increases expression of cytokine receptors. In neutrophils, SAA activates FPR2/ALX and induces IL-8 secretion.[116] In monocytes, SAA shows a peculiar pattern of cytokine induction via FPR2/ALX. Moreover, the cells respond to low concentrations of SAA by producing TNF-α while releasing IL-10 in response to high concentrations of SAA.[155] The synovial tissues of patients with inflammatory arthritis express high levels of SAA and FPR2/ALX and the protein induces the expression of matrix metalloproteinase-1 and -3 in fibroblast-like synoviocytes.[156,157] Furthermore, SAA promotes synovial hyperplasia and angiogenesis through activation of FPR2/ALX and,[158] in addition to using the formyl peptide receptors, SAA activates neutrophil NADPH oxidase through a different receptor.[159]

Another peptide, the 42-amino acid form of β-amyloid peptide ($A\beta_{42}$), which is a cleavage product of the amyloid precursor protein in the brain and a pathologic protein in Alzheimer's disease, was also found to activate FPR2/ALX.[160,161] An additional amyloidogenic disease-associated FPR2/ALX agonist is represented by the prion protein fragment PrP(106–126), which is produced in human brains with prion disease.[162] FPR2/ALX mediates the migration and activation of monocytic phagocytes, including macrophages and brain microglia, induced by β-amyloid (Aβ).[160,163] Moreover, prolonged exposure of FPR2/ALX to this fragment peptide results in accumulation of the $A\beta_{42}$ and culminates in progressive fibrillary aggregation of $A\beta_{42}$ and macrophage death.[164] Therefore, FPR2/ALX not only mediates the proinflammatory activity of the peptide agonists associated with amyloidogenic diseases, but it also participates to the regulation of fibrillary peptide formation and deposition, which contribute to tissue and organ destruction.[128] The in vivo significance of this action for the pathogenesis of Alzheimer's disease is not yet known.

Humanin is a peptide encoded by a cDNA cloned from a relatively healthy region of an Alzheimer's disease brain.[165] Both secreted and synthetic humanin peptides protect neuronal cells from damage by $A\beta_{42}$. Humanin uses human FPR2/ALX and FPR3 as functional receptors to induce chemotaxis of mononuclear phagocytes.[166,167] In addition, humanin reduces aggregation and fibrillary formation by suppressing the interaction of $A\beta_{42}$ with mononuclear phagocytes through FPR2/ALX. Moreover, human neuroblastoma cell lines express functional FPR2/ALX but not FPR1. In these cells, although humanin and $A\beta_{42}$ both activate FPR2/ALX, only $A\beta_{42}$ causes apoptotic death of the cells, a process blocked by humanin. These observations suggest that humanin may exert its neuroprotective effects by competitively inhibiting the access of $A\beta_{42}$.[167]

Urokinase-type plasminogen activator (uPA) is a serine protease known for its ability to regulate fibrinolysis binding to a specific high affinity surface receptor (uPAR). In addition to this action, the uPA-uPAR system is crucial for cell adhesion, migration and tissue repair. A cleaved soluble uPAR

fragment (D2D388–274) binds and activates FPR2/ALX in monocytes, inducing cell migration.[77] The ability of cleaved soluble uPAR (c-suPAR) to activate other members of the FPR family has been reported. For instance, a peptide corresponding to residues 88 to 92 of uPAR, binds to and activates FPR1.[168] Another fragment induces basophil migration by activating both FPR2/ALX and FPR3.[136] Recent studies showed that pretreatment of monocytes with the FPR2/ALX agonist D2D388–274 strongly decreases chemokine-induced integrin-dependent rapidcell adhesion,[169] indicating that FPRs regulate leukocyte chemotaxis at multiple levels and other than mediate cell migration, they may suppress cell responses to chemokines by desensitizing chemokine receptors.

Furthermore, FPRs interact with several bactericidal peptides contained in human neutrophil granules. LL-37, an enzymatic cleavage fragment of the neutrophil granule protein cathelicidin, is an agonist for FPR2/ALX.[170] LL-37 is expressed by leukocytes and epithelial cells and secreted into wounds and into the airway surface. In addition to its microbicidal activity, LL-37 induces directional migration of human monocytes, neutrophils, and T lymphocytes, a function mediated by FPR2/ALX. Recent studies showed that LL-37-induced angiogenesis is mediated by FPR2/ALX in vascular endothelial cells.[171] LL-37 seems to be a multifunctional peptide with a central role in innate immunity against bacterial infection and in the induction of arteriogenesis.

Another antibacterial granule protein, cathepsin G, which is a serine protease and participates in wound healing, is identified as a specific FPR1 agonist and it is considered responsible for chemotactic activity in phagocytes.[172]

Annexin A1 (ANXA1) and its N-terminal peptides have interesting properties in activating formyl peptide receptors by playing a dual role in inflammatory host responses. ANXA1 (also termed lipocortin I) is a glucocorticoid-regulated, phospholipid-binding protein of 37 kDa that possesses both pro- and anti-inflammatory activity, in part mediated by FPR1.[173] Expressed in a variety of cell types, ANXA1 is particularly abundant in neutrophils. The protein is primarily cytosolic, but it may also be secreted through a non classic secretory process and found on the outer cell surface, causing leukocyte detachment and thereby inhibiting their transendothelial migration. At low concentrations, both ANXA1 holoprotein and its N-terminal peptides (Ac2–26 and Ac9–25) elicit Ca^{2+} transients through FPR1 without fully activating the MAPK pathway, causing neutrophil desensitization and inhibition of transendothelial migration induced by other chemoattractants such as the chemokine IL-8. In contrast, at high concentrations, the ANXA1 peptides fully activate neutrophils in vitro and they become potent proinflammatory stimulants. The antimigratory activity of exogenous and endogenous ANXA1 has been shown in both acute and chronic models of inflammation.[118] Other studies have shown that the ANXA1 N-terminal peptides use FPR2/ALX for its anti-inflammatory actions.[76] These peptides are also ligands for FPR3.[173] The utilization of the FPRs by ANXA1 and its amino terminal peptides for their various

function is a complex issue. To this end, one published report demonstrates that Ac9–25 stimulates neutrophil NADPH oxidase activation through FPR1, but its inhibitory effect is mediated through a receptor other than FPR1 or FPR2/ALX,[174] suggesting the presence of additional receptors for ANXA1 and its peptides.

F2L is a highly potent and efficacious human FPR3 agonist peptide.[86] It is an amino-terminally acetylated peptide resulting from the natural cleavage of human heme-binding protein, an intracellular tetrapyrrole-binding protein. The peptide binds and activates FPR3 in the low nanomolar range, triggering typical G protein-mediated intracellular calcium release, inhibition of cAMP accumulation and phosphorylation of the ERK 1/2MAPKs. F2L also chemoattracts and activates monocyte-derived DCs. Thus, F2L seems to be a novel and unique natural chemotactic peptide for FPR3 in DCs and monocytes, in agreement with the selective expression of FPR3 in these cells.[62] F2L may play a role in linking innate and adaptive immune responses by activating antigen-presenting DCs, which express little FPR1 and FPR2/ALX.

D) Host-derived nonpeptide agonists. LXA4 (5*S*,6*R*,15*S*-trihydroxy-7,9,13-*trans*-11-eicosatetraenoic acid, **figure 1.6**) is a potent mediator biosynthesized from arachidonic acid. It is a small molecule with physical and chemical properties that differ from the most part of lipids: it has a unique structure and belongs to a class of conjugated tetraene containing eicosanoids that display stereoselective and highly potent anti-inflammatory effects in vivo, together with pro-resolving activities in many mammalian systems.[175]

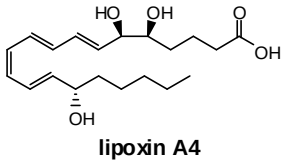


Figure 1.6. Chemical structures of LXA4, a highly selective agonist for FPR2/ALX.

This aspect is unusual since the most part of eicosanoids are pro-inflammatory. As an endogenous mediator, LXA4 displays multilevel control of relevant processes in acute inflammation through specific and selective actions on multiple cell types via specific receptors.[176] In particular, LXA4 has been reported to directly interact with human FPR2/ALX and in addition it is the first identified endogenous ligand for this receptor (**table 1.3**). LXA4 has showed to be responsible for the specific LXA4 functions in neutrophils.[177] In fact, it stimulates rapid (within seconds) phospholipase activation in these cells and this event directly correlates with the induction time course of specific LXA4 binding.[178,179] In cell line HL-60, when differentiated into neutrophils, LXA4 gives high-affinity binding with FPR2/ALX as well as ligand selectivity, compared with other eicosanoids including LXB4, leukotriene B4, leucotriene D4, and PGE2. Each of the actions of LXA4 proved to be stereoselective, because double bond isomerization

and alcohol chirality (*R* or *S*), as well as dehydrogenation of alcohols and reduction of double bonds, are associated with changes in potency. Elimination of the carbon 15 position alcohol from LXA4, denoted 15-deoxy-LXA4, is essentially inactive in vivo and does not stop either neutrophil transmigration or reduce adhesion.[175] Moreover, several in vivo studies have shown that nanogram amounts of LXA4 stops neutrophil infiltration and blocks human neutrophil transmigration across mucosal epithelial cells and vascular endothelial cells.[79,180-186] One of the mechanisms by which it inhibits neutrophil infiltration is the induction of NO production, which suppresses leukocyte-endothelial cell interaction.[187] LXA4 inhibits as well TNFα-induced production of cytokines (IL-1β and IL-6),[188] chemokines (IL-8),[189] and consequently decreases LPS induced secretion of IL-1β, IL-6, and IL-8.[190] Dendritic cell production of the immunomodulatory cytokine IL-12 is also regulated by LXA4.[191,192] In addition to its inhibition of proinflammatory cytokine production, LXA4 plays a role in regulating inflammation-induced pain.[193] This function is probably mediated through alteration of spinal nociceptive processing via astrocyte activation. LXA4 also stimulates non phlogistic phagocytosis of apoptotic neutrophils, which accelerates the clearance of neutrophils and promotes resolution of inflammation.[194] These examples show that, in general terms, LXA4 actively inhibits many endogenous processes which can amplify local acute inflammation, leading to potent anti-inflammatory as well as pro-resolving actions in vivo. The multilevel control by LXA4 on relevant processes of acute inflammation raises the intriguing question of how LXA4, binding to FPR2/ALX, might translate into the anti-inflammatory and pro-resolving activities and whether other receptors contribute to these activities.[49]

1.8.2 Agonists from peptide library

Using combinatorial peptide library screens, a number of peptides have been identified as potent agonists for the formyl peptide receptors (**table 1.3**). WKYMVm, a hexapeptide representing a modified sequence isolated from a random peptide library, was found to be a highly efficacious stimulant for human B lymphocytes, monocytic cell lines and blood neutrophils.[195] WKYMVm binds to FPR1, FPR2/ALX, and FPR3 for activation of human phagocytic cells.[196,197] WKYMVm is by far the most potent peptide agonist for FPR2/ALX, with an EC_{50} well within the picomolar range in chemotaxis assays. WKYMVM, a derivative of WKYMVm with a L-methionine at the carboxyl terminus, is a highly selective agonist for FPR2/ALX and it is also a weaker activator of FPR3.[84] A recent study investigated the relationship between FPR1 and FPR2/ALX, both found in neutrophils, in mediating the WKYMVm-stimulated cellular functions. It was found that WKMYVm activates neutrophils through FPR1 only when the signal through FPR2/ALX is blocked,[198] suggesting its affinity for one type of receptor despite the presence of two different types of receptors in neutrophils. WKYMVm also inhibits LPS-induced maturation of human monocyte-derived dendritic cells via FPR1 and FPR3.[197]

The peptide MMK-1 was identified from a library screen and it was found to be a highly selective FPR2/ALX chemotactic agonist. [199-200] Furthemore, an additional peptide FPR1 agonist was identified from a library based on the activation mechanism of the thrombin receptor PAR-1.[201] This peptide becomes 1000 times more potent when N-formylated, confirming the preferential recognition of *N*-formylmethionine-containing peptides by FPR1.

1.8.3 Agonists from nonpeptide library: synthetic small molecules

Several laboratories have recently identified ligands for the FPRs through the screening of combinatorial libraries consisting of synthetic, non-peptide compounds. These synthetic small molecules belong to different chemical classes (**figure 1.7**) and are highly selective for either FPR1 or FPR2/ALX, providing useful tools for the characterization of formyl peptide receptors. A small molecule named Quin-C1 (**figure 1.7**) with a quinazolinone scaffold was the first reported as a highly selective FPR2/ALX agonist.[61] Quin-C1 (4-butoxy- *N*-[2-(4-methoxy-phenyl)-4-oxo-1,4-dihydro-2*H*quinazolin-3-yl]-benzamide) induces chemotaxis and secretion of β-glucuronidase in peripheral blood neutrophils with a potency approximately 1000 fold lower in comparison with WKYMVm, which is the most potent FPR2/ALX agonist identified till now. However, unlike most FPR2/ALX peptidic agonists, Quin-C1 did not induce substantial neutrophil superoxide generation, even at high concentrations (up to 100 μM). The structural basis for this particular agonistic activity is still unknown but it appeared attractive for its potential use as a therapeutic agent.

A series of pyrazolone compounds were initially identified from a cell-based assay for high-throughput screening and subsequently modified.[202,203] These ligands are also highly selective for FPR2/ALX and exhibit anti-inflammatory properties in mouse. Some pyrazolones belonging to this series were able to stimulate calcium flux in transfected cells expressing FPR2/ALX and the most interesting term, compound 43 (**table 1.3** and **figure 1.7**), was formulated for oral administration and was found to significantly reduce inflammation in a mice model assay.

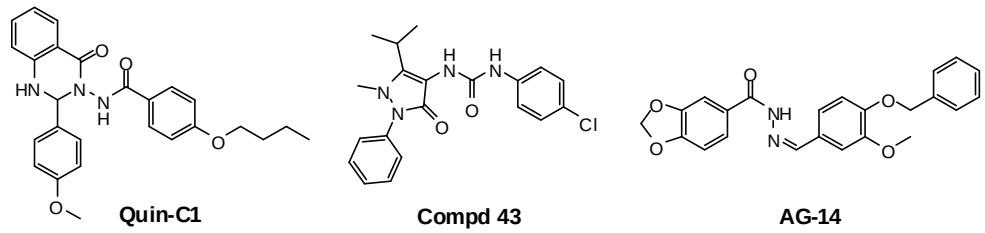


Figure 1.7. Chemical structures of selected small-molecule ligands for the formyl peptide receptors. Despite their abilities to bind to FPR1 and/or FPR2/ALX, these ligands have quite different structures. Quin-C1, and compound 43 are highly selective agonists for FPR2/ALX, whereas AG-14 is selective for FPR1.

Using a strategy combining computer model-based virtual screening and high-throughput no-wash cytometry screening, from an initial pool of 480,000 compounds, 30 leads were identified as FPR1 partial

agonists or antagonists.[139] The pharmacophore model for FPR1 developed in this study may be useful in future identification of agonists and antagonists for this class of receptors.

Starting from a neutrophil superoxide production assay, combined with substructure screen, fragment focusing and structure-activity relationship analyses, *t*-butyl benzene and thiophene-2-amide-3-carboxylic ester derivatives were identified as potential agonists for neutrophil chemoattractant receptors.[204] Among these compounds it emerged AG-14 (**figure 1.7** and **table 1.3**) which was found to be able to activate neutrophils at nanomolar concentrations. Based on desensitization and antagonist inhibition data, the investigators concluded that AG-14 is a FPR1 agonist.

Arylcarboxylic acid hydrazide derivatives were identified by the same investigators as agonists for FPR2/ALX that induce de novo production of TNF-α through activation of macrophages.[205]

All these studies further demonstrate that agonists of FPR1/FPR2 include compounds with wide chemical diversity. The computational studies regarding both the pharmacophore models of FRRs and the structure-activity relationships analysis, [206-207] widely confirm the hypothesis that analysis of such diverse compounds can help to better understand the ligand/receptor interaction features.

1.8.4 FPRs antagonists

Early studies showed that replacing the formyl group of fMLF with tertiary butyloxycarbonyl group (*t*-Boc) renders the peptide of antagonistic activity (**table 1.5**). *t*-Boc-Met-Leu-Phe (Boc1) and *t*-Boc-Phe-D-Leu-Phe-DLeu-Phe (Boc2) are two frequently used antagonists for FPR1, with pIC50 values of 6.19 and 6.59 respectively.[56] In several recent studies, Boc2 was used at high concentrations (e.g., 100 μM) for inhibition of FPR2/ALX.[208,209] A different study has shown that, when used at low micromolar concentrations, both Boc1 and Boc2 are selective FPR1 antagonists; at high micromolar concentrations, Boc2 partially inhibits FPR2/ALX in addition to FPR1.[210] Therefore, the antagonistic effect of Boc2 at high concentrations is not specific for FPR2/ALX.

Cyclosporin H (CsH) is a cyclic undecapeptide produced by fungi and it displays selective antagonistic activity at human FPR1.[211] Studies have shown that CsH is 14-fold more potent than the tertiary butyloxycarbonyl analogs of formyl peptides such as Boc2 in FPR1 binding assays, and approximately 5-fold more potent than Boc2 in the inhibition of fMLF-induced calcium flux and enzyme release.[212] CsH is an inverse agonist (negative antagonist) that suppresses the constitutive activity of FPR1.[213] The biological significance of constitutive activity for FPR is not established. Both Boc2 and CsH competitively displace FPR1-bound fMLF, indicating that its antagonistic activity is mediated through inhibition of fMLF binding. CsH did not displays any detectable inhibitory effect on FPR2/ALX-mediated cellular functions.

Ligand	Assay	Potency	Selectivity
Chemotaxis inhibitory protein of *S. aureus* (CHIPS)	Binding	$pK_d = 7.46$	FPR1
FPRL1-inhibitor protein (FLIPr)	Binding, Ca^{2+} flux	N.D.	FPR2/ALX ≫ FPR1
Trp-Arg-Trp-Trp-Trp-Trp (WRW^4)	Ca^{2+} flux	$pIC_{50} = 6.64$	FPR2/ALX ≫ FPR1 ≈ FPR3
CsH	Binding	$pK_i = 7.00$	FPR1
CsA	Enzyme release	$pK_i = 6.22$	FPR1
i-Boc-Met-Leu-Phe	O_2^- generation	$pIC_{50} = 6.60$	FPR1
t-Boc-Met-Leu-Phe	Enzyme release	$pIC_{50} = 6.19$	FPR1
t-Boc-Phe-Leu-Phe-Leu-Phe	Enzyme release	$pIC_{50} = 6.59$	FPR1 ≫ FPR2/ALX
Quin-C7	Binding	$pK_i = 5.19$	FPR2/ALX
CDCA	Binding	$pK_i = 4.76–4.52$	FPR1 > FPR2/ALX
DCA	Binding	$pK_i = 4.00$	FPR1
Spinorphin	O_2^- generation	$pIC_{50} = 4.30$	FPR1

Table 1.5. Antagonists for the human formyl peptide receptors. Antagonists for the FPRs are listed in the order of their approximate potency, except that antagonists of same types are listed together. *t*-Boc, *N-tert*-butoxycarbonyl group; *i*-Boc, -butoxycarbonyl group; pIC_{50}, negative logarithm of the IC_{50}; p*K*i, negative logarithm of *K*i; N.D., binding affinity or potency was not determined. Adapted from Ye et al. (2009).[49]

Endogenous FPR1 antagonists have been as well identified. Spinorphin, an opioid, is an endogenous peptide antagonist for FPR1 with a pIC_{50} of 4.30.[214,215] The bile acids deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) are two other identified FPR1 antagonists.[216,217] Chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a bacteria-derived protein of 14.1 kDa found in more than half of the clinical strains of *S. aureus*. CHIPS showed antagonistic activity for FPR1.[218] The identification of a bacteria-derived FPR1 antagonist suggests a mechanism used by microorganisms to thwart host defenses. Indeed, the same researchers in a subsequent study reported the identification from *S. aureus* of a 105-amino acid protein, termed FPRL1 inhibitory protein (FLIPr), that selectively inhibits the binding of and activation by FPR2/ALX agonists including MMK-1, WKYMVM, the prion fragement PrP106–126, and amyloid peptide Aβ1–42.[123] At higher concentrations, FLIPr also inhibits fMLF binding to FPR1. FLIPr was found to bind directly FPR2/ALX and FPR1, but not FPR3. The biological function of this inhibition has not been identified.

Quin-C7 (**table 1.5** and **figure 1.8**) is a synthetic, nonpeptide FPR2/ALX antagonist, developed through chemical modification of the FPR2/ALX agonist Quin-C1.[219]

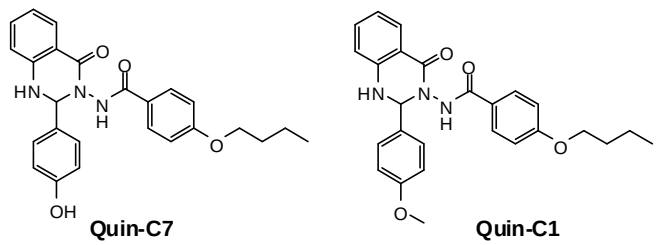


Figure 1.8. Chemical structures of Quin-C7 and Quin-C1, a synthetic pair of antagonist/agonist ligands for FPR2/ALX.

In binding assays, Quin-C7 inhibited iodinated WKYMVm binding to FPR2/ALX. This antagonist showed to be highly selective for FPR2/ALX. In summary, several FPR1 and FPR2/ALX antagonists have been identified and characterized. It is noteworthy that, in most cases, these antagonists differ considerably from the identified FPR1 and FPR2/ALX agonists. The observation that *t*-Boc peptides are antagonists while *N*-formyl peptides of the same or similar composition are agonists may be helpful to define the binding pocket of these peptides in FPR1. For the same reason, the synthetic nonpeptide antagonist Quin-C7 (**figure 1.8**), differing from the agonist Quin-C1 (**figure 1.7**) only in the *para* position of the phenyl ring,[219] provides a potentially useful tool in study the binding properties of FPR2/ALX.

1.9 FPRs: future

It is clear that FPR family can convey a large variety of signals, thereby affecting an ever-increasing number of biological functions. The study of endogenous ligands for FPRs provides evidence of both activatory and inhibitory responses in experimental settings and validate endogenous anti-inflammation for successful drug discovery programs. However, the characterisation of the pharmacological properties of synthetic agonists for these receptors strongly suggests a prominent role in inflammation and in general in host defence context. The overarching hypothesis is that compounds that are able to mime specific pathways operative in the host to down-regulate inflammation could present beneficial reductions in side effects of potential therapeutics.

In this scenario, the identification and development of small-molecules represent an ideal strategy to clarify FPR structures and functions, since such molecules are well defined and can be easily modified for structure-activity relationships (SAR) analysis. Moreover, this approach may provide a basis for construction of useful pharmacophore models of FPR ligands and contribute to the development of an innovative approach to modulate host defence in inflammatory pathologies.

2. BACKGROUND AND AIMS OF THE PROJECT

The overall aim of this project is to exploit the versatility and usefulness of heterocyclic chemistry to generate a library of compounds able to act as FPRs agonists. Over the past thirty years, Prof. Dal Piaz and co-workers have shown that heterocyclic compounds such as isoxazoles, pyridazines, pyridazinones, pyrimidines, phtalazinones and pyrazoles, are good candidates to develop small molecules which achieve a high interaction with different biological systems.[220-232] Taking advantage of these previous experiences acquired in these researches, a similar approach was undertaken to generate a number of FPRs agonists. To achieve this goal, it was initially necessary to identify in literature a lead compound working as FPRs agonist, and then to synthesize some different series of heterocyclic compounds.

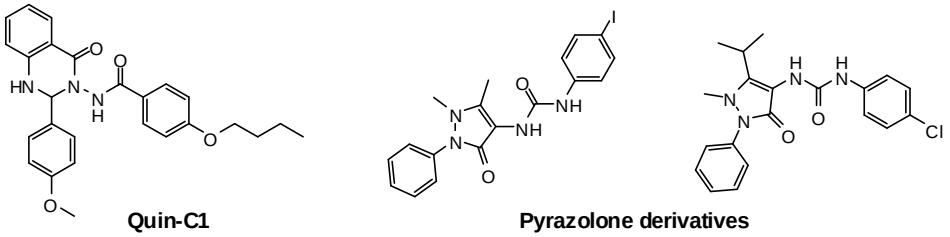


Figure 2.1. The key reference molecules used as leads in ligand-based drug design approach for the synthesis of FPRs agonists.

Thus, in the first instance, our project was based on the structural analysis of "Quin-C1" (**figure 2.1**),[61,219] the first synthetic compound reported in literature as FPR2 selective ligand; moreover, we took into account also some pyrazolone derivatives, which were among the most potent small molecules discovered as FPRs agonists (**figure 2.1**).[202,203]

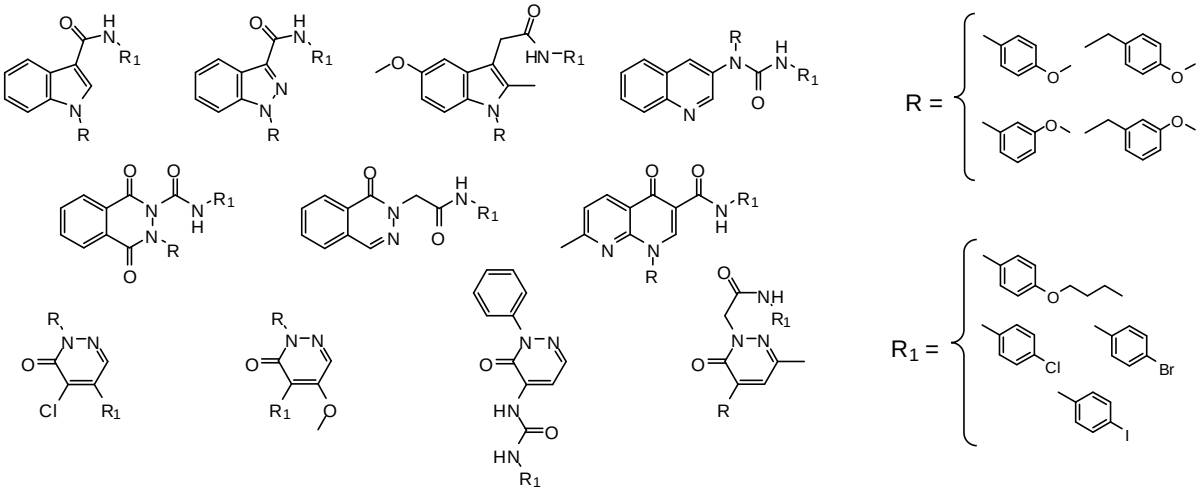


Figure 2.2. Some examples of compounds designed and synthesized using different nitrogen heterocyclic scaffold and bearing the functional groups strictly required for the activity of the reference molecules.

Thus, following a ligand-based drug design approach, we synthesized several series of nitrogen heterocyclic derivatives (**figure 2.2**) characterized by geometric, electronic, steric and lipophilic properties similar to that of the reference compounds. The aim of this approach was to allow the rapid

identification of the appropriate scaffold bearing the suitable substituents able to interact with the FPRs system. Thus the activity of the synthesized compounds was tested on HL-60 cells transfected with FPR1, FPR2 and FPR3 respectively. The biological tests were performed by Prof. Quinn and co-workers of Veterinary Molecular Biology Department, Montana State University. In this initial screening we luckily were able to identify compound **46a** (**scheme 12** in **section 3** and **table 4.1** in **section 4**), a potent mixed FPR1/FPR2 agonist.[233] Thus, **46a** became our lead compound, on which we planned to perform extensive structure-activity relationship (SAR) studies by modifying the nature and the length of the functionalized side chain and by changing as well the substituents and their position on the pyridazinone ring (**figure 2.3**). Moreover, several groups were introduced to replace Br and OCH_3 on both the aromatic systems of the lead compound.

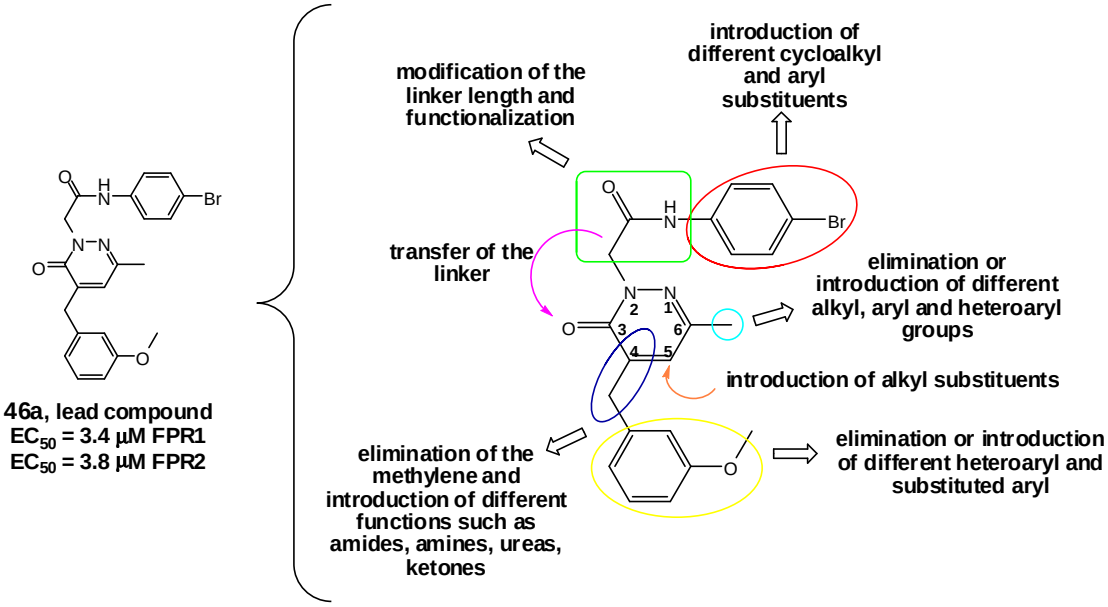


Figure 2.3. Modification performed on the lead compound (**46a**, **scheme 12**, **table 4.1**).

Once the reactions on the pyridazinone ring have been optimized, a small library of analogues was designed and synthesized in order to achieve a convenient chemical diversity on this scaffold (**figure 2.3**). The introduction of a chiral center on the phenylacetamide linker at N-2 position of the lead compound (**figure 2.4**), represent an additional project which led to the development of a new series of chiral and branched derivatives.

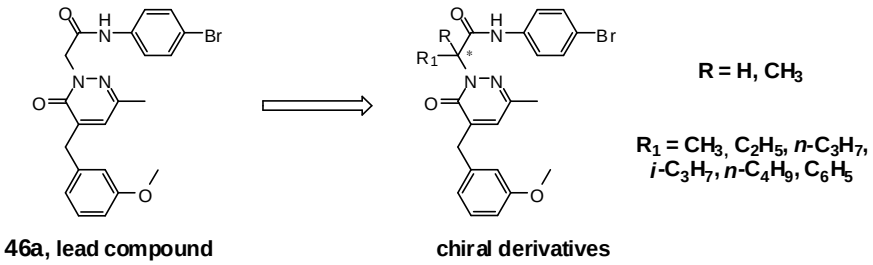


Figure 2.4. Homologue series of chiral derivatives.

During this research, the preliminary biological results prompted us to prepare and test as well the pure enantiomers in order to see if the enantioselectivity could produce an increase of the activity and/or selectivity. Since asymmetric synthesis did not allow us to obtain the pure enantiomers, chiral HPLC purification was performed to obtain both enantiomerically pure compounds. The analytical work was carried out in collaboration with Dr. Bartolucci and co-workers of Pharmaceutical Sciences Department, University of Florence.

In the present thesis are reported the results of these chemical efforts as well as the biological activity of the novel synthesized molecules.

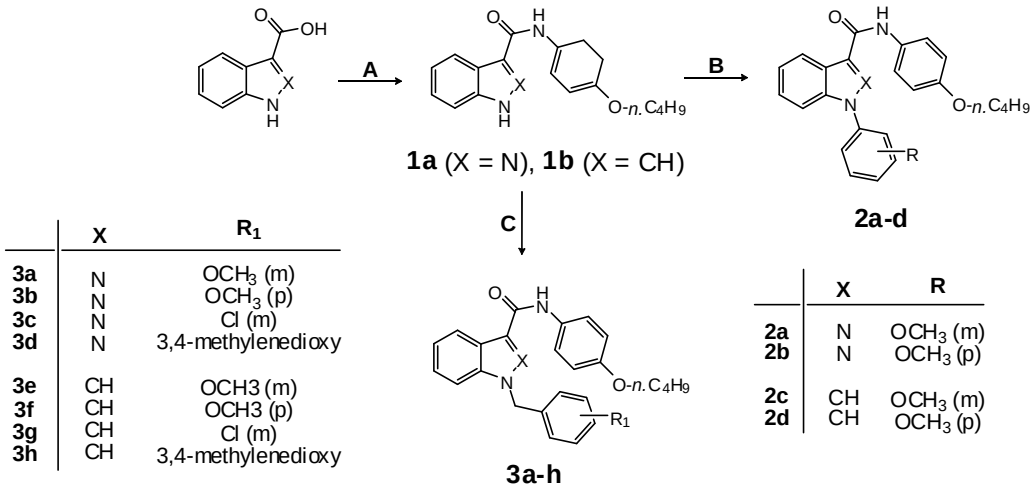
3. CHEMISTRY

Initial investigations in the FPRs agonists field have been focused on the synthesis of small molecules based on different heterocyclic scaffolds, such as indole, indazole, quinoline, naphtyridone, phthalazinone, phthalhydrazide and pyridazinone (**section 3.1**). Using this approach a relevant number of functionalised aryl-substituted heterocycles were prepared in moderate to good yields. Among them a pyridazinone derivative (**46a**, **scheme 12**) was found to be an interesting FPR1/FPR2 mixed agonist (**section 3.2**).[233] Therefore, the next progress of the project, concerned the optimization of the selected lead compound by modifying the position of the substituents on the pyridazinone ring and the nature and the length of the functionalized side chain (**section 3.3**).

3.1 Investigating different heterocyclic scaffolds

3.1.1 Synthesis of indole and indazole derivatives

Indoles and indazoles **2a-d** and **3a-h** can be easily prepared in two steps using respectively the commercially available indole-3-carboxylic acid and indazole-3-carboxylic acid as starting material (**scheme 1**). These compounds were first treated with $SOCl_2$, in presence of Et_3N, to afford the intermediate acid chlorides. Addition of 4-butoxyaniline generated the corresponding amides **1a,b** which in turn were transformed into the final compounds **2a-d**, under coupling conditions with the appropriate phenylboronic acid in presence of $Cu(OAc)_2$. Finally, the target compounds **3a-h** were prepared by classical alkylation with the opportune benzyl halide.



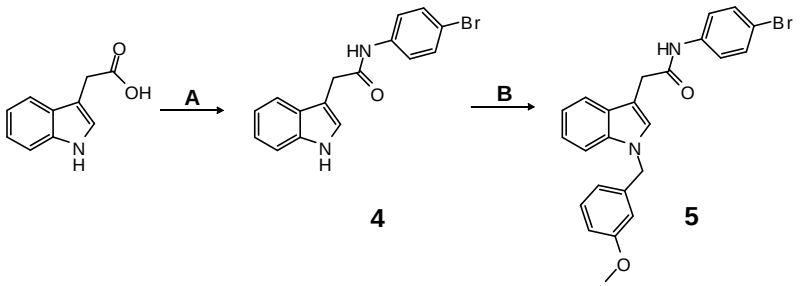
	X	R_1
3a	N	OCH_3 (m)
3b	N	OCH_3 (p)
3c	N	Cl (m)
3d	N	3,4-methylenedioxy
3e	CH	OCH3 (m)
3f	CH	OCH3 (p)
3g	CH	Cl (m)
3h	CH	3,4-methylenedioxy

	X	R
2a	N	OCH_3 (m)
2b	N	OCH_3 (p)
2c	CH	OCH_3 (m)
2d	CH	OCH_3 (p)

Scheme 1. Reagents and conditions: A) $SOCl_2$ (27 equiv), Et_3N (catalytic), 1 h, 60 °C, then 4-*n*.butoxyaniline (2 equiv), anhydrous THF, 12 h, rt; **B)** 3- or 4-methoxy-phenylboronic acid (2 equiv), $Cu(OAc)_2$ (1.5 equiv), Et_3N (2 equiv), CH_2Cl_2, 5-12 h, rt; **C)** substituted benzyl halide (1.1 equiv), K_2CO_3 (2 equiv), anhydrous acetone, 2-10 h, reflux.

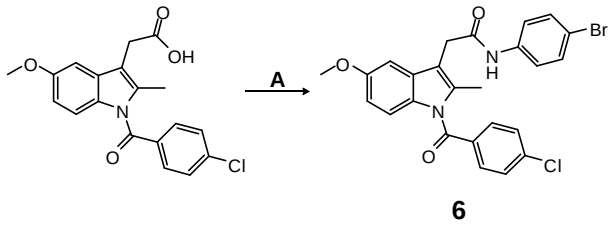
In **schemes 2** and **3** are depicted the synthetic pathways to prepare two "indomethacin-like" analogues. The amide intermediate **4** was obtained by coupling the 3-indoleacetic acid with 4-bromoaniline,[234] both

commercially available, using DCC to activate the carboxylic group. The following alkylation with 3-methoxybenyl chloride gave the final compound **5**.



Scheme 2. Reagents and conditions: A) 4-bromoaniline (1 equiv), DCC (1 equiv), anhydrous CH_2Cl_2, 24 h, rt; **B)** 3-methoxybenzyl chloride (1.5 equiv), K_2CO_3 (2 equiv), anhydrous acetone, 4 h, reflux.

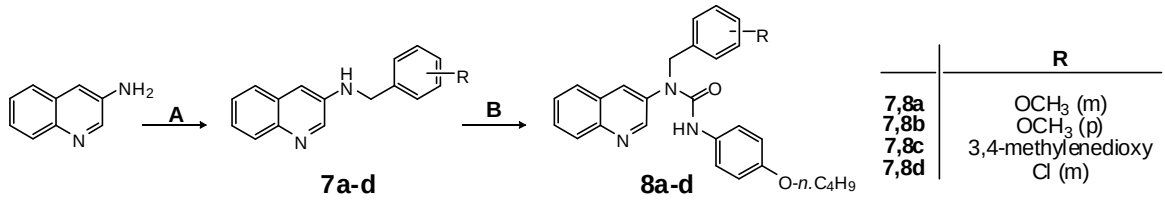
The same coupling reaction with 4-bromoaniline was also carried out on indomethacin to give the final compound **6**.



Scheme 3. Reagents and conditions: A) 4-Bromoaniline (1 equiv), DCC (1 equiv), anhydrous CH_2Cl_2 , 24 h, rt.

3.1.2 Synthesis of quinoline derivatives

Final compounds **8a-d** with quinoline scaffold were prepared in two steps (**scheme 4**): the commercially available 3-aminoquinoline was firstly alkylated with the suitable substituted benzyl halide in standard conditions to give intermediates **7a-d** which, in turn, by treatment with the 4-*n*.butoxyphenyl isocyanate afforded the desired urea derivatives **8a-d**.

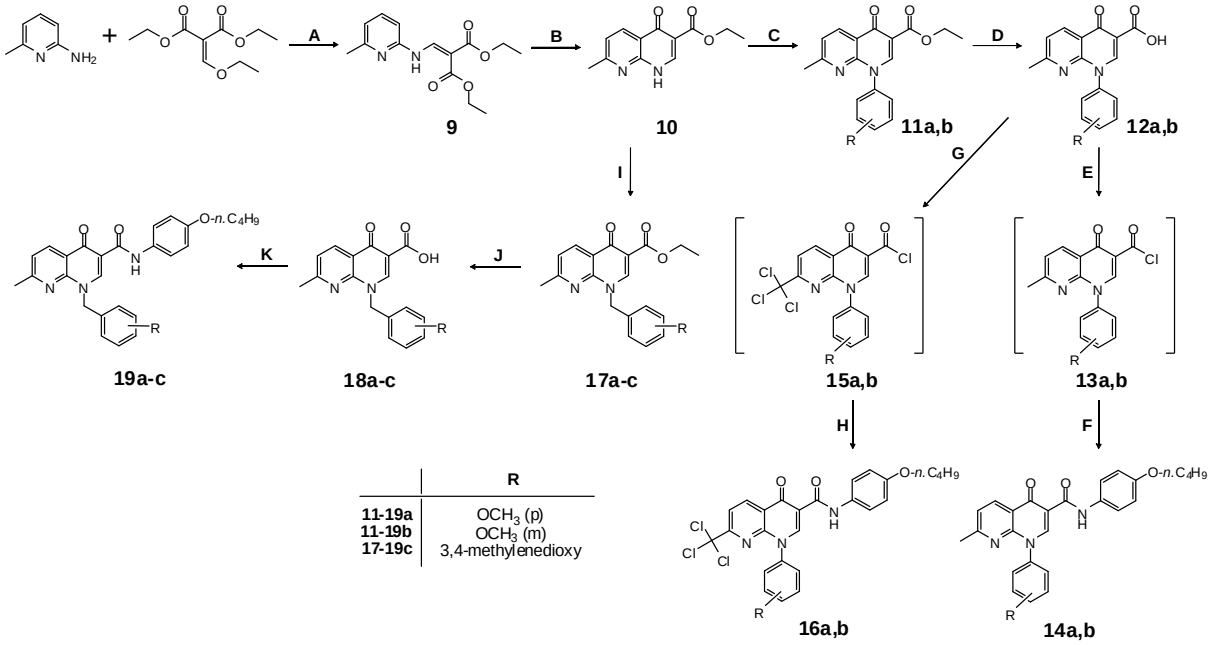


Scheme 4. Reagents and conditions: A) substituted benzyl halide (2 equiv), K_2CO_3 (2 equiv), anhydrous acetone, 3-7 h, 60 °C; **B)** 4-*n*.butoxyphenyl isocyanate (1.1 equiv), anhydrous CH_2Cl_2, 3-8 h, rt.

3.1.3 Synthesis of naphtyridone derivatives

The synthesis of naphtyridone scaffold **10** (**scheme 5**),[235] was performed starting from commercially available 2-amino-6-methylpyridine and diethyl-2-(ethoxymethylene)malonate, followed by thermal ring closure of the diethyl-(6-methyl-2-pyridylaminomethylene)malonate **9**.[236] To prepare the final compounds **14a,b** and **16a,b**, the intermediate **10** was coupled with the appropriate phenylboronic acid in presence of

$Cu(OAc)_2$. An alkaline hydrolysis of the ester group afforded the carboxylic acids **12a,b** which were converted in the acid chlorides using $SOCl_2$. 4-Butoxyaniline was then added to intermediates **13a,b** to give the final amides **14a,b**.

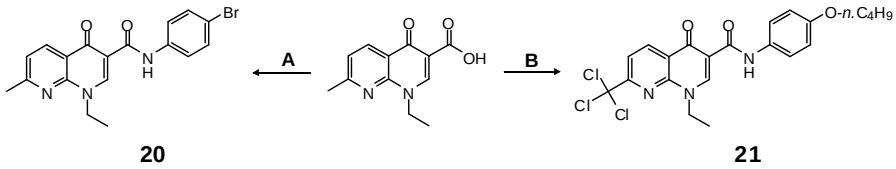


Scheme 5. Reagents and conditions: A) 6 h, 100 °C; **B)** diphenyl ether, 0.5 h, 270 °C; **C)** 3- or 4-methoxyphenylboronic acid (2 equiv), $Cu(OAc)_2$ (1.5 equiv), Et_3N (2 equiv), CH_2Cl_2, 15-20 h, rt; **D)** NaOH 6N, EtOH, 0.5-1 h, rt; **E)** $SOCl_2$ (27 equiv), Et_3N (catalytic), 2-3 h, rt; **F)** 4-*n*.butoxyaniline (2 equiv), anhydrous THF, 2 h, rt; **G)** $SOCl_2$ (27 equiv), Et_3N (catalytic), 2-3 h, 60 °C; **H)** see F; **I)** substituted benzyl halide (1.1 equiv), K_2CO_3 (2 equiv), anhydrous acetone, 5-7 h, 60 °C; **J)** see D; **K)** 4-*n*.butoxyaniline (1 equiv), DCP (4 equiv), Et_3N (catalytic), anhydrous DMF, 15 h, rt.

It is worth noting that treatment of the acids **12a,b** with $SOCl_2$, followed by heating at 60 °C afforded the corresponding trichloromethyl acid chlorides **15a,b**. Indeed, it is well known in the literature that compounds such as 2-methylpyridines,[237] 2-methylquinolines,[238] 2-methyl-4(1*H*)-quinolones,[239] react with different chlorinating agents to give the corresponding trichloromethyl derivatives.[240] In our case, **15a,b**, were transformed into the final **16a,b** (**scheme 5**) in moderate yields, monitoring the reactions by TLC, and the products were characterized by ^1H NMR and MS spectroscopy. The synthesis of the final compounds **19a-c** was performed in standard condition starting from the intermediate **10**, through alkylation and alkaline hydrolysis, to give the carboxylic acids **18a-c**. In the last step **18a-c** were treated with the appropriate amine at room temperature in anhydrous DMF, using a catalytic amount of Et_3N and ***diethyl cyanophosphonate (DCP)*** to activate the carboxylic group (**scheme 5**).

In **scheme 6** it is showed the synthetic pathway to prepare two nalidixic acid derivatives. The treatment of nalidixic acid with ethyl chloroformate, in THF and in presence of triethylamine, afforded the intermediate mixed anhydride, which was transformed into the final amide **20**. On the contrary,

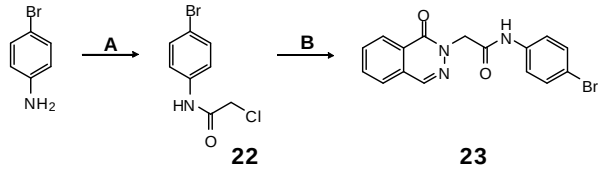
compound **21** was obtained using the same reaction sequence performed for the trichloromethyl analogues **16a,b** (**scheme 5**).



Scheme 6. Reagents and conditions: A) ethyl chloroformate (1.1 equiv), Et_3N (3.5 equiv), 4-bromoaniline (2 equiv), anhydrous THF, 12 h, -5 °C → rt; **B)** $SOCl_2$ (27 equiv), Et_3N (catalytic), 2 h, 60 °C, then 4-*n*.butoxyaniline (2 equiv), anhydrous THF, 2 h, rt.

3.1.4 Synthesis of phthalazinone derivative

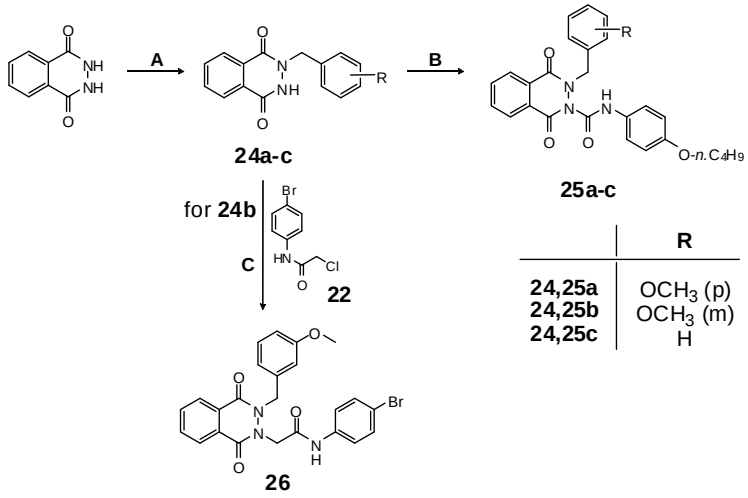
The synthetic route employed to obtain the phthalazinone derivative **23** is depicted in **scheme 7**. The 4-bromoaniline was converted in good yield to the known phenylamide intermediate **22** following a procedure reported in literature.[241] Alkylation in standard conditions of the phthalazin-1*(2H)*-one with compound **22** gave rise the final compound **23**.



Scheme 7. Reagents and conditions: A) c***hloroacetyl chloride*** (1.2 equiv), K_2CO_3 (1.2 equiv), anhydrous CH_2Cl_2, 2 h, reflux; **B)** 1-(2*H*)-**p*hthalazinone*** (0.9 equiv), K_2CO_3 (1.8 equiv), anhydrous CH_3CN, 3 h, reflux.

3.1.5 Synthesis of phthalhydrazide derivatives

Scheme 8 outlines the synthetic procedure performed to get the final compounds **25a-c** and **26**.

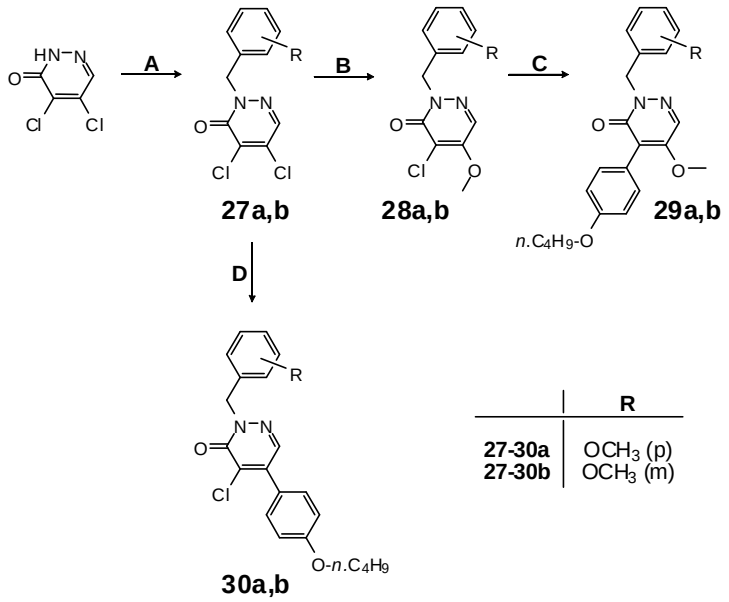


Scheme 8. Reagents and conditions: A) substituted benzyl halide (1.1 equiv), K_2CO_3 (2 equiv), anhydrous DMF, 2-4 h, 80 °C; **B)** 4-*n*.butoxyphenyl isocyanate (2 equiv), anhydrous CH_2Cl_2, 14 h, 0 °C → rt; **C) 22** (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 3 h, reflux.

The first step is the alkylation in standard conditions of the commercially available 2,3-diidrophthalazin-1,4-dione with the suitable benzyl halide. For this reaction, anhydrous DMF gave the best results in terms of mono- and bi-alkylated ratio, although traces of product alkylated at both nitrogens were recovered in all reactions, as showed by chromatographic and ^{1}H NMR analysis. The previously described intermediate **24c**,[242] and the new **24a,b** were converted in the final urea derivatives **25a-c** using 4-*n*-butoxyphenyl isocyanate in anhydrous CH_2Cl_2. The final compound **26** was instead synthesized through the further alkylation of compound **24b**, using the previously described intermediate **22** (**scheme 7**).

3.1.6 Synthesis of *N*-2-benzyl pyridazinone derivatives

In **scheme 9** is depicted the synthesis of final compounds **29a,b** and **30a,b** starting from the cheap and commercially available ***4,5-dichloro***-3*(2H)*-***pyridazinone. Following a procedure of*** **N-*benzylation at 2-position of the pyridazinone ring reported in the literature,*[243]** **N**-2-benzyl derivatives **27a,b** were prepared in good yields by treatment with 3- or 4-methoxybenzyl bromide in anhydrous acetonitrile in the presence of potassium carbonate and tetrabutylammonium bromide.[244] To synthesize final compounds **30a,b** starting from the ***4,5-dichloro***-3(2*H*)-***pyridazinone***, it was not possible to obtain selective monoarylation under Suzuky conditions. Interestingly, selective C-5 coupling with 4-buthoxyphenyloronic acid was achieved using trans-dichlorobis(triethylphosphine)palladium (II) as catalist and 1M Na_2CO_3 as base in DMF at room temperature, as reported in the literature.[245] An important strategy to achieve high mono- (versus di-) substitution is the use of a two fold excess of pyridazin-3(2*H*)-one versus boronic acid.

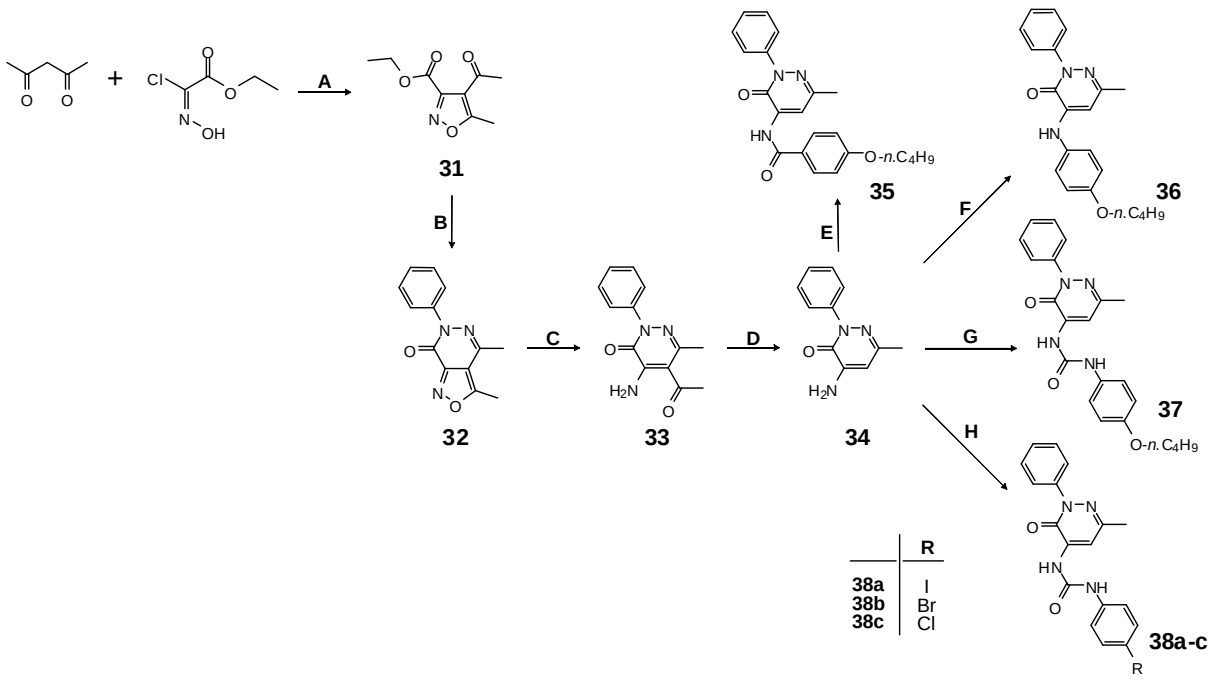


Scheme 9. Reagents and conditions: A) 3 or 4-methoxybenzyl chloride (1.5 equiv), K_2CO_3 (2 equiv), Bu_4NBr (0.1 equiv), anhydrous CH_3CN, 5-7 h, reflux; **B)** $NaOCH_3$ (2 equiv), anhydrous CH_3OH, 1 h, rt; **C)** 4-*n*.butoxyphenylboronic acid (3 equiv), $Pd(PPh_3)_4$ (0.03 equiv), Na_2CO_3 2M in H_2O (1 equiv), toluene, 5-8 h, reflux; **D)** 4-*n*.butoxyphenylboronic acid (0.5 equiv), $PdCl_2[(C_2H_5)_3P]_2$ (0.1 equiv), Na_2CO_3 1M in H_2O (1 equiv), DMF, 6-12 h, rt.

The final compounds **29a,b** were instead obtained in two steps starting from **27a,b** (**scheme 9**) where the first nucleophilic reaction involved a selective displacement of the chlorine at C-5 of the pyridazinone ring,[246] using sodium methoxide in anhydrous methanol; the second step was an oxidative addition to tetrakis(triphenylphosphine)palladium(0) catalist to give, in good yields, the final 4-arylated-5-methoxypyridazinones using the 4-butoxyphenylboronic acid under classical Suzuki conditions.[247]

3.1.7 Synthesis of *N*-2-phenyl pyridazinone derivatives[233]

The synthetic pathway affording the final compounds **35-37** and **38a-c** is depicted in **scheme 10**. The isoxazole **31**, the isoxazolo[3,4-*d*]-pyridazinone **32** and the pyridazinones **33,34** were synthesized following the procedures previously described.[248-251] Subsequently, the amide **35** was obtained from the primary amine **34** by treatment with the opportune aroylchloride in toluene at reflux, while coupling of **34** with 4-butoxyphenylboronic acid in CH_2Cl_2 in presence of $Cu(Ac)_2$ gave derivative **36**.



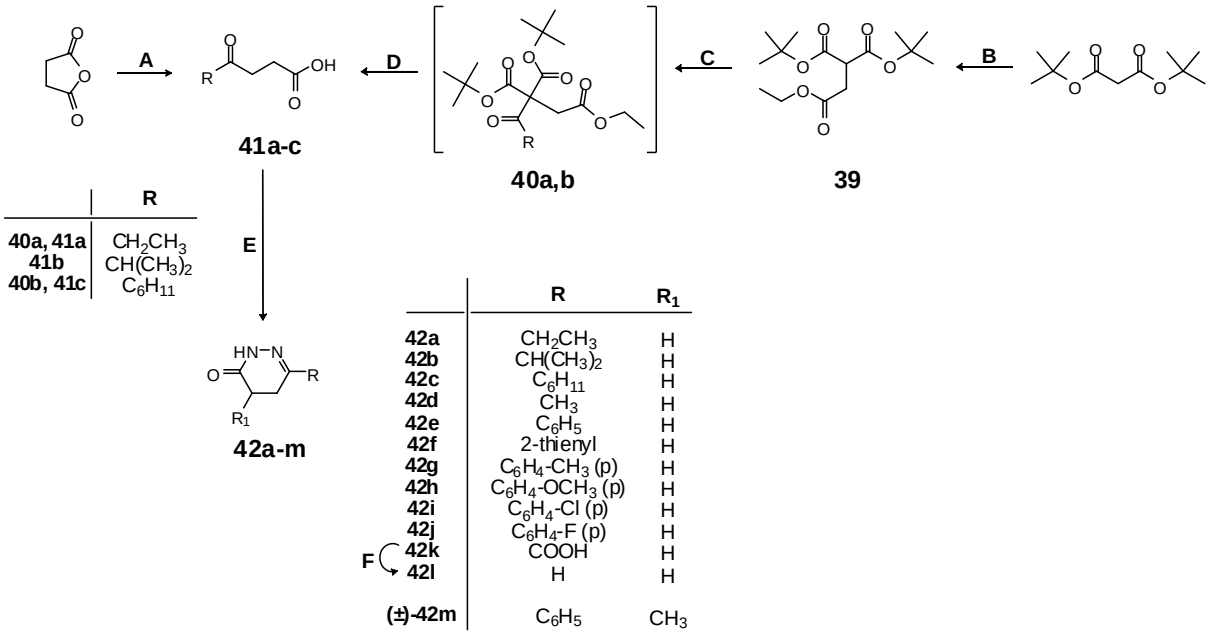
Scheme 10. Reagents and conditions: A) NaOEt (1 equiv), anhydrous EtOH, 2 h, -5 °C → rt; **B)** NH_2-NH-C_6H_5 (2 equiv), PPA, anhydrous EtOH, 3.5 h, reflux; **C)** $HCOONH_4$ (2.5 equiv), Pd/C 10% (catalytic), anhydrous EtOH, 1.5 h, reflux; **D)** HBr 48%, 1 h, 140 °C; **E)** 4-*n*.butoxybenzoyl chloride (0.9 equiv), Et3N (4.1 equiv), anhydrous toluene, 4 h, reflux; **F)** 4-*n*.butoxyphenylboronic acid (2 equiv), $Cu(OAc)_2$ (1.5 equiv), Et_3N (2 equiv), CH_2Cl_2, 12 h, rt; **G)** NaOAc (2.4 equiv), $CO(OCCl_3)_2$ (3.5 equiv), anhydrous THF, 2 h, reflux, then 4-*n*.butoxyaniline (2.4 equiv), 12 h, rt; **H)** substituted aryl isocyanate (1.1 equiv), anhydrous toluene, 4-7 h, reflux.

Compound **34** in anhydrous toluene was refluxed with the appropriate aryl isocyanate to afford the urea derivatives **38a-c**. The 4-butoxy analogue **37** was synthesized with an alternative procedure,[2] starting from the same precursor **34** and using triphosgene in anhydrous THF, followed by treatment with the appropriate aniline.

3.2 6-Methyl-2,4-Disubstituted Pyridazin-3*(2H)*-ones: synthesis of the lead compound 46a[233]

3.2.1 Synthesis of dihydropyridazinone scaffolds

The dihydropyridazinones **42a-m** (**scheme 11**) are the key building blocks for the synthesis of the pyridazinone class of compounds examined in the **sections 3.2** and **3.3**. As extensively reported in the literature, the γ-keto acids are the common starting material to easily achieve them.[252-254] Some of the γ-keto acids useful for the synthesis of the dihydropyridazinone scaffold are not commercially available. Thus, the first goal for this second part of the project was the development of an efficient synthetic route to obtain this starting material.



Scheme 11. Reagents and conditions: A) $Fe(acac)_3$ (3 equiv), *i*PrMgCl (for compound **41b**) 2M in diethyl ether (3 equiv), anhydrous THF, 12 h, rt; **B)** NaH 60% in mineral oil (1.3 equiv), ethyl bromoacetate (1 equiv), anhydrous THF, 15 h, rt; **C)** propionyl chloride (for compound **41a**) or cyclohexylcarbonyl chloride (for compound **41c**) (1 equiv), NaH 60% in mineral oil (1.05 equiv), anhydrous THF, 3 h, rt; **D)** *p*-toluenesulfonic acid • H_2O (0.1 equiv), anhydrous toluene, 2.5 h, reflux, then EtOH, NaOH 1N, 4 h, rt; **E)** NH_2NH_2 • H_2O (1 equiv), EtOH, 1-3 h, 60 °C; **F)** 0.5 h, 160 °C.

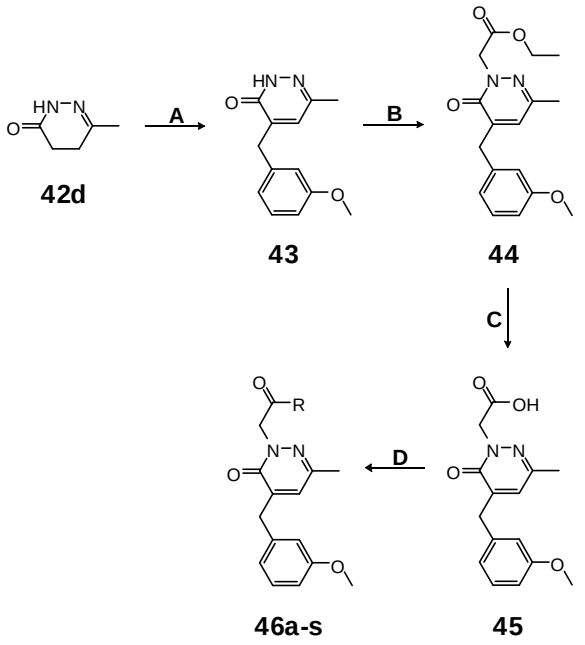
The synthesis of the γ-keto acid **41b** was achieved following a procedure reported in the literature (**A** in **scheme 11**) starting from the cheap and commercially available succinic anhydride which was reacted with isopropyl magnesium chloride in anhydrous THF at 0 °C in presence of iron(III) acetylacetonate.[255] Since the obtained yields were lower compared to that reported in literature, to prepare quickly and efficiently the key starting materials another route was explored, in which the di-tert-butyl malonate was firstly alkylated with ethyl bromoacetate in the presence of NaH and the following acylation of **39** with the appropriate acid chlorides gave intermediates **40a,b**. These intermediates were not isolated and were directly decarboxylated in presence of *p*-toluenesulfonic acid as catalist. Hydrolysis of the ethyl esters under basic conditions afforded the previously described γ-keto acids **41a,c** in good yields.[256-257] This

route resulted amenable for the synthesis of a large number of γ-keto acid analogues, using a wide range of cheap and commercially available acid chlorides.

The following condensation of both commercially available and synthesized γ-keto acids with ***hydrazine hydrate*** afforded the previously described C-6 substituted dihydropyridazinones **42a-i,k,m**,[252,258-264] and the new **42j** in good yields. A further synthetic step was required to obtain the compound **42l** generated by melting and spontaneous decarboxylation of the carboxylic acid **42k** at 160° C.[265]

3.2.2 Synthesis of substituted *N*-arylacetamide pyridazinones[233]

In **scheme 12** is depicted the synthesis of compounds **46a-s**. The dihydropyridazinone **42d** was firstly converted into the 4-benzyl derivative **43** by Knoevenagel condensation using the appropriate aromatic aldehyde in the presence of KOH and then alkylated with ethyl bromoacetate to give **44**. Alkaline hydrolysis of the ester afforded the carboxylic acid **45**, which is the key intermediate for the synthesis of a range of substituted N-phenylacetamide pyridazinones. Indeed treatment with ethyl chloroformate in THF in presence of triethylamine, afforded the intermediate mixed anhydrous, which was transformed in good yields into the final amides by treatment with the appropriate aryl (**46a-r**) or cycloalkyl amine (**46s**).



	R
46a	NH-C_6H_4-Br (p)
46b	NH-C_6H_4-Br (m)
46c	NH-C_6H_4-Br (o)
46d	NH-C_6H_4-F (p)
46e	NH-C_6H_4-Cl (p)
46f	NH-C_6H_4-I (p)
46g	NH-C_6H_5
46h	NH-C_6H_4-CH_3 (p)
46i	NH-C_6H_4-$C(CH_3)_3$ (p)
46j	NH-C_6H_4-OCH_3 (p)
46k	NH-C_6H_4-O-*n*.C_4H_9 (p)
46l	NH-C_6H_3-$(OCH_3)_2$ (m, p)
46m	NH-C_6H_3-3,4-methylenedioxy
46n	NH-C_6H_4-CF_3 (p)
46o	NH-C_6H_4-OCF_3 (p)
46p	NH-C_6H_4-SCH_3 (p)
46q	NH-C_6H_4-CN (p)
46r	NH-C_6H_4-NO_2 (p)
46s	1-methylpiperazine

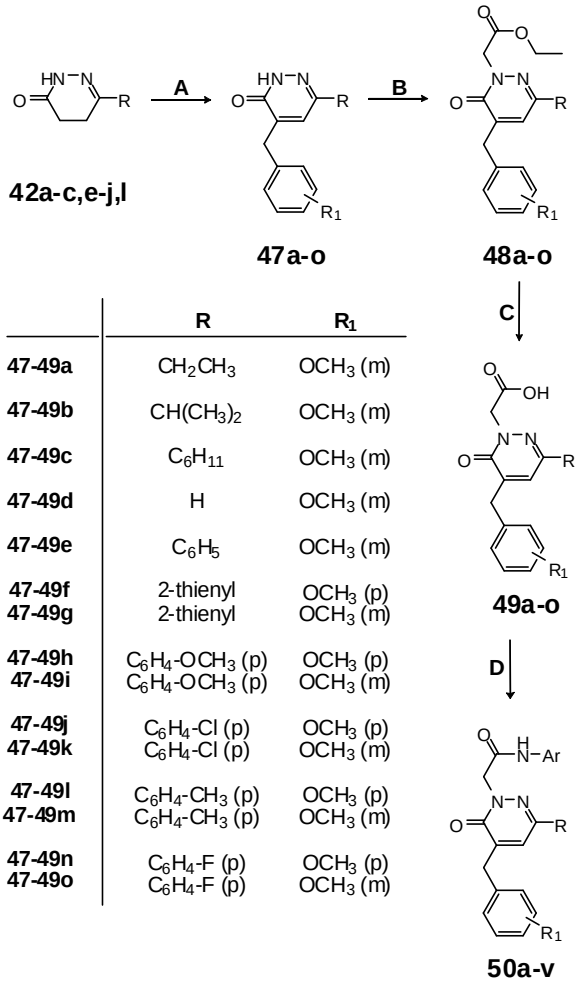
Scheme 12. Reagents and conditions: A) 3-methoxybenzaldehyde (1 equiv), KOH 5% (w/v) in anhydrous EtOH, 3 h, reflux; **B)** ethyl bromoacetate (3 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 6 h, reflux; **C)** NaOH 6N, EtOH, 1 h, reflux; **D)** ethyl chloroformate (1.1 equiv), Et_3N (3.5 equiv), substituted aryl(cycloalkyl)-amine (2 equiv), anhydrous THF, 12 h, -5 °C → rt.

3.3 Optimization of the lead: 2,3,4,5,6-substituted pyridazinones

3.3.1 Synthesis of C-6 modified *N*-arylacetamide pyridazinones

The C-6 modified pyridazinones **50a-v** (**scheme 13**) were prepared in four steps starting from the dihydropyridazinones **42a-c,e-j,l** synthesized as shown in **scheme 11** and using the same procedure

already described to get the final compounds **46a-s** (**scheme 12**). Through Knoevenagel condensation with the commercially available 3- or 4-methoxybenzaldehyde, the previously described derivatives **47h,j,l**,[266] and the new **47a-g,i,k,m-o** were obtained in good yields. The following alkylation and alkaline hydrolysis were performed in standard conditions. The reaction of the carboxylic acids **49a-o** with ethyl chloroformate, to give the anhydride intermediate, and then with the appropriate aryl amines, furnished the final compounds **50a-v**.



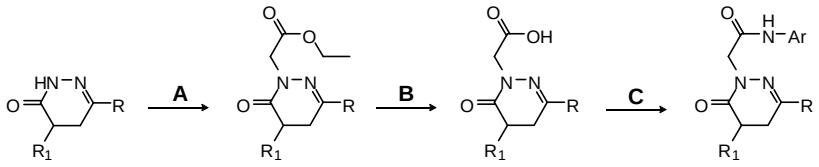
	R	R_1
47-49a	CH_2CH_3	OCH_3 (m)
47-49b	$CH(CH_3)_2$	OCH_3 (m)
47-49c	C_6H_{11}	OCH_3 (m)
47-49d	H	OCH_3 (m)
47-49e	C_6H_5	OCH_3 (m)
47-49f	2-thienyl	OCH_3 (p)
47-49g	2-thienyl	OCH_3 (m)
47-49h	C_6H_4-OCH_3 (p)	OCH_3 (p)
47-49i	C_6H_4-OCH_3 (p)	OCH_3 (m)
47-49j	C_6H_4-Cl (p)	OCH_3 (p)
47-49k	C_6H_4-Cl (p)	OCH_3 (m)
47-49l	C_6H_4-CH_3 (p)	OCH_3 (p)
47-49m	C_6H_4-CH_3 (p)	OCH_3 (m)
47-49n	C_6H_4-F (p)	OCH_3 (p)
47-49o	C_6H_4-F (p)	OCH_3 (m)

	R	R_1	Ar
50a	CH_2CH_3	OCH_3 (m)	C_6H_4-I (p)
50b	$CH(CH_3)_2$	OCH_3 (m)	C_6H_4-Br (p)
50c	C_6H_{11}	OCH_3 (m)	C_6H_4-Br (p)
50d	H	OCH_3 (m)	C_6H_4-Br (p)
50e	C_6H_5	OCH_3 (m)	C_6H_4-Br (p)
50f	C_6H_5	OCH_3 (m)	C_6H_3-3,4-methylenedioxy
50g	C_6H_5	OCH_3 (m)	C_6H_4-F (p)
50h	2-thienyl	OCH_3 (p)	C_6H_4-Br (p)
50i	2-thienyl	OCH_3 (m)	C_6H_3-3,4-methylenedioxy
50j	2-thienyl	OCH_3 (m)	C_6H_4-F (p)
50k	C_6H_4-OCH_3 (p)	OCH_3 (p)	C_6H_4-Br (p)
50l	C_6H_4-OCH_3 (p)	OCH_3 (m)	C_6H_3-3,4-methylenedioxy
50m	C_6H_4-OCH_3 (p)	OCH_3 (m)	C_6H_4-F (p)
50n	C_6H_4-Cl (p)	OCH_3 (p)	C_6H_4-Br (p)
50o	C_6H_4-Cl (p)	OCH_3 (m)	C_6H_3-3,4-methylenedioxy
50p	C_6H_4-Cl (p)	OCH_3 (m)	C_6H_4-F (p)
50q	C_6H_4-CH_3 (p)	OCH_3 (p)	C_6H_4-Br (p)
50r	C_6H_4-CH_3 (p)	OCH_3 (m)	C_6H_3-3,4-methylenedioxy
50s	C_6H_4-CH_3 (p)	OCH_3 (m)	C_6H_4-F (p)
50t	C_6H_4-F (p)	OCH_3 (p)	C_6H_4-Br (p)
50u	C_6H_4-F (p)	OCH_3 (m)	C_6H_3-3,4-methylenedioxy
50v	C_6H_4-F (p)	OCH_3 (m)	C_6H_4-F (p)

Scheme 13. Reagents and conditions: A) 3 or 4-methoxybenzaldehyde (1 equiv), KOH 5% (w/v) in anhydrous EtOH, 1-3 h, reflux; **B)** ethyl bromoacetate (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 1-3 h, reflux; **C)** NaOH 6N, 1-2 h, 60-80 °C; **D)** ethyl chloroformate (1.1 equiv), Et_3N (3.5 equiv), substituted aniline (2 equiv), anhydrous THF, 12 h, -5 °C → rt.

3.3.2 Synthesis of C-4 modified *N*-arylacetamide pyridazinones

The C-4 modified dihydropyridazinones **53a-d** (**scheme 14**) were obtained in good yields carrying out the same reaction sequence (alkylation/hydrolysis/amide bond formation) shown in **schemes 12** and **13** starting from compounds **42d-m** whose synthesis is reported in **scheme 11**.

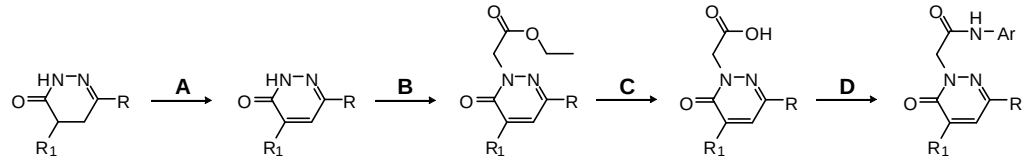


	R	R_1
42d and 51,52a	CH_3	H
(±)-42m and (±)-51,52b	C_6H_5	CH_3

	R	R_1	Ar
53a	CH_3	H	C_6H_4-I (p)
(±)-53b	C_6H_5	CH_3	C_6H_4-Br (p)
(±)-53c	C_6H_5	CH_3	C_6H_4-F (p)
(±)-53d	C_6H_5	CH_3	C_6H_3-3,4-methylenedioxy

Scheme 14. Reagents and conditions: A) ethyl bromoacetate (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 2-3 h, reflux; **B)** NaOH 6N, 3-5 h, 80 °C; **C)** ethyl chloroformate (1.1 equiv), Et_3N (3.5 equiv), substituted aniline (2 equiv), anhydrous THF, 12 h, -5 °C → rt.

In **scheme 15** is depicted the synthesis of compounds **57a-f**, where the starting dihydropyridazinines **42c,d,(±)-m** (**scheme 11**) were, as first step, converted in the previously described pyridazinones **54a-c**. The reaction was carried out using selenium dioxide or bromine in acetic acid as oxidizing agents.[263,267,268] Then, the alkylation in standard condition with ethyl bromoacetate afforded the known **55c**,[269] and the new **55a,b** which were subjected to the usual hydrolysis and amide bond formation steps, performed as already described (**schemes 12,13**).



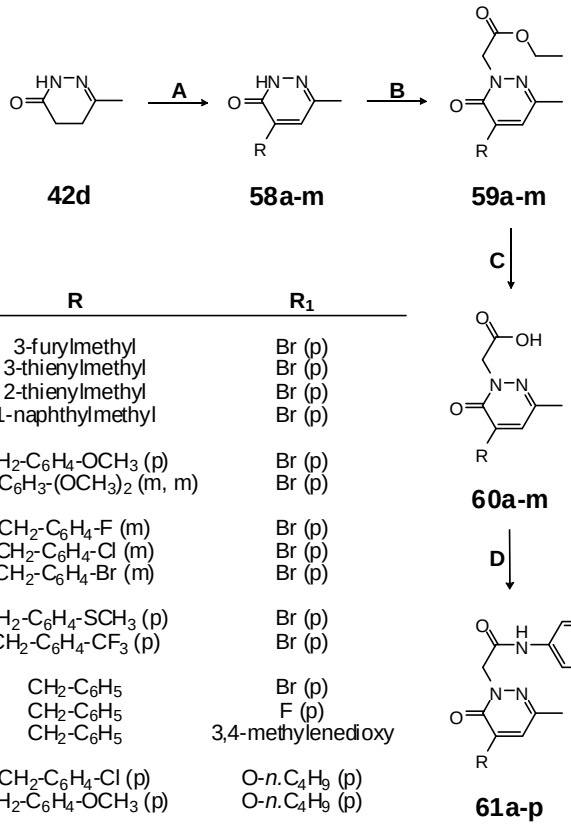
	R	R_1
42c and 54-56a	C_6H_{11}	H
42d and 54-56b	CH_3	H
(±)-42m and 54-56c	C_6H_5	CH_3

	R	R_1	Ar
57a	C_6H_{11}	H	C_6H_4-F (p)
57b	C_6H_{11}	H	C_6H_3-3,4-methylenedioxy
57c	CH_3	H	C_6H_4-I (p)
57d	C_6H_5	CH_3	C_6H_4-Br (p)
57e	C_6H_5	CH_3	C_6H_4-F (p)
57f	C_6H_5	CH_3	C_6H_3-3,4-methylenedioxy

Scheme 15. Reagents and conditions: A) for compound **54c**: Br_2 (4 equiv), CH_3COOH 100%, 4-5 h, reflux; for compunds **54a,b**: SeO_2 (3 equiv), anhydrous EtOH, 5-7 h, reflux; **B)** ethyl bromoacetate (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 2-3 h, reflux; **C)** NaOH 6N, 2 h, 80 °C; **D)** ethyl chloroformate (1.1 equiv), Et_3N (3.5 equiv), substituted aniline (2 equiv), anhydrous THF, 12 h, -5 °C → rt.

The synthesis of final compounds **61a-p**, where the 4-methoxybenzyl at the C-4 position of the pyridazinone ring was replaced with several heterocyclic or substituted benzyl moieties, is shown in **scheme 16**. The dihydropyridazinone **42d** (**scheme 11**) was converted into the previously described derivatives **58b-e,i**,[270-273] and the new **58a,f-h,j-m** by Knoevenagel condensation with the appropriate

aromatic aldehyde in the presence of KOH. Compounds **58a-m**, in turn, were alkylated with ethyl bromoacetate to give the esters **59a-m**, whose **59d,e,i** were previously reported.[271,272,274] Alkaline hydrolysis of compounds **59a-m** gave the known **60i**,[270] and the new carboxylic acid derivatives **60a-h,j-m**. These compounds were treated with ethyl chloroformate in THF in presence of triethylamine, affording the mixed anhydrides, which in turn were transformed into the final amides **61a-p** by treatment with the appropriate aryl amine.

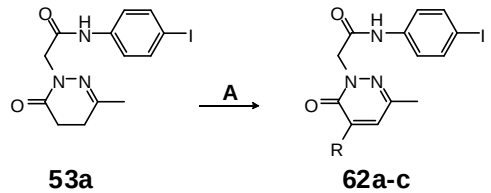


	R	R_1
61a	3-furylmethyl	Br (p)
61b	3-thienylmethyl	Br (p)
61c	2-thienylmethyl	Br (p)
61d	1-naphthylmethyl	Br (p)
61e	CH_2-C_6H_4-OCH_3 (p)	Br (p)
61f	CH_2-C_6H_3-$(OCH_3)_2$ (m, m)	Br (p)
61g	CH_2-C_6H_4-F (m)	Br (p)
61h	CH_2-C_6H_4-Cl (m)	Br (p)
61i	CH_2-C_6H_4-Br (m)	Br (p)
61j	CH_2-C_6H_4-SCH_3 (p)	Br (p)
61k	CH_2-C_6H_4-CF_3 (p)	Br (p)
61l	CH_2-C_6H_5	Br (p)
61m	CH_2-C_6H_5	F (p)
61n	CH_2-C_6H_5	3,4-methylenedioxy
61o	CH_2-C_6H_4-Cl (p)	O-*n*.C_4H_9 (p)
61p	CH_2-C_6H_4-OCH_3 (p)	O-*n*.C_4H_9 (p)

	R
58-60a	3-furylmethyl
58-60b	3-thienylmethyl
58-60c	2-thienylmethyl
58-60d	1-naphthylmethyl
58-60e	CH_2-C_6H_4-OCH_3 (p)
58-60f	CH_2-C_6H_3-$(OCH_3)_2$ (m, m)
58-60g	CH_2-C_6H_4-F (m)
58-60h	CH_2-C_6H_4-Cl (m)
58-60i	CH_2-C_6H_4-Cl (p)
58-60j	CH_2-C_6H_4-Br (m)
58-60k	CH_2-C_6H_4-SCH_3 (p)
58-60l	CH_2-C_6H_4-CF_3 (p)
58-60m	CH_2-C_6H_5

Scheme 16. Reagents and conditions: A) substituted benzaldehyde (1 equiv), KOH 5% (w/v) in anhydrous EtOH, 3-5 h, reflux; **B)** ethyl bromoacetate (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 2-4 h, reflux; **C)** NaOH 6N, 1-2 h, 80 °C; **D)** ethyl chloroformate (1.1 equiv), Et_3N (3.5 equiv), substituted aniline (2 equiv), anhydrous THF, 12 h, -5 °C → rt.

In **scheme 17** is shown the synthesis of final compounds **62a-c** modified at the C-4 of the pyridazinone ring and bearing a 4-iodophenylacetamide chain at position 2. To get this compounds, the Koevenagel condensation with the appropriate aromatic aldehyde was performed on the intermediate **53a** (**scheme 14**).

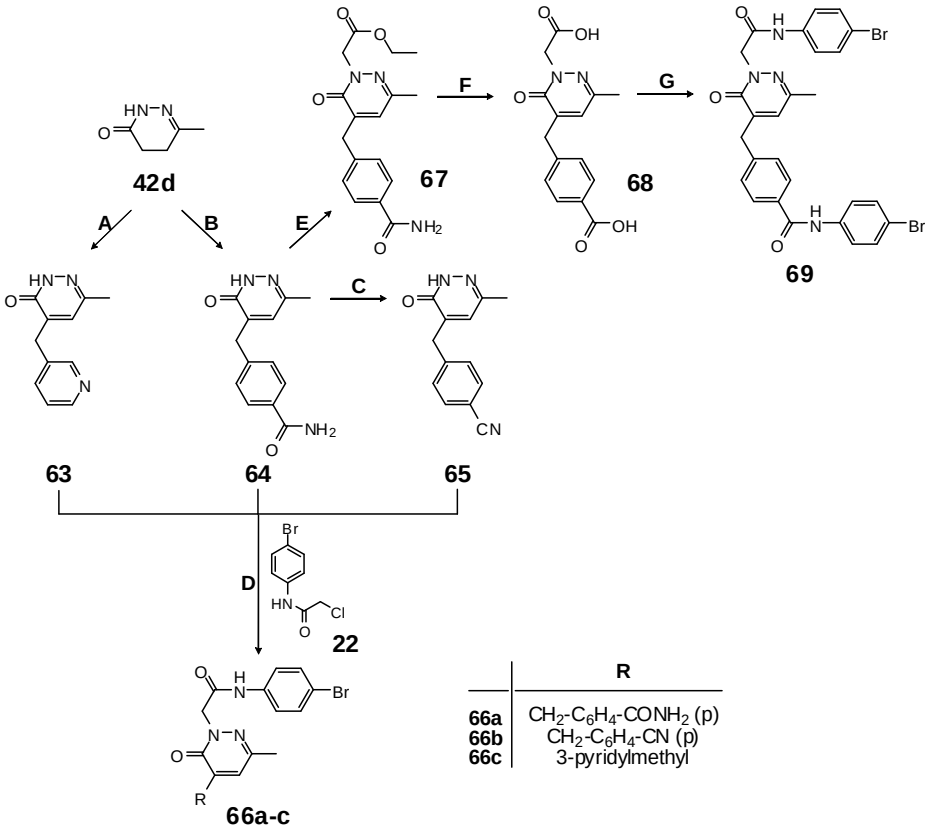


	R
62a	CH_2-C_6H_4-SCH_3 (p)
62b	CH_2-C_6H_3-$(OCH_3)_2$ (m, m)
62c	CH_2-C_6H_4-Cl (m)

Scheme 17. Reagents and conditions: A) substituted benzaldehyde (1 equiv), KOH 5% (w/v) in anhydrous EtOH, 1-5 h, reflux.

This alternative methodology was chosen over the previous synthetic pathway (**scheme12**) due to the exciting possibility to synthesize the suitable building block (**53a**, **scheme 14**) and then to condensate it with the convenient aromatic aldehyde. Despite the fact that Knoevenagel condensation appeared relatively simple and high-yielding on the C-4 of the pyridazinone ring (**schemes 12** and **13**), the *N*-(phenylacetamido)pyridazinone **53a** didn't result suitable for the synthesis of a various range of compounds, probably due to the instability of the phenylacetamido moiety. Indeed, in this conditions, a mixture of products resulted and just three aromatic aldehydes were able to react in low to moderate yields. For these reasons the alternative route was not efficient for the preparation of analogues as we supposed.

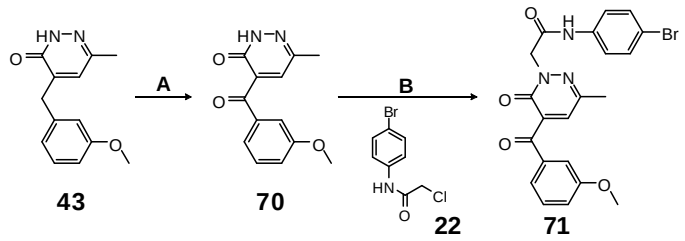
Moreover, a further procedure was required to get the final compounds **66a-c** (**scheme 18**). Intermediates **63** and **64** were obtained by Knovenagel condensation in the same conditions already described (**scheme 12**), using the commercially available 3-pyridinecarboxaldehyde and 4-cyanobenzaldehyde respectively. Compound **65** was instead prepared from **64** by dehydration of the amide with phosphorus oxychloride. Due to their instability to strong base treatment, the intermediates **63-65** were directly converted in the final compounds **66a-c** by alkylation in standard condition with compound **22** (**scheme 7**).



Scheme 18. Reagents and conditions: A) 3-pyridinecarboxaldehyde (1 equiv), KOH 5% (w/v) in anhydrous EtOH, 5 h, reflux; **B)** 4-cyanobenzaldehyde (2 equiv), KOH 5% (w/v) in anhydrous EtOH, 4 h, reflux; **C)** $POCl_3$, 3 h, 60 °C; **D) 22** (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 2-3 h, reflux; **E)** ethyl bromoacetate (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 2 h, reflux; **F)** NaOH 6N, 2 h, 60 °C; **G)** ethyl chloroformate (1.1 equiv), Et_3N (3.5 equiv), 4-bromoaniline (2 equiv), anhydrous THF, 12 h, -5 °C → rt.

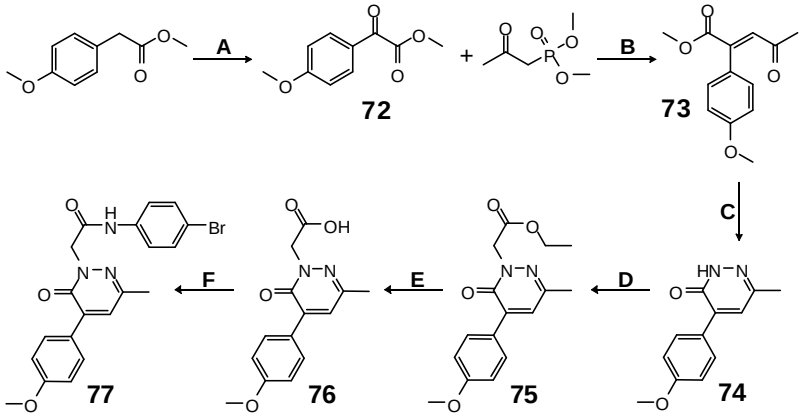
To get the pyridazinone **69** (**scheme 18**), the intermediate **64** was alkylated with ethyl bromoacetate to give **67** which, after alkaline hydrolysis of the ester moiety afforded the bicarboxylic acid **68**. Treating **68** firslty with ethyl chloroformate and then with 4-bromoaniline, in the usual conditions (**scheme 12**), it was possible to obtain in good yields compound **69**, bearing two amide functions.

In **scheme 19** is depicted the synthesis of the 4-arylketone derivative **71** prepared from **44** (**scheme 12**) by oxidation of the benzylic methylene (**70**) with cerium ammonium nitrate (CAN) and subsequent alkylation in standard conditions with the previously described intermediate **22** (**scheme 7**).



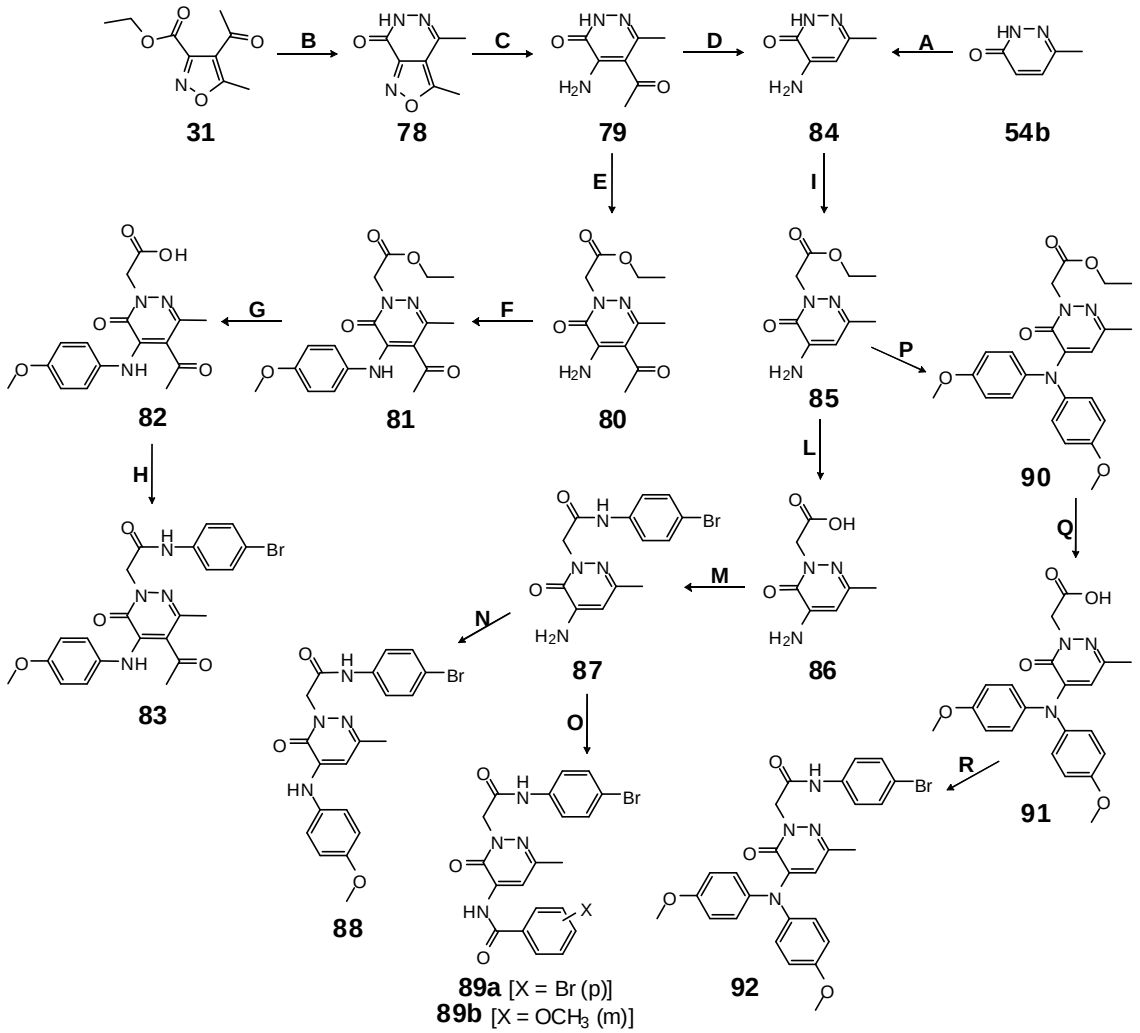
Scheme 19. Reagents and conditions: A) CAN (3 equiv), CH_3COOH 50%, 1.5 h, 60 °C; **B) 22** (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 6 h, reflux.

The synthesis of compound **77** whit the 4-methoxyphenyl group directly bonded to the C-4 of the pyridazinone ring is shown in **scheme 20**. As reported in literature,[275] the commercially available methyl 4-methoxyphenylacetate can be easily converted in the previously described keto ester **72**,[276] by oxidation with tert-butyl hydroperoxide (TBHP) and a catalytic amount of iron trichloride in pyridine at 85 °C. The intermediate **72** was then processed to Wittig reaction,[277,278] using dimethyl acetylmethylphosphonate as phosphonium ylide source and sodium methoxyde in methanol at 0 °C, to afford the known γ-keto ester **73**.[279] Condensation with hydrazine hydrate afforded the intermediate **74**, which as usual, was subjected to alkylation with ethyl bromoacetate, alkaline hydrolysis and finally coupling with 4-bromoaniline, through the intermediate mixed anhydride to give the final amide **77** in good yield.



Scheme 20. Reagents and conditions: A) TBHP (3 equiv), $FeCl_3 \cdot 6\ H_2O$, pyridine, 8 h, 85 °C; **B)** $NaOCH_3$ (2 equiv), anhydrous CH_3OH, 3.5 h, 0 °C → rt; **C)** $NH_2NH_2 \cdot H_2O$ (2 equiv), anhydrous toluene, 2 h, reflux; **D)** ethyl bromoacetate (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 1.5 h, reflux; **E)** NaOH 6N, 0.5 h, 60 °C; **F)** ethyl chloroformate (1.1 equiv), Et_3N (3.5 equiv), 4-Bromoaniline (2 equiv), anhydrous THF, 12 h, -5 °C → rt.

The synthetic pathway affording the final compounds **83, 87-89** and **92** is depicted in **scheme 21**. The isoxazoles **31** (**scheme 10**) and the isoxazolo[3,4-*d*]pyridazinones **78** were previously described.[227, 280] Reductive opening of the isoxazole ring carried out on the intermediate **78** with ammonium formate and a catalytic amount of carbon-supported palladium catalyst (Pd/C) in anhydrous ethanol at reflux, afforded the known 4-amino-5-acetyl pyridazinone derivative **79**.[223] Performing the usual alkylation/alkaline hydrolysis/amide bond formation reaction sequence (**schemes 12** and **13**) the final amide **83** was synthesized in good yields. The already described intermediate **84** was obtained by deacetylation of **79** using 48% hydrobromic acid under pressure at high temperature (140 °C).[231]

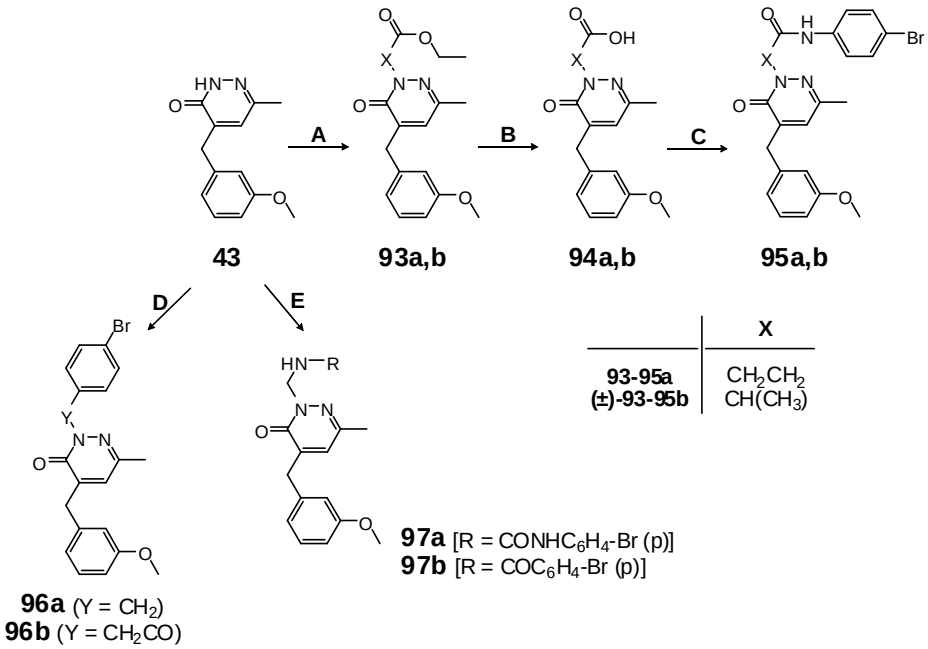


Scheme 21. Reagents and conditions: A) $NH_2NH_2 \cdot H_2O$, 4 h, 180 °C; **B)** NH_2-NH2 • H_2O (2 equiv), EtOH, 10 min, rt; **C)** $HCOONH_4$ (2.5 equiv), Pd/C 10% (catalytic), anhydrous EtOH, 1.5 h, reflux; **D)** HBr 48%, 1 h, 140 °C; **E)** ethyl bromoacetate (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 3 h, reflux; **F)** 4-methoxyphenylboronic acid (2 equiv), $Cu(OAc)_2$ (1.5 equiv), Et_3N (2 equiv), CH_2Cl_2, 12 h, rt; **G)** NaOH 6N, 1.5 h, 80 °C; **H)** ethyl chloroformate (1.1 equiv), Et_3N (3.5 equiv), 4-bromoaniline (2 equiv), anhydrous THF, 12 h, -5 °C → rt; **I)** see E; **L)** see G; **M)** see H; **N)** 4-methoxyphenylboronic acid (1 equiv), $Cu(OAc)_2$ (1.5 equiv), Et_3N (2 equiv), CH_2Cl_2, 12 h, rt; **O)** substituted benzoyl chloride (2.4 equiv), Et_3N (catalytic), anhydrous CH_2Cl_2, 16 h, 0 °C → rt; **P)** see F; **Q)** see G; **R)** see H.

To get **84** in high yields, a previously described procedure starting from **54b** (**scheme 15**) and using hydrazine hydrate at 180 °C was as well tried.[281] The high temperature of reaction afforded a mixture difficult to purify and the resulting yield was lower than that stated in the literature. Thus, the previous methodology based on deacetylation of **79** was elected to get the intermediate **84.** This compound was processed to standard alkylation, hydrolysis and amidation reactions to generate the final 4-amino pyridazinone **87**. Subsequently, the coupling of the amine group of **87** with 4-methoxyphenylboronic acid in presence of $Cu(Ac)_2$ in CH_2Cl_2 gave the 4-arylamine **88**, while the 4-arylamide analogues **89a,b** were obtained from **87** by treatment with the opportune aroylchloride and triethylamine in CH_2Cl_2 at 0 °C. To synthesize the biarylamine **90** the intermediate **85** was coupled with a two-fold amount of 4-methoxyphenylboronic acid in usual conditions and then, after hydrolysis of the ester group and following usual amidation, the final compound **92** was obtained in good yields.

3.3.3 Synthesis of *N*-arylacetamide modified pyridazinone derivatives

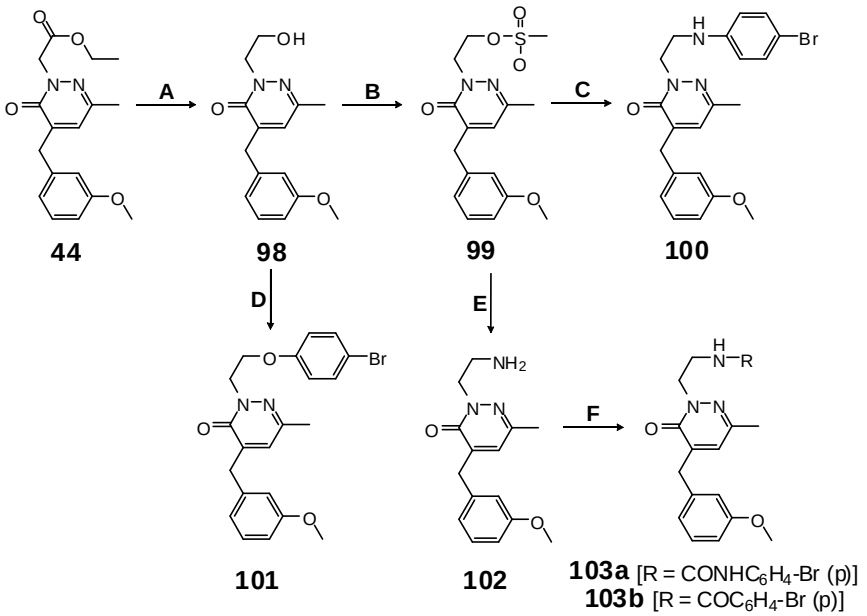
Scheme 22 depict the synthetic procedure for compounds **95-97**. The precursor **43** was alkylated with the appropriate bromo ester to give compounds **93a,b**, which, in turn, were converted in the corresponding acids **94a,b**. The final step was the transformation of these compounds into the final amides **95a,b** using the same procedure extensively described in the present section of the thesis.



Scheme 22. Reagents and conditions: A) ethyl 3-bromopropionate (for compound **93a**) or (±)-ethyl-2-bromopropionate (for compound **93b**) (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 6 h, reflux; **B)** NaOH 6N, 1 h, 80 °C; **C)** ethyl chloroformate (1.1 equiv), Et_3N (3.5 equiv), 4-Bromoaniline (2 equiv), anhydrous THF, 12 h, -5 °C → rt; **D)** alkyl halide (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 6 h, reflux; **E)** CH_2O 40%, NH_3 33%, dioxane, 1 h, 50 °C, then 4-bromophenyl isocyanate (for compound **97a**) (1.1 equiv) or 4-bromobenzoyl chloride (for compound **97b**) (1.5 equiv), CH_2Cl_2, 6-12 h, rt (**97a**), 0 °C (**97b**).

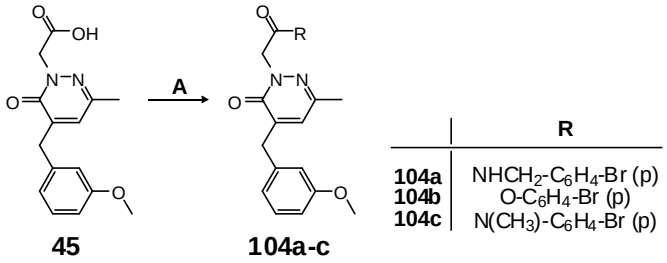
On the other hand, compounds **96a,b** were obtained by alkylation of **43** with the appropriate halide in standard conditions (**scheme 22**). Moreover the precursor **43** was converted into the final **97a,b** through a multicomponent Mannich reaction (CH_2O + NH_3).[282] The intermediate amine was not isolated and it was converted one-pot into the urea **97a** by treatment with 4-bromophenyl isocyanate and into the amide **97b** with 4-bromobenzoyl chloride.

The final compounds **100, 101** and **103a,b** were synthesized as shown in **scheme 23**. The intermediate **44** was reduced with sodium borohydride in THF/MeOH to generate the primary alcohol **98**. This compound was the starting material for the synthesis both of the ether **101**, through a coupling reaction with the 4-bromophenylboronic acid in presence of $Cu(Ac)_2$, and of compound **100**, through the mesylate **99**,[283] which, in turn, was then converted into the final compound **100** by nucleophilic replacement with 4-bromo aniline. Treatment of **99** with ammonia gave the intermediate **102**, from which the urea **103a** and the amide **103b** were obtained using 4-bromophenyl isocyanate or 4-bromobenzoyl chloride respectively in CH_2Cl_2.



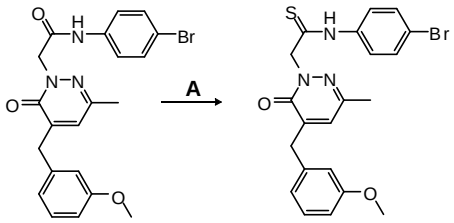
Scheme 23. Reagents and conditions: A) $NaBH_4$ (5.6 equiv), anhydrous THF, anhydrous CH_3OH, 1 h, 60 °C; **B)** methanesulfonyl chloride (1.5 equiv), pyridine (1.1 equiv), anhydrous CH_2Cl_2, 4 h, 0 °C → rt; **C)** 4-bromoaniline (2 equiv), 2-propanol, 6 h, 60 °C; **D)** 4-bromophenylboronic acid (2 equiv), $Cu(OAc)_2$ (1.5 equiv), Et_3N (2 equiv), CH_2Cl_2, 12 h, rt; **E)** NH_3 33%, 2-propanol, 3 h, 60 °C; **F)** 4-bromophenyl isocyanate (for compound **103a**) (1.1 equiv) or 4-bromobenzoyl chloride (for compound **103b**) (1.1 equiv), Et_3N (2 equiv), anhydrous CH_2Cl_2, 6 h, 0 °C.

To obtain the analogues **104a-c** (**scheme 24**), the mixed anhydride generated as usual by treatment of the intermediate **45** (**scheme 12**) with ethyl chloroformate was transformed in good yields into the final compounds by treatment respectively with commercially available 4-bromobenzylamine (for **104a**), 4-bromophenol (for **104b**) and *N*-methyl-4-bromobenzylamine (for **104c**).



Scheme 24. Reagents and conditions: A) ethyl chloroformate (1.1 equiv), Et_3N (3.5 equiv), 4-bromobenzylamine or 4-bromo-*N*-methylaniline or 4-bromophenol (2 equiv), anhydrous THF, 12 h, -5 °C → rt.

In **scheme 25** is shown the synthesis of the thioamide analogue **105** directly generated from **46a** (**scheme 12**) using Lawesson's reagent in toluene at 80 °C.[284]



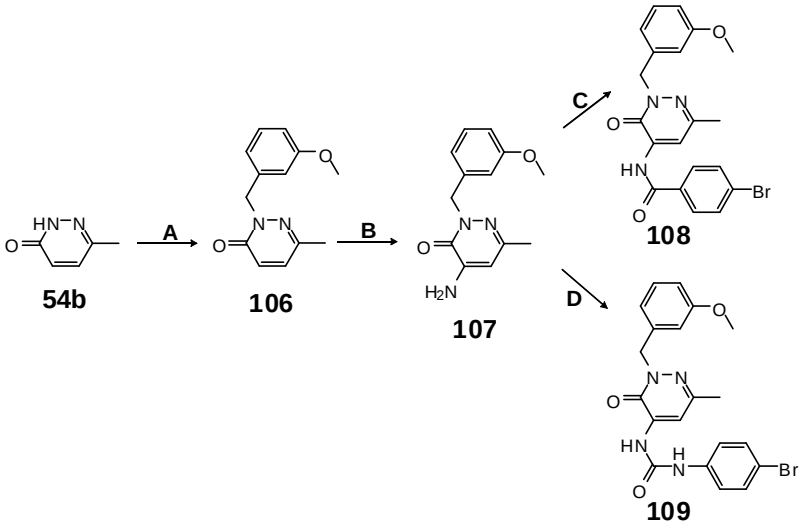
Scheme 25. Reagents and conditions: A) Lawesson's Reagent (2 equiv), anhydrous toluene, 3 h, 80 °C.

There are two possible achievable mono-tioderivatives from this reaction: the first one, where the sulphur replace the oxygen of carbonyl group at C-3 of the pyridazinone ring, and the second, where the sulphur replaced the oxygen of CO in the exocyclic amide side chain (**105**). Thus, the relative position of tionation needed to be attributed, and the structure of **105** was confirmed by both ^{1}H NMR and MS(ESI) analysis. ^{1}H NMR experiments showed a clear difference in the chemical shift between the methylene bonded to the carbonyl group in **46a** and that bonded to the thiocarbonyl group in **105**. In addition, LCMS experiments showed that it is evident a correlation between the fragments generated during the analysis and the structure **105**. Indeed, leaving aside the fact that the exact mass was exactly the one expected for **105**, it resulted evident the presence of the pyridazinone fragment without the *N*-phenylacetamide or the *N*-phenylethanethioamide moiety. This result was a proof that the pyridazinone ring was unchanged and the sulphur is on the side chain as drawn (**scheme 25**). Thus, it is reasonable to presume that reaction via sulphur substitution by Lawesson's reagent is favoured on the carbonyl group of the side chain.

3.3.4 Synthesis of N-2/C-4 inverted pyridazinone analogues

In **scheme 26** is depicted the synthesis of compounds **108** and **109**. Alkylation in standard condition of the 6-methylpyridazinone **54b** (**scheme 15**) with 3-methoxybenzyl chloride in acetonitrile resulted in compound **106** which gave the corresponding 4-amino derivative **107** by heating with $N_2H_4 \cdot H_2O$ in hard

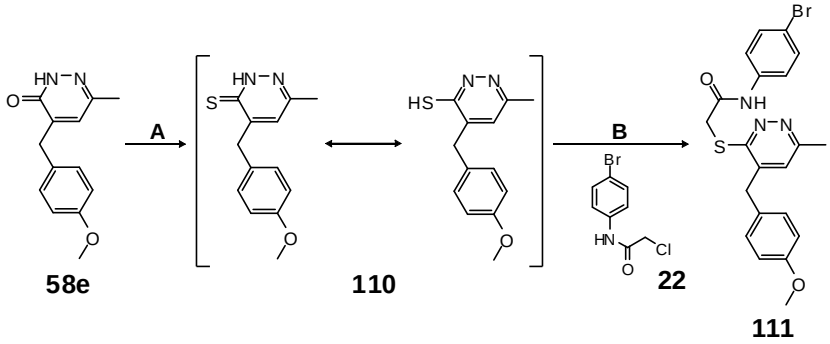
conditions. The amide **108** and the urea **109** were obtained from **107** by treatment with the opportune aroyl chloride or the aryl isocyanate following the same conditions reported for **89a,b** (**scheme 21**) and **38a-c** (**scheme 10**).



Scheme 26. Reagents and conditions: A) 3-methoxybenzyl chloride (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 6 h, reflux; **B)** $NH_2NH_2 \cdot H_2O$, 12 h, 180 °C; **C)** 4-bromobenzoyl chloride (2.4 equiv), Et_3N (catalytic), anhydrous CH_2Cl_2, 10 h, 0 °C; **D)** 4-bromophenyl isocyanate (2.2 equiv), anhydrous toluene, 7 h, reflux.

3.3.5 Synthesis of C-3 susbstituted pyridazine analogue

Scheme 27 outlines the synthetic procedure for compound **111**. Using Lawesson's reagent, as previously described, the precursor **58e** (**scheme 16**) was transformed into intermediate **110** (**scheme 25**) which, in turn, was alkylated in standard condition using **22** (**scheme 7**) to afford the corresponding analogue **111**, having the phenylacetamide moiety at the C-3 of the pyridazinone ring.



Scheme 27. Reagents and conditions: A) Lawesson's Reagent (1 equiv), anhydrous toluene, 2 h, 80 °C; **B) 22** (1.5 equiv), K_2CO_3 (2 equiv), anhydrous CH_3CN, 3 h, reflux.

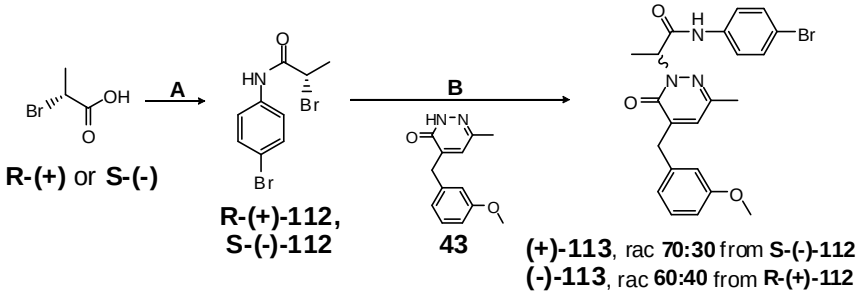
3.3.6 Synthesis of chiral pyridazinone analogues

A further development of the project was the introduction of stereogenic center on the *N*-phenylacetamide linker of the lead compound **46a** (**scheme 12**). The modification introduces stereochemical complexity into the products and, at the same time, it should be interesting to see the effect of the presence of a

stereogenic center on the biological activity, maintaining however the essential conformational shape of the molecules. The development of an homologous series of chiral compounds, possibly through enantioselective reaction, would be particularly desirable in the case of the synthesis of a group of biologically-useful small molecules due to the well known advantages of chirality when considering the interactions of small-molecules with biological systems.

3.3.6.1 Enantioselective synthesis of the *N*-arylpropanamide analogue

In the first instance, the asymmetric synthesis of enantiomers **(+)-113** and **(-)-113** had been tried (**scheme 28**). Commercially available R-(+)- and S-(-)-bromopropionic acids were firstly treated with $SOCl_2$ affording the intermediate acid chlorides which in turn were transformed in the amides **R-(+)-112** and **S-(-)-112** using 4-bromoaniline.[285] As expected and as confirmed by chiral HPLC and polarographic analysis, the amide bond formation occurred by retention of the relative configuration of the starting carboxylic acids due to the missing participation of the chiral center in the reaction and to the not basic reaction conditions.[286-287] The amides **R-(+)-112** and **S-(-)-112** were then used to alkylate in standard conditions the intermediate **43** (**scheme 12**) to give respectively the two pure enantiomers. Precedent literature described the alkylation on the N-2 of the pyridazinone ring as a classical S_N2 reaction,[288] so that this methodology could be theoretically applied to the synthesis of both enantiomers **(+)-113** and **(-)-113**, with complete inversion of the configuration of starting amides. Despite the fact that this reaction is simple and well known,[289-292] it was found that it is not suitable for asymmetrical synthesis because the inversion of configuration was not complete and consequent racemization occurred. Inspection by chiral HPLC analysis of the chromatographically pure compounds showed the achievement of a mixture 70:30 starting from **S-(-)-112** and a mixture 60:40 from **R-(+)-112**. All attempts at doing this stereoselective alkylation reaction proved unsuccessful.

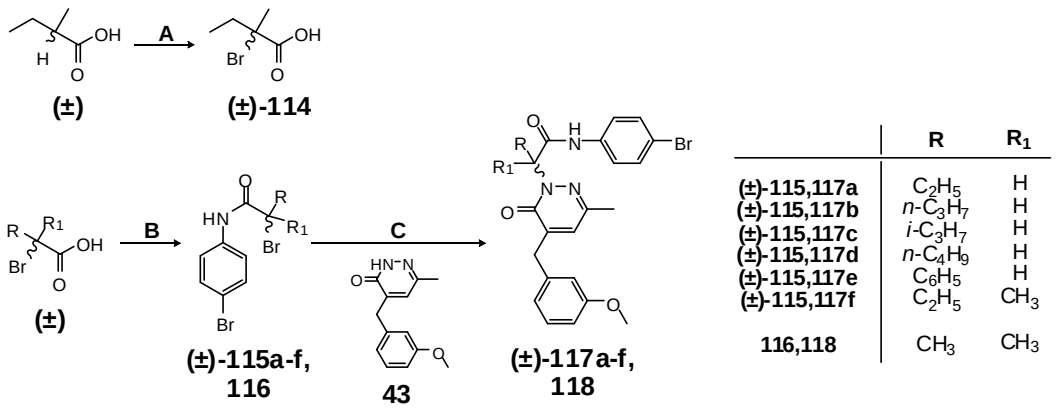


Scheme 28. Reagents and conditions: A) $SOCl_2$ (6 equiv), anhydrous CH_2Cl_2, 4 h, reflux, then 4-bromoaniline (2 equiv), anhydrous THF, 4 h, rt; **B) 43** (0.9 equiv), K_2CO_3 (1.8 equiv), anhydrous CH_3CN, 2 h, reflux.

3.3.6.2 Synthesis of the homologous series of *N*-aryl-(alkyl)-amide derivatives

To get the chiral compounds **(±)-117a-f** as racemates and the dimethyl analogue **118**, not optically active, the same synthetic pathway used for **(+)-113/(-)-113** (**scheme 28**) has been performed as shown

in **scheme 29**. For the synthesis of the compound **(±)-117f** it was previously required the preparation of the starting bromo-derivative **(±)-114**, not commercially available and obtained as reported in literature by treatment with *N*-bromosuccinimide in CCl_4 at reflux.[293] After treatment with $SOCl_2$ of the appropriate chiral carboxylic acids (**scheme 29**) the commercially available 4-bromophenyl amides **(±)-115a,c**, the already described **(±)-115e,f**, **116**,[294-298] and the new **(±)-115b,d** were obtained in low yields as useful intermediates for the synthesis of the final compounds **(±)-117a-f** and **118**.

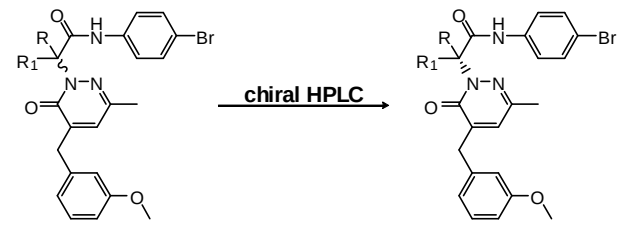


Scheme 29. Reagents and conditions: A) NBS (1.05 equiv), CCl_4, N_2, 8 h, reflux; **B)** $SOCl_2$ (6 equiv), anhydrous CH_2Cl_2, 4 h, reflux, then 4-bromoaniline (2 equiv), anhydrous THF, 4 h, rt; **C) 43** (0.9 equiv), K_2CO_3 (1.8 equiv), anhydrous CH_3CN, 4-5 h, reflux.

3.3.6.3 Chiral Chromatographic resolution of racemates (±)-95b and (±)-117a-f

Due to the significant racemization during nucleophilic displacement of bromide with the pyridazinone intermediate **43** (**scheme 28**, **section 3.3.6.2**), an alternative strategy was required to get the pure enantiomers of **(±)-95b** and **(±)-117a-f** (**schemes 29** and **30**). Chiral HPLC is one of the most valid and efficient methods for obtaining both enantiomers of a chiral compound in high optical purity [> 97% enantiomeric excess (ee)]. This approach has now become an established procedure for *in vitro* comparative biological testing, where few milligrams of both enantiomers are required. In addition, the isolation of single enantiomers represents a strategy for rational drug design due to the role of stereochemistry in molecular interactions involving biological macromolecules.

Compounds **(±)-95b** and **(±)-117a-f** (**scheme 30**) were then isolated as pure (ee = 97.4-99.9%) by semi-preparative chiral HPLC (**scheme 30**) and two distinct polysaccharide based chiral stationary phases (CSPs) had to be employed to separate the different racemates. Indeed in the early stage of the chromatographic resolution a Chiralcel OD® column [cellulose tris(3,5-dimethylphenylcarbamate)] was used to separate racemates **(±)-95b** and **(±)-117a** (**table 3.1**), according to the literature for a series of pyridazinone like compounds.[221] An isocratic elution mode was employed and detection by UV was carried out at 250 nm.



	R	R_1
(+)-95b	CH_3	H
(-)-95b	CH_3	H
(+)-117a	C_2H_5	H
(-)-117a	C_2H_5	H
(+)-117b	*n*-C_3H_7	H
(-)-117b	*n*-C_3H_7	H
(+)-117c	*i*-C_3H_7	H
(-)-117c	*i*-C_3H_7	H
(+)-117d	*n*-C_4H_9	H
(-)-117d	*n*-C_4H_9	H
(+)-117e	C_6H_5	H
(-)-117e	C_6H_5	H
(+)-117f	C_2H_5	CH_3
(-)-117f	C_2H_5	CH_3

Scheme 30. Chromatographic resolution using Chiralcel OD®column for **(±)-95b** and **(±)-117a** (eluent: *n*-hexane/2-propanol 95:5); Lux Amylose-2® column for **(±)-117b-f** (eluent: *n*-hexane/2-propanol 60:40).

Unexpectedly, during the analytical work resulted a lack of selectivity of the Chiralcel OD® column by increasing the steric hindrance on the chiral center. Indeed, already the resolution of racemate **(±)-117b,** having a propyl group on the chiral center, resulted not possible due to poor resolution between two enantiomers (**figure 3.1**).

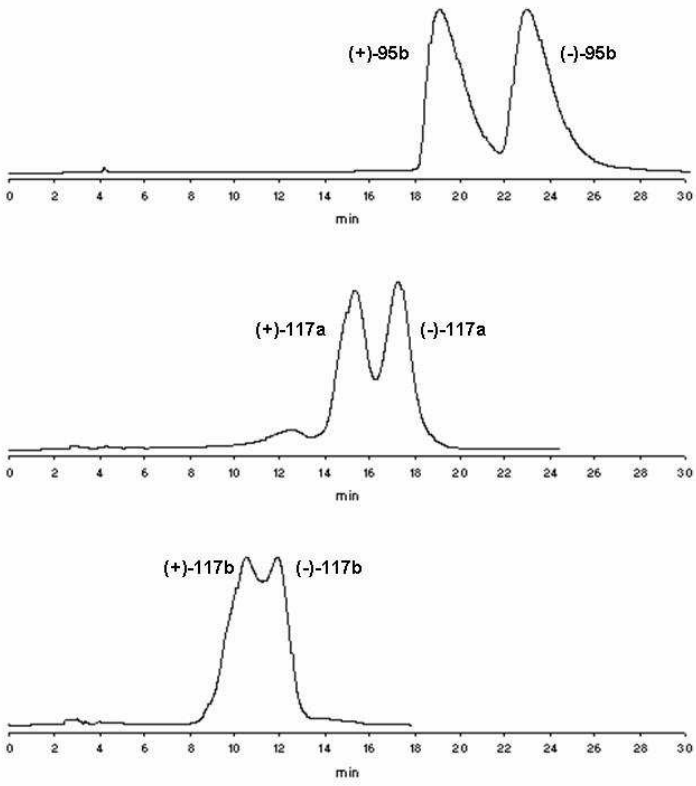


Figure 3.1. Chiral semi-preparative HPLC resolution of racemates **(±)-95b** and **(±)-117a**. **Conditions:** Chiralcel OD column (10 µm, 4.6 mm I.D. x 250 mm), *n*-hexane/IPA 95:5 (v/v) as eluent, flow rate 1.2 mL/min, detection by UV at 250 nm, temperature 25 °C, . At the bottom it is shown the lack of selectivity resulted using Chiralcel OD column in the same conditions to separate racemate **(±)-117b**.

Enantiomer	Stationary Phase	Eluent (*n*-hexane/IPA)	Flow rate (mL/min)	Temperature (°C)	Retention Time (t_R, min)
(+)-95b	Chiralcel OD	95:5	1.2	25	19.2
(-)-95b					23.2
(+)-117a	Chiralcel OD	95:5	1.2	25	15.9
(-)-117a					17.9
(+)-117b	Amylose-2	60:40	1.5	40	5.1
(-)-117b					9.4
(+)-117c	Amylose-2	60:40	1.5	40	3.9
(-)-117c					10.1
(+)-117d	Amylose-2	60:40	1.5	40	5.4
(-)-117d					9.7
(+)-117e	Amylose-2	60:40	1.5	40	12.3
(-)-117e					7.6
(+)-117f	Amylose-2	60:40	1.5	40	8.6
(-)-117f					6.7

Table 3.1. Data of the chiral HPLC separations for the different racemates **(±)-95b** and **(±)-117a-f** related to semi-preparative conditions, column types (250 mm x 4.6 mm I.D.), mobile phases, flow rates, temperature and retention times (t_R) detected by UV at 250 nm.

In order to develop the semi-preparative resolution it was required to find another chiral stationary phase. As it shown in **table 3.1** and in **figure 3.2** a very good separation of the (+) and (-) enantiomers of **117b-f** was achieved using Lux Amylose-2® column [amylose tris(5-chloro-2-methylphenyl-carbamate)] and the optically pure compounds were obtained as required. In all cases, an isocratic elution mode was employed and detection by UV was carried out at 250 nm.

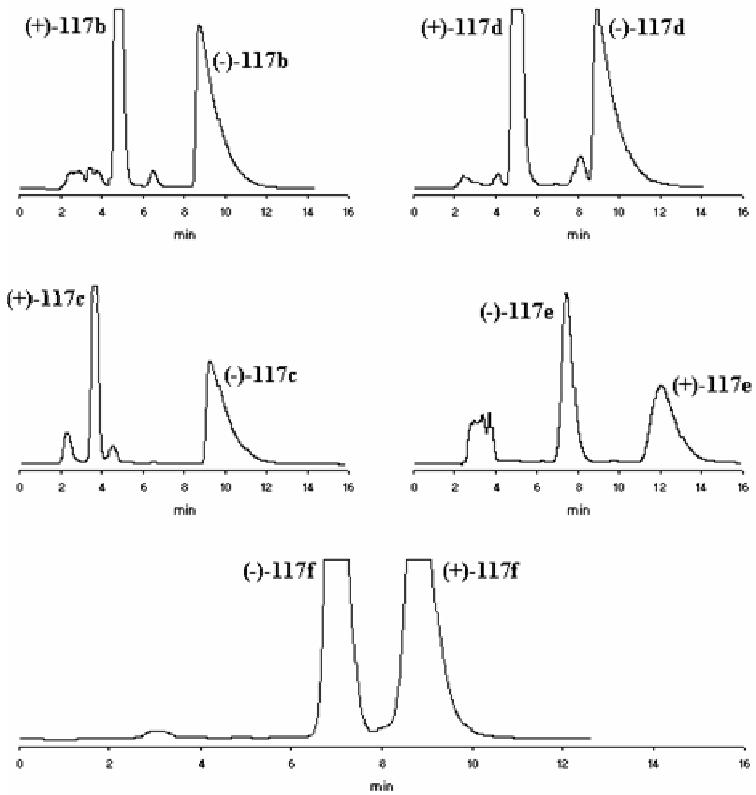


Figure 3.2. Chiral semi-preparative HPLC resolution of racemates **(±)-117b-f**. **Conditions:** Lux Amylose-2 column (5 µm, 4.6 mm I.D. x 250 mm), *n*-hexane/IPA 60:40 (v/v) as eluent, flow rate 1.5 mL/min, detection by UV at 250 nm, temperature 40 °C.

After semipreparative HPLC the fractions collected were dried under nitrogen and analyzed on chiral analytical Lux Amylose-2® (3 µm, 4.6 mm I.D. x 50 mm) column to determine their enantiomeric excess (ee) (**figure 3.3** and **table 3.2**).

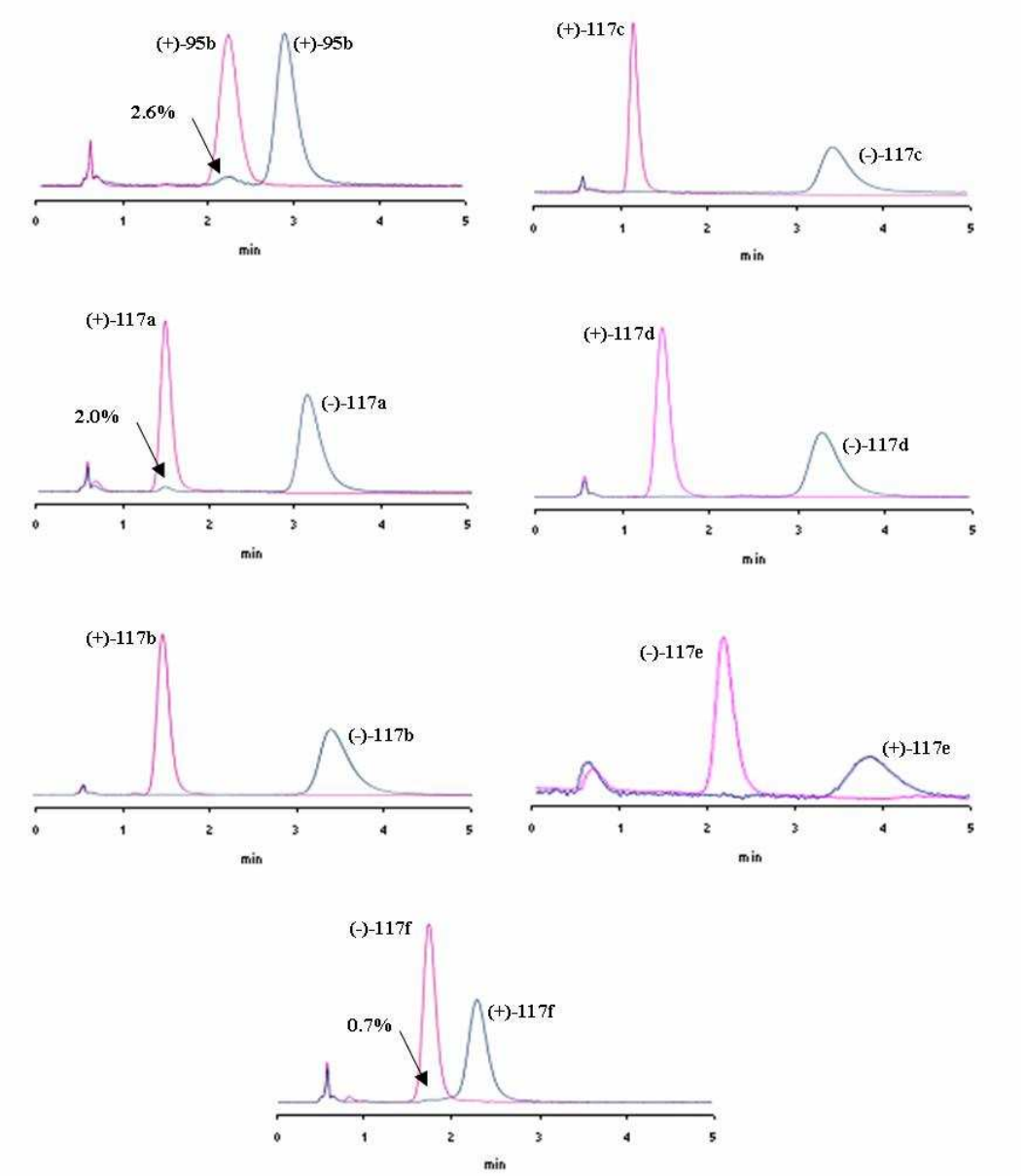


Figure 3.3. Chiral analytical HPLC analysis of the chromatographically resolved enantiomers **(+)-95b/(-)-95b** and **(+)-117a-f/(-)-117a**. **Conditions:** Lux Amylose-2 column (3 µm, 4.6 mm I.D. x 50 mm), *n*-hexane/IPA 60:40 (v/v) as eluent, flow rate 1.0 mL/min, detection by UV at 250 nm, temperature 40 °C, injection volume of 10 µL. For analytical enantioseparations a standard solution was prepared by dissolving 0.1 mg of each enantiomer into 1 mL of ethanol. The ee values were calculated from relative peak areas (**table 3.2**). Arrows indicate the percentage of the enantiomeric impurities for **(+)-95b**, **(-)-117a**, **(+)-117f** whereas all the other enantiomers were obtained with a purity > 99.9%.

Polarimetric analysis were as well performed by dissolving 20 mg of pure compounds into 2 mL of chloroform (c = 1) to establish the specific rotations ($[\alpha]^{20}_{D}$) of enantiomers (**table 3.2**). The signal was measured at 589 nm using a polarimeter equipped with a Na lamp.

Enantiomer	$[\alpha]^{20}_D$ ($CHCl_3$, *c* = 1)	Enantiomeric excess (ee, %)
(+)-95b	+ 80°	> 99.9
(-)-95b	- 78°	97.4
(+)-117a	+ 129°	> 99.9
(-)-117a	- 129°	98.0
(+)-117b	+ 139°	> 99.9
(-)-117b	- 137°	> 99.9
(+)-117c	+ 99°	> 99.9
(-)-117c	- 97°	> 99.9
(+)-117d	+ 111°	> 99.9
(-)-117d	- 110°	> 99.9
(+)-117e	+ 16°	> 99.9
(-)-117e	- 17°	> 99.9
(+)-117f	+ 17°	99.3
(-)-117f	- 18°	> 99.9

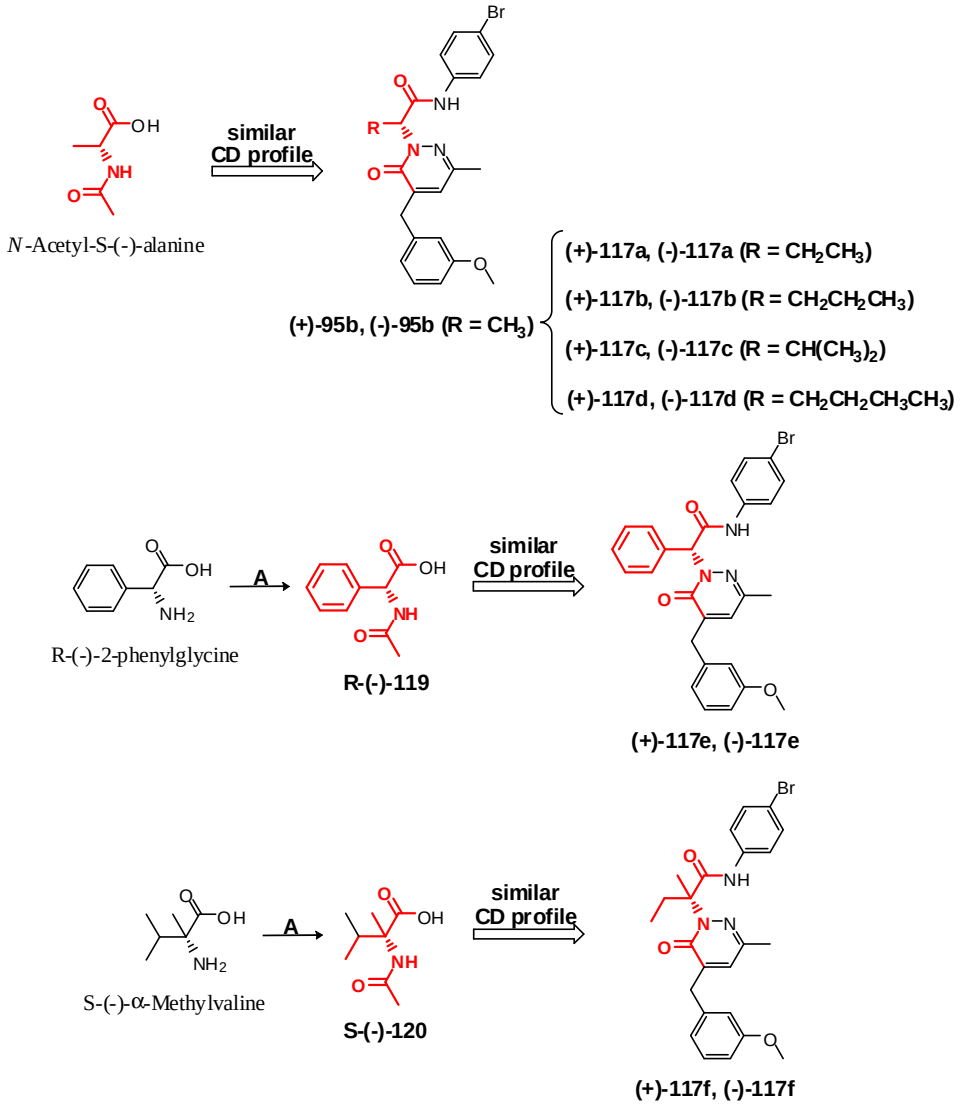
Table 3.2. Data for the different racemates **(±)-95b** and **(±)-117a-f** of specific rotation factor ($[\alpha]^{20}_D$), determined by polarimeter in $CHCl_3$ (*c* = 1 mg/1 mL), and enantiomeric excess, determined by chiral analytical HPLC separations.

Chiral HPLC and polarimetric analysis indicated that for racemates **(±)-95b** and **(±)-117a-d** the first eluted enantiomers using both Chiralcel OD or Lux Amylose-2 columns rotated polarized light in the positive direction, accordingly to the comparable nature of substituents on the stereogenic center (R = alkyl, R_1 = H, **schemes 29** and **30**). Oppositely, the elution sequence was reversed in the case of racemates **(±)-117e,f** where the enantiomers **(-)-117e,f** eluted faster then the corresponding (+)-forms during the separating process. Enantiomer elution order is a very important topic in the determination of enantiomeric purity of chiral compounds as well as in the study of enantiorecognition mechanism.[299] The polysaccharide-type CSPs are based on natural materials and are available only in one configuration. Although it is difficult to control the enantiomer elution order with these CSPs, examples of inversion enantiomer retention order have been reported by alteration of the mobile phase composition or enantioseparation temperature.[300] In our case, these results appear to be consistent with the assumption that structural differences on the chiral center between the analytes determined the reversal of the enantiomer elution order. Probably the bigger hindrance of the phenyl group in **(±)-117e** and the absence of H on the chiral center in **(±)117f** are the key determinants in this inversion of chromatographic elution. Thus, from seven separated compounds five [**(±)-95b** and **(±)-117a-d**] exhibited higher affinity of the (-)-form for the stationary phase over the (+)-form and for the remaining two [**(±)-117e,f**] it was the opposite. It is as well interesting to note that enantiomer elution order was not influenced by changing the amount of alcohol (2-propanol) used in the mobile phase in both colums.

3.3.6.4 Assignment of the absolute configurations

To assign the absolute configuration to the pure enantiomers **(+)-95b**, **(-)-95b**, **(+)-117a-f** and **(-)-117a-f**, we made several attempts to get crystals suitable for single crystal X-ray analysis but, unluckily, none of

these analogues were amenable for configurational analysis by crystallization. Since this approach failed, for the configurational assignment we took into account the methodology based on the comparison of CD curve analysis of the unknown enantiomers and the CD profile of commercially available molecules or well known compounds whose absolute configuration was already been established. Examples of assignment of the absolute configuration using this approach have been extensively reported in the literature.[301-308]



Scheme 31. ***N*-Acetyl-S-(-)-alanine** and acetyl derivatives of R-(-)-***2-phenylglycine*** ***[*R-(-)-119**] and S-(-)-α-Methylvaline [**S-(-)-120**] used as reference compounds in CD experiments in comparison to enantiomeric pairs **(+)-95b/(-)-95b**, **(+)-117e/(-)-117e**, **(+)-117f/(-)-117f** due to their comparable substituents on the stereogenic center. The result obtained for **(+)-95b/(-)-95b** can be extended as well for enantiomers **(+)-117a-d/(-)-117a-d**. **Reagents and conditions: A)** acetic anhydride (7.5 equiv), H_2O, 0.5 h, 70 °C.

Commercially available *N*-acetyl-S-(-)-alanine, R-(-)-***2-phenylglycine*** and S-(-)-α-methylvaline were chosen as reference molecules considering that they are characterized by comparable substituents on the stereogenic centre. Indeed, as it shown in **scheme 31**, *N*-acetyl-S-(-)-alanine bearing a methyl group on the chiral center, an hydrogen, a carboxylic CO and an amidic NH can be used in CD analysis as

reference for **(+)-95b/(-)-95b** and the result can be extended as well for **(+)-117a-d/(-)-117a-d** enantiomeric pairs. R-(-)-***2-phenylglycine*** and S-(-)-α-methylvaline were instead firstly converted into their acetyl derivatives **R-(-)-119** [commercially available, *N*-acetyl-R-(-)-phenylglycine] and **S-(-)-120** [*N*-acetyl-S-(-)-α-methylvaline] to make the chiral center more similar to that of **(+)-117e/(-) -117e** and **(+)-117f/(-)-117f** respectively (**scheme 31**). The acetylation reaction was performed in H_2O using acetic anhydride at 70 °C and it occurred by complete retention of the absolute configuration as reported in literature.[309] Both enantiomers of **(±)-95b** were analyzed in comparison with *N*-acetyl-S-(-)-alanine ($[\alpha]^{23}_D$ – 62° (*c* = 1, H_2O) (**scheme 31** and **figure 3.4**). The solutions of **(+)-95b** and **(-)-95b** in methanol (concentration about 0.25 mg/mL, optical pathway 0.1 cm) and of *N*-acetyl-S-(-)-alanine (concentration about 1 mg/mL, optical pathway 0.1 cm) were analyzed in nitrogen atmosphere. CD spectra were scanned at 50nm/min with a spectral band width of 1 nm and data resolution of 0.2 nm. The spectra were averaged over five instrumental scans and the intensities are presented in terms of ellipticity values (mdeg) (**figure 3.4**). The CD spectrum of the **(+)-95b** enantiomer (**figure 3.4**) displays a broad positive Cotton effect around 300 nm. The **(-)-95b** enantiomer exhibited the corresponding mirror-image CD. In comparison, the positive Cotton effect of the *N*-acetyl-S-(-)-alanine is in the range of wavelength between 215 and 250 nm. Therefore, (S)-absolute configuration of the reference compound may also be assigned to **(+)-95b** and (R)-absolute configuration to **(-)-95b** that oppositely showed a negative Cotton effect around 300 nm. The differences in the profile of the curves could depend on the different type of chromophore between **(+)-95b/(-)-95b** and the *N*-acetyl-S-(-)-alanine without any aromatic substituent.

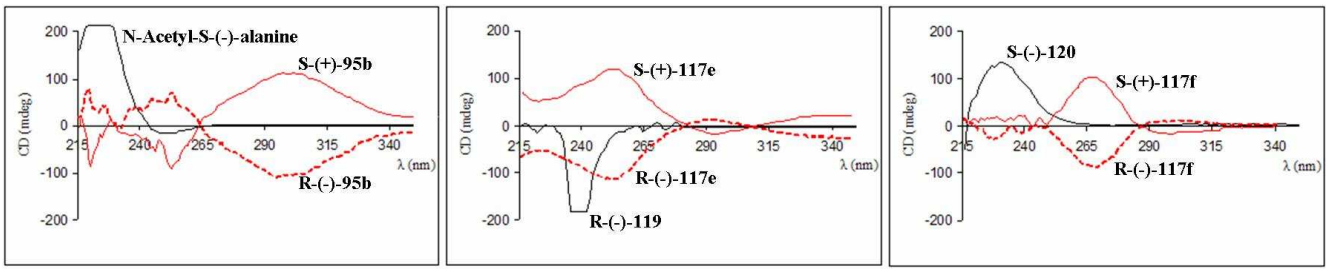


Figure 3.4. Experimental Circular dichroism (CD) spectra recorded in methanol at 25 °C of the pure enantiomeric pairs **(+)-95b/(-)-95b**, **(+)-117e/(-)-117e**, **(+)-117f/(-)-117f** and the reference compounds ***N*-acetyl-S-(-)alanine**, **R-(-)-119**, **S-(-)-120** (**scheme 31**). In each spectra black traces refers to the reference compounds, whereas solid and dashed red traces correspond respectively to the (S)- and (R)-enantiomers analyzed.

Due to the similarity on the chiral center between compounds **S-(+)-95b/R-(-)-95b** and enantiomeric pairs **(+)-117a-d/(-)-117a-d** (**scheme 31**), it seems then possible that enantiomers of the same sign of optical rotation may have the same absolute configuration. Some support for such a reasoning is in the fact that a correlation between the absolute configuration of these optically active analogues could be deduced as well from the same elution order showed during chiral HPLC analysis (**section 3.3.6.3**, **figure 3.2** and **3.3**). Indeed analogues **(+)-117a-d/(-)-117a-d** exhibited same-sense chiral recognition mechanism

and, consequently, the same enantiomer elution order on both stationary phases used (Chiralcel OD and Lux Amylose-2) with preferential retention of the (-)-enantiomer. These data support the presumption that all the enantiomers **(+)-117a-d** possess the same absolute (S)-configuration and, obviously, the absolute (R)-configuration is for levorotatory isomers **(-)-117a-d**.

Similarity of CD spectra of reference molecule **(R)-(-)-119** and **(-)-117e** (**figure 3.4**) is certainly in line with the rule that enantiomers with the same sign of Cotton effect may have the same absolute configuration. Enantiomers **(-)-117e/(+)-117e** display the Cotton effects between 230 and 270 nm and a maximum around 250 nm. Also in this case, it is noteworthy that differences in the profile of the curves could depend from different type of chromophore between **(-)-117e/(+)-117e** and the **(R)-(-)-119**. CD spectra of **(-)-117e** and **(+)-117e**, recorded in methanol, clearly are mirror images (**figure 3.4**), demonstrating again the enantiomeric relationship. The negative Cotton effect of **(-)-117e** is in agreement with negative Cotton effects of the previously described **(R)-(-)-119**,[310-312] supporting the absolute R-(-)-configuration assigned to this enantiomer (**scheme 31**).

The configurational assignment for the last enantiomeric pair **(-)-117f/(+)-117f** was performed by comparing their circular dichroism spectra with that of **S-(-)-120** (**scheme 31**), always characterized by similar substituents on the stereogenic centers (two alkyl groups, a carboxylic CO and an amidic NH), whose configuration was already established. The spectra showed once more analogous profiles and comparable Cotton effects between **S-(-)-120** and **(+)-117f** in the spectral region between 215 and 300 nm (**figure 3.4**). The bathochromic shift of the **S-(-)-120** curve could depend on the differences between the different chromophores. Therefore the absolute configurations of **S-(-)-120** may be proposed also for **(+)117f** and, consequently, the absolute R-configuration was assigned to **(-)-117f** (**scheme 31**).

In conclusion, on the basis of combined chromatographic and chiroptical studies, the absolute configuration assignment was unambiguously completed, and configurations were assigned as follow to the seven enantiomeric pairs synthesized: **S-(+)-95b/R-(-)-95b** and **S-(+)-117a-f/R-(-)-117a-f**. Due to the comparable chiral centers in this group of homologue molecules, the results showed as well that (S)-absolute configuration is for all dextrorotatory enantiomers of the series and (R)-absolute configuration is for the corresponding levorotatory isomers.

4. RESULTS AND CONCLUSIONS

4.1 Results

In the present study, we synthesized a library of 162 heterocyclic compounds which were screened in order to identify novel molecules able to activate human neutrophil through FPRs interaction. Thus the final compounds were evaluated for their ability to induce intracellular Ca^{2+} flux in HL-60 (Human promyelocytic leukemia) cells transfected with FPR1, FPR2, or FPR3. In fact it is well known that it is possible to estimate FPRs affinity by means of evaluation in Ca^{2+} flux changing.[313-314] All compounds were also evaluated in WT (wild-type non-transfected HL-60 cells) and they where inactive. Moreover, both EC_{50} values and relative efficacy, compared to the peptide agonists fMLF and WKYMVm, were determined. Finally, the compounds that showed the best activity profile were selected to evaluate the activity as chemotactic agents and the capacity to mobilize Ca^{2+} in human neutrophils.

4.1.1 Screening different nitrogen heterocyclic derivatives as FPRs agonists

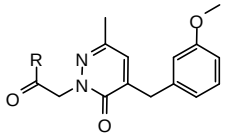
In the early phase of our project, we synthesized several compounds where functionalized side chains similar to that of the references compounds (Quin-C1 and pyrazolone derivatives in **figure 2.1**) are bonded to different heterocyclic scaffolds such as indazole, indole, quinoline, naphtyridone, phthalazinone and phthalhydrazide (**schemes 1-8**, **section 3.1**). Any activity was found for these compounds towards the three FPR subtypes. Some of them showed low activity as chemotactic agent (data not shown) probably due to the interaction with a different biological system or other receptors in the neutrophils. Likewise pyridazin-3(2H)-ones bearing a methoxybenzyl or a phenyl group at position 2 and a functionalized side chain at positions 4 or 5 (**schemes 9,10**, **section 3.1**) were inactive as FPRs agonists. Performing further modifications on the same scaffold as the insertion of substituted benzyl at position 4 together with a functionalized chain at N-2 (**scheme 12**, **section 3.1**), we identified compound **46a** (**table 4.1**), as a potent mixed FPR1 and FPR2 agonist.[233] Thus, this first active compound **46a** was selected as lead and extensive structure-activity relationship (SAR) studies on this prototype were performed.

During the development of the project, it became necessary to replace the initial cell line RBL-2H3 (Rat basophilic leukemia) cells, used to test agonistic activity of the compounds, with HL-60 cells, having higher expression of FPRs and as well higher response to reference molecule fMLF. In the meantime some modifications of the lead compound had been already performed, such as the introduction of a phenyl group in the position C-6 of the pyridazinone and a iodine on the phenyl of the acetamidic spacer. The biological tests carried out on the first cell line (RBL-2H3) showed for these compounds a very interesting activity and, thus, several analogues were designed and synthesized on the basis of this evidence. Unluckily, the same results were not confirmed by testing the same molecules in HL-60 cell.

4.1.2 EC_{50} and efficacy of *N*-arylacetamide pyridazinones

The nature and the position of the substituent on the phenyl group of the side chain proved to play a crucial role in ligand activity as it is shown in **table 4.1**. Moving Br of lead compound **46a** from position para to meta (**46b**) and ortho (**46c**) resulted in a complete loss of FPR1/FPR2 activity. Among halo-derivatives, the 4-chloro analogue **46e** exhibited the same profile as **46a**, while the corresponding 4-iodo derivative **46f** was two times less potent for FPR2. For this compound, a weak effect at FPR3 was also observed. The 4-fluoro analogue **46d** was less potent compared to **46a**, but specificity for FPR1 was demonstrated.

Table 4.1. Activity of the compounds **46a-s** (**scheme 12**) in HL-60 cells expressing human FPR1, FPR2, or FPR3.



compd	R	Ca^{2+} Mobilization EC_{50} (μM) and Efficacy (%)[a]		
		FPR1	FPR2	FPR3
46a	NH-C_6H_4-Br (p)	3.4 ± 1.6 (75)	3.8 ± 1.5 (70)	N.A.
46b	NH-C_6H_4-Br (m)	N.A.	N.A.	N.A.
46c	NH-C_6H_4-Br (o)	N.A.	N.A.	N.A.
46d	NH-C_6H_4-F (p)	**7.6 ± 0.2 (40)**	**N.A.**	**N.A.**
46e	NH-C_6H_4-Cl (p)	2.6 ± 0.3 (110)	4.0 ± 1.6 (35)	N.A.
46f	NH-C_6H_4-I (p)	2.8 ± 0.2 (90)	6.8 ± 2.2 (40)	13.0 ± 3.1 (30)
46g	NH-C_6H_5	N.A.	N.A.	N.A.
46h	NH-C_6H_4-CH_3 (p)	7.2 ± 2.2 (120)	10.9 ± 3.4 (50)	N.A.
46i	NH-C_6H_4-*t*C_4H_9 (p)	N.A.	N.A.	N.A.
46j	NH-C_6H_4-OCH_3 (p)	7.7 ± 2.5 (65)	14.4 ± 2.0 (35)	N.A.
46k	NH-C_6H_4-OC_4H_9 (p)	N.A.	N.A.	N.A.
46l	NH-C_6H_3-$(OCH_3)_2$ (3, 4)	15.5 ± 2.9 (25)	16.8 ± 3.2 (25)	N.A.
46m	NH-C_6H_3-3,4-methylenedioxy	**2.3 ± 1.1 (50)**	**N.A.**	**N.A.**
46n	NH-C_6H_4-CF_3 (p)	5.7 ± 1.8 (50)	8.8 ± 2.3 (95)	N.A.
46o	NH-C_6H_4-OCF_3 (p)	N.A.	N.A.	N.A.
46p	NH-C_6H_4-SCH_3 (p)	26.4	14.7	N.A.
46q	NH-C_6H_4-CN (p)	N.A.	N.A.	N.A.
46r	NH-C_6H_4-NO_2 (p)	10.5 ± 2.9 (60)	12.3 ± 2.5 (55)	N.A.
46s	1-methylpiperazine	N.A.	N.A.	N.A.
fMLF		0.01	20.4	1.9
WKYMVm		0.5	0.001	0.01

[a]N.A., no activity was observed (no response was observed during first 2 min after addition of compounds under investigation) considering the limits of efficacy > 20 % and EC_{50} < 50 μM. The EC_{50} values are presented as the mean ± S.D. of three independent experiments, in which median effective concentration values (EC_{50}) were determined by nonlinear regression analysis of the dose-response curves (5-6 points) generated using GraphPad Prism 5 with 95% confidential interval ($p < 0.05$). Efficacy (in bracket) is expressed as percent of the response induced by 5 nM fMLF (FPR1) or 5 nM WKYMVm (FPR2 and FPR3).

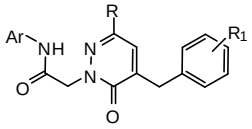
The elimination of Br (compound **46g**) was associated with a complete loss of activity. Replacement of Br in **46a** with substituents having similar steric properties, such as *t*-But (**46i**), OCF_3 (**46o**), and CN (**46q**), led to loss of activity at both FPR1 and FPR2. The 4-trifluoromethyl and the 4-nitro analogues (**46n** and **46r**, respectively) as well as compound **46h**, bearing a methyl group at position 4, had relatively low activity. Introduction of alkoxy groups gave interesting results: the 4-methoxy derivative **46j** and the 3,4-dimethoxy derivative **46l** had low activity at both receptors, whereas of the 3,4-methylendioxy derivative **46m** showed specificity for FPR1. Differently compound **46p**, with a methylthio group in the para position of the phenyl group showed a drastic decrease of activity. It is worth noting that compounds **46k**, where Br in the para position is substituted with a buthoxy, and **46s**, where the amidic nitrogen is included in a piperazine nucleus, were found to be completely devoid of activity.

4.1.3 EC_{50} and efficacy of C-6 modified *N*-arylacetamide pyridazinones

SAR studies at position 6 of the pyridazinone ring were planned by modifying the methyl group of the lead compound **46a** (**table 4.1**) as follow: elimination or replacement with superior homologues, cyclohexyl group, thiophene and (substituted)aryls (**table 4.2**).

As it is possible to see from the biological results, the ethyl analogue (**50b**) displayed a very similar behaviour with respect to the lead compound (**46a**), whereas the isopropyl derivative (**50a**) resulted a mixed agonist for FPR1, FPR2 and FPR3. On the contrary, the introduction of a cyclohexyl at position C-6 (**50c**) was associated with FPR1 selectivity. The elimination of the methyl group (**50d**) led to a quite potent but not selective compound, since it was able to activate all the three receptor subtypes. Among compounds having a phenyl group at C-6 only **50e**, bearing Br in the phenylacetamide chain showed a relevant activity, mainly at FPR1 level. The 2-thienyl derivatives **50h-j** were completely inactive, as well as all compounds bearing OCH_3 (**50k-m**), Cl (**50n-p**), CH_3 (**50q-s**), F (**50t-v**), in para position of the phenyl at C-6 of pyridazinone nucleous. It is worth nothing the inactivity of 4-F analogue **50t**, in comparison with the above seen **50e** unsubstituted analogue. This finding suggest that for these compounds electronic features play a more important role than steric properties.

Table 4.2. Activity of the compounds **50a-v** (**scheme 13**) in HL-60 cells expressing human FPR1, FPR2, or FPR3.



compd	R	R_1	Ar	Ca^{2+} Mobilization EC_{50} (μM) and Efficacy (%)[a]		
				FPR1	FPR2	FPR3
50a	CH_2CH_3	OCH_3 (m)	NH-C_6H_4-I (p)	5.0 (95)	7.2 (75)	N.A.
50b	$CH(CH_3)_2$	OCH_3 (m)	NH-C_6H_4-Br (p)	4.5 (135)	7.2 (90)	17.4 (30)
50c	C_6H_{11}	OCH_3 (m)	NH-C_6H_4-Br (p)	**10.8 (80)**	**N.A.**	**N.A.**
50d	H	OCH_3 (m)	NH-C_6H_4-Br (p)	6.1 (125)	7.7 (60)	14.6 (25)
50e	C_6H_5	OCH_3 (m)	NH-C_6H_4-Br (p)	9.0 (110)	4.3 (25)	N.A.
50f	C_6H_5	OCH_3 (m)	NH-C_6H_3-3,4-methylenedioxy	N.A.	N.A.	N.A.
50g	C_6H_5	OCH_3 (m)	NH-C_6H_4-F (p)	N.A.	N.A.	N.A.
50h	2-thienyl	OCH_3 (p)	NH-C_6H_4-Br (p)	N.A.	N.A.	N.A.
50i	2-thienyl	OCH_3 (m)	NH-C_6H_3-3,4-methylenedioxy	N.A.	N.A.	N.A.
50j	2-thienyl	OCH_3 (m)	NH-C_6H_4-F (p)	N.A.	N.A.	N.A.
50k	C_6H_4-OCH_3	OCH_3 (p)	NH-C_6H_4-Br (p)	N.A.	N.A.	N.A.
50l	C_6H_4-OCH_3	OCH_3 (m)	NH-C_6H_3-3,4-methylenedioxy	N.A.	N.A.	N.A.
50m	C_6H_4-OCH_3	OCH_3 (m)	NH-C_6H_4-F (p)	N.A.	N.A.	N.A.
50n	C_6H_4-Cl	OCH_3 (p)	NH-C_6H_4-Br (p)	N.A.	N.A.	N.A.
50o	C_6H_4-Cl	OCH_3 (m)	NH-C_6H_3-3,4-methylenedioxy	N.A.	N.A.	N.A.
50p	C_6H_4-Cl	OCH_3 (m)	NH-C_6H_4-F (p)	N.A.	N.A.	N.A.
50q	C_6H_4-CH_3	OCH_3 (p)	NH-C_6H_4-Br (p)	N.A.	N.A.	N.A.
50r	C_6H_4-CH_3	OCH_3 (m)	NH-C_6H_3-3,4-methylenedioxy	N.A.	N.A.	N.A.
50s	C_6H_4-CH_3	OCH_3 (m)	NH-C_6H_4-F (p)	N.A.	N.A.	N.A.
50t	C_6H_4-F	OCH_3 (p)	NH-C_6H_4-Br (p)	N.A.	N.A.	N.A.
50u	C_6H_4-F	OCH_3 (m)	NH-C_6H_3-3,4-methylenedioxy	N.A.	N.A.	N.A.
50v	C_6H_4-F	OCH_3 (m)	NH-C_6H_4-F (p)	N.A.	N.A.	N.A.
46a				3.4 (75)	3.8 (70)	N.A.
fMLF				0.01	20.4	1.9
WKYMVm				0.5	0.001	0.01

[a]N.A., no activity was observed (no response was observed during first 2 min after addition of compounds under investigation) considering the limits of efficacy > 20 % and EC_{50} < 50 μM. The EC_{50} values are presented as the mean of three independent experiments, in which median effective concentration values (EC_{50}) were determined by nonlinear regression analysis of the dose-response curves (5-6 points) generated using GraphPad Prism 5 with 95% confidential interval ($p < 0.05$). Efficacy (in bracket) is expressed as percent of the response induced by 5 nM fMLF (FPR1) or 5 nM WKYMVm (FPR2 and FPR3).

4.1.4 EC_{50} and efficacy of C-4 modified *N*-arylacetamide pyridazinones

SAR studies at position 4 were performed on the lead compound **46a** (**table 4.1**) as function of the following criteria:

a) complete elimination of the substituents of the benzyl or replacement with a methyl group (**table 4.3**);

b) replacement of the methoxyphenyl in benzyl group with heterocycles or different aromatic groups (**table 4.4**);

c) introduction of different substituents in meta position of the benzyl group (**table 4.4**);

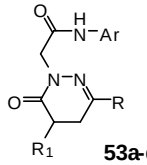
d) introduction of substituents in para position of the benzyl group (**table 4.4**);

e) elimination of OCH_3 on the benzyl group (**table 4.4**);

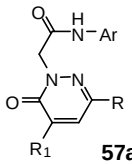
f) elimination of the CH_2-spacer of the benzyl group or introduction of different functionalized linkers (**table 4.5**).

a) With the exception of low active agonists **(±)-53b** and **57c,d** (**table 4.3**), the derivatives in which the methoxybenzyl is eliminated or replaced by a methyl group were completely devoid of activity and this behaviour is evident in both series of 4,5-dihydropyridazinones (**53a-d**) and pyridazinones (**57a-f**).

Table 4.3. Activity of compounds **53a-d** and **57a-f** (**schemes 14,15**) in HL-60 cells expressing human FPR1, FPR2, or FPR3.



53a-d



57a-f

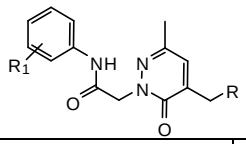
compd	**R**	**R_1**	**Ar**	**Ca^{2+} Mobilization EC_{50} (μM) and Efficacy (%)[a]**		
				FPR1	**FPR2**	**FPR3**
53a	CH_3	H	NH-C_6H_4-I (p)	N.A.	N.A.	N.A.
(±)-53b	C_6H_5	CH_3	NH-C_6H_4-Br (p)	23.5 (55)	7.0 (65)	N.A.
(±)-53c	C_6H_5	CH_3	NH-C_6H_4-F (p)	N.A.	N.A.	N.A.
(±)-53d	C_6H_5	CH_3	NH-C_6H_3-3,4-methylenedioxy	N.A.	N.A.	N.A.
57a	C_6H_{11}	H	NH-C_6H_4-F (p)	N.A.	N.A.	N.A.
57b	C_6H_{11}	H	NH-C_6H_3-3,4-methylenedioxy	N.A.	N.A.	N.A.
57c	CH_3	H	NH-C_6H_4-I (p)	30.4 (45)	19.7	N.A.
57d	C_6H_5	CH_3	NH-C_6H_4-Br (p)	21.5 (50)	10.1 (45)	N.A.
57e	C_6H_5	CH_3	NH-C_6H_4-F (p)	N.A.	N.A.	N.A.
57f	C_6H_5	CH_3	NH-C_6H_3-3,4-methylenedioxy	N.A.	N.A.	N.A.
46a				3.4 (75)	3.8 (70)	N.A.
fMLF				0.01	20.4	1.9
WKYMVm				0.5	0.001	0.01

[a]N.A., no activity was observed (no response was observed during first 2 min after addition of compounds under investigation) considering the limits of efficacy > 20 % and EC_{50} < 50 μM. The EC_{50} values are presented as the mean of three independent experiments, in which median effective concentration values (EC_{50}) were determined by nonlinear regression analysis of the dose-response curves (5-6 points) generated using GraphPad Prism 5 with 95% confidential interval ($p < 0.05$). Efficacy (in bracket) is expressed as percent of the response induced by 5 nM fMLF (FPR1) or 5 nM WKYMVm (FPR2 and FPR3).

b) Useful informations originated by replacement of methoxyphenyl group whit 5- and 6-membered heterocycles (**table 4.4**). Indeed, both thienyl derivatives (**61b,c**) showed an interesting level of potency

but a weak selectivity for FPR1, as well as furyl (**61a**) and piridyl (**66c**) analogues which had a similar profile of activity without relevant subtype-selectivity. On the contrary, the insertion of a naphtylmethyl (**61d**) and a 3,5 dimethoxybenzyl (**61f**, **62b**) groups in the position 4 was associated with decrease or complete loss of the activity.

Table 4.4. Activity of compounds **61a-p**, **62a-c** and **66a-c** (**schemes 16-18**) in HL-60 cells expressing human FPR1, FPR2, or FPR3.



compd	R	R_1	Ca^{2+} Mobilization EC_{50} (μM) and Efficacy (%)[a]		
			FPR1	**FPR2**	**FPR3**
61a	3-furyl	Br (p)	5.8 (100)	6.3 (75)	N.A.
61b	3-thienyl	Br (p)	4.5 (100)	14.1 (65)	N.A.
61c	2-thienyl	Br (p)	8.1 (140)	11.4 (60)	10.2 (25)
61d	1-naphthyl	Br (p)	13.8 (20)	N.A.	N.A.
66c	3-pyridyl	Br (p)	9.3 (85)	2.8 (90)	N.A.
61e	C_6H_4-OCH_3 (p)	Br (p)	**N.A.**	**2.4 ± 0.9 (70)**	**N.A.**
61f	C_6H_3-$(OCH_3)_2$ (3,5)	Br (p)	N.A.	N.A.	N.A.
62b	C_6H_3-$(OCH_3)_2$ (3,5)	I (p)	11.1 (90)	9.7	N.A.
61g	C_6H_4-F (m)	Br (p)	6.6 (110)	N.A.	N.A.
61h	C_6H_4-Cl (m)	Br (p)	10.5 (100)	N.A.	N.A.
62c	C_6H_4-Cl (m)	I (p)	6.8 (65)	10.6 (30)	N.A.
61i	C_6H_4-Br (m)	Br (p)	N.A.	N.A.	N.A.
61j	C_6H_4-SCH_3 (p)	Br (p)	N.A.	N.A.	N.A.
62a	C_6H_4-SCH_3 (p)	I (p)	19.7 (60)	15.9 (90)	30.1
61k	C_6H_4-CF_3 (p)	Br (p)	N.A.	N.A.	N.A.
66a	C_6H_4-CN (p)	Br (p)	N.A.	N.A.	N.A.
66b	C_6H_4-$CONH_2$ (p)	Br (p)	29.3 (40)	27.2 (80)	N.A.
61 l	C_6H_5	Br (p)	5.5 (50)	11.6 (20)	N.A.
61m	C_6H_5	F (p)	N.A.	N.A.	N.A.
61n	C_6H_5	3,4-methylenedioxy	6.9 (55)	N.A.	N.A.
61o	C_6H_4-Cl (p)	O-C_4H_9-*n* (p)	N.A.	N.A.	N.A.
61p	C_6H_4-OCH_3 (p)	O-C_4H_9-*n* (p)	N.A.	N.A.	N.A.
46a			3.4 ± 1.6 (75)	3.8 ± 1.5 (70)	N.A.
fMLF			0.01	20.4	1.9
WKYMVm			0.5	0.001	0.01

[a]N.A., no activity was observed (no response was observed during first 2 min after addition of compounds under investigation) considering the limits of efficacy > 20 % and EC_{50} < 50 μM. The EC_{50} values are presented as the mean ± S.D. of three independent experiments, in which median effective concentration values (EC_{50}) were determined by nonlinear regression analysis of the dose-response curves (5-6 points) generated using GraphPad Prism 5 with 95% confidential interval ($p < 0.05$). Efficacy (in bracket) is expressed as percent of the response induced by 5 nM fMLF (FPR1) or 5 nM WKYMVm (FPR2 and FPR3).

c) Replacement of 3-methoxy group, on the benzyl at position 4 of the pyridazinone nucleous of the lead compound **46a**, with different substituents showed as well attractive results (**table 4.4**). In fact,

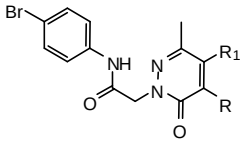
introduction of F in meta position gave compound **61g** which is a selective FPR1 agonist and a similar behaviour was evident for compound with Cl (**61h**). Differently, analogue **62c** bearing a 4-iodophenyl in the acetamidic spacer was a mixed FPR1/FPR2 agonist. Introduction of a Br (**61i**) in the place of OCH_3 group of lead compound **46a** was associated with loss of activity. All these data suggest that the presence of a substituents of limited hindrance in the meta position is an essential requirement for binding at FPR1 and FPR2.

d) Moving OCH_3 from the meta to the para position (**61e**) surprisingly resulted in high activity (EC_{50} = 2.4 μM) and selectivity for FPR2 (**table 4.4**). Oppositely, introduction of SCH_3 (**61j**, **62a**), CF_3 (**61k**), CN (**66a**) and $CONH_2$ (**66b**) in the para position was generally detrimental. However compound **62a**, bearing a 4-iodophenyl moiety on the acetamidic linker, and the benzamide derivative **66b** showed a weak mixed agonistic activity. These data confirmed that the methoxy group is the optimum for steric and electronic properties in the aromatic moiety at position 4 of the pyridazinone nucleous, as both biological results of old (**46a**, **table 4.1**) and new (**61e**, **table 4.4**) lead compounds showed. On the contrary, compounds **61o** and **61p** where a 4-Cl or a 4-OCH_3 were introduced in the benzyl group and bearing a buthoxy group on the phenylacetamide spacer were completely devoid of activity.

e) In comparison to lead compound **46a** (**table 4.1**), when OCH_3 group was eliminated from the benzyl fragment at position 4 of the pyridazinone scaffold (**61 l**) the activity lowered particularly as FPR2 agonist (**table 4.4**), while the concomitant presence of an unsubstituted benzyl at C-4 and F (**61m**) or methylenedioxy (**61n**) in the aryl acetamide side chain was associated in the first case with a complete loss of activity and in the second with a weak agonistic FPR1 activity.

f) Introduction of carbonyl group as spacer in the place of CH_2 of the benzyl group at position 4 of the pyridazinone ring resulted in compound **71** which is a potent mixed agonist of both FPR1 and FPR2 (**table 4.5**), while elimination of the methylenic linker originated a very selective ligand (**77**) at FPR1. When CH_2 was replaced by NH (**88**) an interesting activity was found mainly at FPR2 and the same behaviour was shown by the 5-acetyl analogue (**83**), whereas the synthetic precursor **87** resulted less potent as FPR2 ligand. Substitution of CH_2 with an amidic group was detrimental for activity, since compound **89a** was totally inactive and the 3-methoxy analogue **89b** showed low activity as FPR1/FPR2 agonist. Lastly, when more hindered substituents were introduced in the position 4, the activity disappeared completely, being compound **69** and the biaryl derivative **92** completely devoid of activity.

Table 4.5. Activity of compounds **69**, **71**, **77**, **83**, **87-89a,b** and **92** (**schemes 18-21**) in HL-60 cells expressing human FPR1, FPR2, or FPR3.



compd	R	R_1	Ca^{2+} Mobilization EC_{50} (μM) and Efficacy (%)[a]		
			FPR1	FPR2	FPR3
69	CH_2-C_6H_4-CONH(p)-C_6H_4-Br (p)	H	N.A.	N.A.	N.A.
71	CO-C_6H_4-OCH_3 (m)	H	**3.0 (140)**	**1.4 (100)**	**N.A.**
77	C_6H_4-OCH_3 (p)	H	**11.2 (55)**	**N.A.**	**N.A.**
83	NH-C_6H_4-OCH_3 (p)	$COCH_3$	13.5 (75)	2.3 (80)	N.A.
87	NH_2	H	8.1 (115)	29.4 (85)	N.A.
88	NH-C_6H_4-OCH_3 (p)	H	12.8 (100)	3.8 (85)	N.A.
89a	NHCO-C_6H_4-Br (p)	H	N.A.	N.A.	N.A.
89b	NHCO-C_6H_4-OCH_3 (m)	H	9.3 (120)	6.4 (70)	N.A.
92	N-(C_6H_4-OCH_3(p))$_2$	H	N.A.	N.A.	N.A.
46a			3.4 (75)	3.8 (70)	N.A.
fMLF			0.01	20.4	1.9
WKYMVm			0.5	0.001	0.01

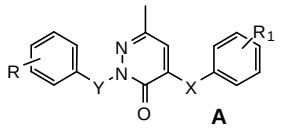
[a]N.A., no activity was observed (no response was observed during first 2 min after addition of compounds under investigation) considering the limits of efficacy > 20 % and EC_{50} < 50 μM. The EC_{50} values are presented as the mean of three independent experiments, in which median effective concentration values (EC_{50}) were determined by nonlinear regression analysis of the dose-response curves (5-6 points) generated using GraphPad Prism 5 with 95% confidential interval ($p < 0.05$). Efficacy (in bracket) is expressed as percent of the response induced by 5 nM fMLF (FPR1) or 5 nM WKYMVm (FPR2 and FPR3).

4.1.5 EC_{50} and efficacy of *N*-arylacetamide modified pyridazinones, N-2/C-4 inverted pyridazinones and C-3 substituted pyridazine analogue

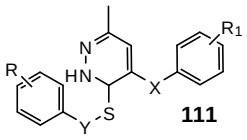
Elongation of carbon chain from one to two methylene groups, at the level of aryl acetamide side chain of lead compound **46a**, gave **95a** which resulted less potent of the lead compound as FPR1/FPR2 ligand (**table 4.6**). More substantial modifications of the functionalized chain were obtained with compounds **96a,b** and were completely detrimental. Further inactive compounds were the urea derivative **97a**, the inverse amide **97b**, as well as their superior homologues **103a,b**. Replacement of CH_2CONH of the lead compound **46a** with a secondary amine and an ether group gave compounds **100** and **101** respectively, which also resulted inactive. Other unproductive modifications of the functionalized chain were performed with the synthesis of compounds **104a,c**, and replacement of CONH with COO (**104b**) gave the same effect. Differently when the amidic group of the lead compound **46a** was changed into the corresponding thioamide (**105**) a low activity toward FPR2 is retained. Moreover, compounds **108** and **109**, in which the substituents at position 2 and 4 were interchanged, were inactive. Finally, moving the 4-

bromo phenylacetamide moiety from the N-2 to the C-3 of the pyridazinone led to a decrease of activity, being the pyridazine derivative **111** a selective but weak FPR2 agonist.

Table 4.6. Activity of compounds **95-97a,b**, **100-102**, **103a,b**, **104a-c**, **105**, **108**, **109** (structure **A**) and **111** (**schemes 22-27**) in HL-60 cells expressing human FPR1, FPR2, or FPR3.



A



111

Compd	Y	R	X	R_1	Ca^{2+} Mobilization EC_{50} (μM) and Efficacy (%)[a]		
					FPR1	FPR2	FPR3
95a	$(CH_2)_2CONH$	Br (p)	CH_2	OCH_3 (m)	9.7 ± 2.7 (30)	5.4 ± 1.2 (25)	N.A.
96a	CH_2	Br (p)	CH_2	OCH_3 (m)	N.A.	N.A.	N.A.
96b	CH_2CO	Br (p)	CH_2	OCH_3 (m)	N.A.	N.A.	N.A.
97a	$CH_2NHCONH$	Br (p)	CH_2	OCH_3 (m)	N.A.	N.A.	N.A.
97b	CH_2NHCO	Br (p)	CH_2	OCH_3 (m)	N.A.	N.A.	N.A.
103a	$(CH_2)_2NHCONH$	Br (p)	CH_2	OCH_3 (m)	N.A.	N.A.	N.A.
103b	$(CH_2)_2NHCO$	Br (p)	CH_2	OCH_3 (m)	N.A.	N.A.	N.A.
							N.A.
100	$(CH_2)_2NH$	Br (p)	CH_2	OCH_3 (m)	N.A.	N.A.	
101	$(CH_2)_2°$	Br (p)	CH_2	OCH_3 (m)	N.A.	N.A.	N.A.
104a	$CH_2CONHCH_2$	Br (p)	CH_2	OCH_3 (m)	N.A.	N.A.	N.A.
104b	CH_2COO	Br (p)	CH_2	OCH_3 (m)	N.A.	N.A.	N.A.
104c	$CH_2CON(CH_3)$	Br (p)	CH_2	OCH_3 (m)	N.A.	N.A.	N.A.
105	CH_2CSNH	Br (p)	CH_2	OCH_3 (m)	N.A.	8.3 (< 25)	N.A.
108	CH_2	OCH_3 (m)	NHCO	Br (p)	N.A.	N.A.	N.A.
109	CH_2	OCH_3 (m)	NHCONH	Br (p)	N.A.	N.A.	N.A.
111	CH_2CONH	Br (p)	CH_2	OCH_3 (p)	N.A.	14.7 (< 25)	N.A.
46a					3.4 (75)	3.8 (70)	N.A.
fMLF					0.01	20.4	1.9
WKYMVm					0.5	0.001	0.01

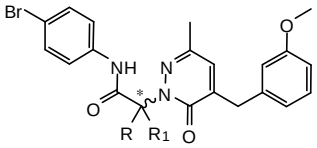
[a]N.A., no activity was observed (no response was observed during first 2 min after addition of compounds under investigation) considering the limits of efficacy > 20 % and EC_{50} < 50 μM. The EC_{50} values are presented as the mean ± S.D. of three independent experiments, in which median effective concentration values (EC_{50}) were determined by nonlinear regression analysis of the dose-response curves (5-6 points) generated using GraphPad Prism 5 with 95% confidential interval ($p < 0.05$). Efficacy (in bracket) is expressed as percent of the response induced by 5 nM fMLF (FPR1) or 5 nM WKYMVm (FPR2 and FPR3).

4.1.6 EC_{50} and efficacy of chiral pyridazinone analogues

The insertion of alkyl or aryl groups at the level of the methylene spacer in the acetamide side chain of the lead compound **46a** (**table 4.1**, **scheme 12**) led to series of branched and chiral compounds which were tested both as racemates and as pure enantiomers. Starting from compound **(±)-95b**, data showed that the racemate did not activate FPR2 but retained activity for FPR1 similar to that of **46a** (**table 4.7**). As soon as the biological tests on the pure enantiomers were ongoing, the higher activity of the R-(-)-

forms of the alkyl derivatives was evident from the beginning. Indeed, the first tested enantiomers **R-(-)-95b** and **R-(-)-117a** showed to be more active at both FPR1 and FPR2 compared to the corresponding S-(+)-forms [**S-(+)-95b** and **S-(+)-117a**].

Table 4.7. Activity of racemates and pure enantiomers (±)-**95b**, **(±)-117a-f** and non chiral homologue **118** (**schemes 22, 29, 30**) in HL-60 cells expressing human FPR1, FPR2, or FPR3.



compd	R	R_1	Ca^{2+} Mobilization EC_{50} (μM) and Efficacy (%)[a]		
			FPR1	FPR2	FPR3
(±)-95b	H	CH_3	3.2 ± 1.5 (90)	N.A.	N.A.
S-(+)-95b	H	CH_3	17.9 (35)	N.A.	N.A.
R-(-)-95b	H	CH_3	8.4 (80)	14.4 (70)	N.A.
(±)-117a	H	C_2H_5	1.4 (160)	1.2 (100)	N.A.
S-(+)-117a	H	C_2H_5	15.7 (130)	23.1 (55)	N.A.
R-(-)-117a	H	C_2H_5	2.8 (135)	3.0 (70)	N.A.
(±)-117b	H	*n*-C_3H_7	**2.8 (75)**	**2.3 (60)**	**N.A.**
S-(+)-117b	H	*n*-C_3H_7	**N.A.**	**N.A.**	**N.A.**
R-(-)-117b	H	*n*-C_3H_7	**0.5 (90)**	**0.6 (90)**	**N.A.**
(±)-117c	H	*i*-C_3H_7	**2.0 (75)**	**13.5 (30)**	**N.A.**
S-(+)-117c	H	*i*-C_3H_7	**N.A.**	**N.A.**	**N.A.**
R-(-)-117c	H	*i*-C_3H_7	**1.9 (95)**	**3.9 (70)**	**N.A.**
(±)-117d	H	*n*-C_4H_9	1.1 (110)	0.20 (110)	N.A.
S-(+)-117d	H	*n*-C_4H_9	21.8 (40)	15.2 (45)	N.A.
R-(-)-117d	H	*n*-C_4H_9	**0.7 (120)**	**0.10 (110)**	**N.A.**
(±)-117e	H	C_6H_5	**0.26 (80)**	**0.24 (45)**	**N.A.**
S-(+)-117e	H	C_6H_5	**0.16 (120)**	**0.18 (50)**	**N.A.**
R-(-)-117e	H	C_6H_5	**N.A.**	**N.A.**	**N.A.**
(±)-117f	CH_3	C_2H_5	**1.5 (70)**	**N.A.**	**N.A.**
S-(+)-117f	CH_3	C_2H_5	**2.1 (80)**	**N.A.**	**N.A.**
R-(-)-117f	CH_3	C_2H_5	**4.8 (50)**	**N.A.**	**N.A.**
118	CH_3	CH_3	**3.7 (50)**	**N.A.**	**N.A.**
46a			3.4 ± 1.6 (75)	3.8 ± 1.5 (70)	N.A.
fMLF			0.01	20.4	1.9
WKYMVm			0.5	0.001	0.01

[a]N.A., no activity was observed (no response was observed during first 2 min after addition of compounds under investigation) considering the limits of efficacy > 20 % and EC_{50} < 50 μM. The EC_{50} values are presented as the mean ± S.D. of three independent experiments, in which median effective concentration values (EC_{50}) were determined by nonlinear regression analysis of the dose-response curves (5-6 points) generated using GraphPad Prism 5 with 95% confidential interval ($p < 0.05$). Efficacy (in bracket) is expressed as percent of the response induced by 5 nM fMLF (FPR1) or 5 nM WKYMVm (FPR2 and FPR3).

This behaviour was mainly confirmed by testing *n*-propyl and *i*-propyl derivatives **(±)-117b** and **(±)-117c**. In this case the (+)-forms were completely inactive, while enantiomers **R-(-)-117b** and **R-(-)-117c**

showed an increase of activity being their EC_{50} in the sub-micromolar and low micromolar range respectively (**table 4.7**). Elongation of carbon chain on the chiral center gave *n*-butyl derivative **(±)-117d** which, even if it showed less stereoselectivity, in his (-)-form resulted the most potent FPR1/FPR2 mixed agonist of the series (EC_{50} = 100 nM for FPR2). Introduction of a phenyl group in the chiral center gave racemate **(±)-117e** having an activity in the sub-micromolar range. In this case, enantiomer **S-(+)-117e** is more potent of its racemate, while **R-(-)-117e** is completely devoid of activity. This is a surprising result in terms of stereoselectivity, also taking into account that the previously discussed alkyl derivatives have always in their R-(-)-forms the most potent compounds of each pair (see enantiomers **(-)-95b** and **(-)-117a-d**, **table 4.7**). Introduction of an additional alkyl group on the chiral center raised a complete loss of stereoselectivity and a total shift of activity toward FPR1. Indeed compounds **(+)-117f** and **(-)-117f**, bering on the chiral center a methyl and an ethyl group, are selective agonists of FPR1 and their racemate **(±)-117f** resulted more potent than the pure enantiomers on the same receptor. In this case the sterical hindrance on the chiral center seemed to be the main determinant for the activity and the selectivity, and this result was confirmed again by testing the non chiral analogue **118**, which is as well a selective and relatively potent FPR1 agonist.

4.1.7 Evaluation of chemotactic activity and Ca^{2+} mobilization

Compounds that showed the best agonistic profiles were as well selected to evaluate their activity as chemotactic agents and their ability to produce Ca^{2+} mobilization in human neutrophils.

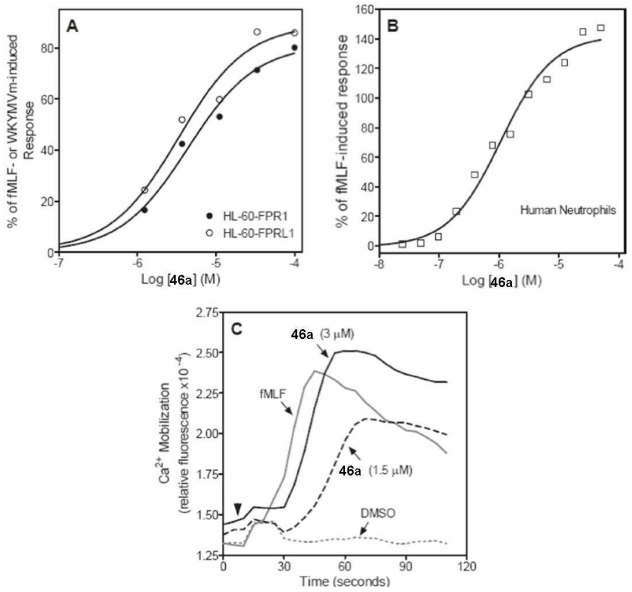


Figure 4.1. Analysis of Ca^{2+} mobilization in phagocytes treated with compound **46a**. HL-60-FPR1 and HL-60-FPR2 cells (A) or human neutrophils (B) were loaded with FLIPR calcium 3 dye, and Ca^{2+} flux was analyzed, as described in **section 6**. Responses were normalized to the response induced by 5 nM fMLF for HL-60-FPR1 cells and neutrophils, or 5 nM WKYMVm for HL-60-FPR2 cells, which were assigned a value of 100%. (C) Representative kinetics of Ca^{2+} mobilization after treatment with compound **46a** or fMLF. Human neutrophils were treated with the compound **46a** (1.5 and 3 μM), 5 nM fMLF (positive control), or 1% DMSO (negative control), and Ca^{2+} flux was monitored for the indicated times. The data are from one experiment that is representative of three independent experiments.

Table 4.8. Ca^{2+} mobilization and chemotactic activity in human neutrophils treated with selected FPR1/FPRL1 agonists.

EC_{50} (μM)		
Compd	**Ca^{2+} mobilization**	**Chemotaxis**
46a	2.6 ± 0.3	2.1 ± 0.8
46d	3.9 ± 0.6	8.2 ± 1.4
46e	6.7 ± 1.1	1.6 ± 0.2
46f	3.2 ± 1.2	1.8 ± 0.3
46h	3.2 ± 0.3	0.6 ± 0.3
46j	1.6 ± 0.8	0.9 ± 0.2
46l	1.1 ± 0.6	1.1 ± 0.6
46m	3.6 ± 1.0	1.2 ± 0.6
46n	3.6 ± 0.8	4.5 ± 2.5
46r	21.7 ± 4.2	1.9 ± 0.6
61e	4.3 ± 1.1	13.1 ± 2.3
95a	11.3 ± 2.8	11.8 ± 2.6
(±)-95b	0.8 ± 0.2	0.6 ± 0.4

The data are presented as the mean ± S.D. of three independent experiments with cells from different donors, in which median effective concentration values (EC_{50}) were determined by nonlinear regression analysis of the dose-response curves (5-6 points) generated using GraphPad Prism 5 with 95% confidential interval ($p < 0.05$).

Some of these FPRs agonists proved to be able to stimulate chemotaxis at sub-micromolar concentrations (**table 4.8**). Furthermore, the effect of selected agonists on Ca^{2+} flux in human neutrophils was also determined to verify if the results coming from HL-60 tests were confirmed in primary phagocytes (**table 4.8**). We usually found that both selective and nonselective agonists identified in HL-60 cell assays also induced Ca^{2+} flux in human neutrophils, with EC_{50} values in the range 0.8-21.7 μM. Among compounds tested to evaluate the chemotactic activity, the most potent resulted **46h** (EC_{50} = 0.6 μM) and **46j** (EC_{50} = 0.9 μM), which were both FPR1/FPR2 nonselective agonists. It is noteworthy that the FPR2-selective agonist (**61e**) showed lower potency (EC_{50} = 13.1 μM) as chemotactic agent.

For lead compound **46a** were also calculated dose-response curves as shown in **figure 4.1**.

4.2 Conclusions

In conclusion, the data acquired and processed till now showed that we have identified a novel chemotype endowed with interesting selective or mixed FPR1/FPR2 agonistic activity in human neutrophils. From biological tests it resulted evident that, by manipulating the chemical structure of a series of *N*-arylacetamide pyridazinones (**46a-s**, **scheme 12**, **table 4.1**), it is possible to achieve potency and selectivity towards FPR1 and FPR2 subtype receptors.

Going to analyse the data in our hands, we can observe that regarding the aromatic system at the end of the functionalized chain in position 2 (**scheme 12**, **table 4.1**), the presence of a lipophilic and/or electronegative substituent, such as F, Br, I or CH_3, in position para is an essential requirement for potency and/or selectivity.

The position 6 (**table 4.2**) of the pyridazinone ring resulted poorly tolerant to modifications. A methyl group is the substituent that gives the best results regarding the activity as agonist, while its elimination or substitution with more hindered moiety produce a deep loss of activity.

Likewise, the presence of an acetamide spacer at N-2 of the pyridazinone ring also plays a crucial role in specificity and potency (**table 4.6**). The role of both CO and NH in the side chain seems to indicate that a hydrogen bond donor (HBD) neighbouring an acceptor (HBA) system is also an essential requirement for binding at FPRs. Moreover, this HBD\HBA system must be placed at an appropriate distance from both the aromatic and the heterocyclic scaffold. The very low activity of the thioamide analogue **105** (**table 4.6**) further support this hypothesis.

Differently, the position 4 (**tables 4.3-4.5**) resulted more amenable to chemical manipulation, indeed heterocycles (e.g. thienyl, piridyl, furyl) or substituted benzyl groups and functionalised spacers (e.g. CO, NH) can be productively introduced at this level, retaining a good agonistic activity.

Lastly, the chiral compounds reported in this work (**table 4.7**) represent a series of homologues differently hindered at stereocenter level and all of them show a different affinity for the three FPR isoforms. Enantioselectivity showed from the R-(-)-enantiomers, among the alkyl derivatives, may be related to the ability of these compounds to establish a better interaction with the receptors, compared to the respective S-(+)-forms. On the other hand, for enantiomers S-**(+)-117e** and **R-(-)-117e** the activity of the pure enantiomers resulted exactly the opposite, probably due to the high hindrance and lipophilicity of the phenyl group. Furthermore, in contrast with compounds having just one alkyl group on the chiral center, dialkylated enantiomers of **(±)-117f** did not show enantioselectivity and in addition, chirality doesn't seem crucial for the activity. This result was evident testing non chiral dimethyl analogue **118**; however both dimethyl and chiral ethyl-methyl derivatives showed selectivity for FPR1.

Work is underway and further modification have been planned to develop new FPRs agonists hoping they may be useful to gain further structure-activity relationships in this class of compounds in order to optimize the potency and selectivity and to increase the knowledge of the pharmacological basis of FPRs binding and signalling.

5. EXPERIMENTAL CHEMISTRY

5.1 Materials and Methods

Reagents and starting materials were obtained from commercial sources. Extracts were dried over Na_2SO_4, and the solvents were removed under reduced pressure. All reactions were monitored by thin layer chromatography (TLC) using commercial plates precoated with Merck silica gel 60 F-254. Visualization was performed by UV fluorescence (λ_{max} = 254 nm) or by staining with iodine or potassium permanganate. Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063-0.200 mm; Merck), flash chromatography (Kieselgel 40, 0.040-0.063 mm; Merck), silica gel preparative TLC (Kieselgel 60 F_{254}, 20 x 20 cm, 2 mm), or CombiFlash® Rf System (using RediSep® Rf Silica Columns, Teledyne Isco, Lincoln, Nebraska, USA). Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. When reactions were performed in anhydrous conditions, the mixtures were maintained under nitrogen atmosphere. Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom 2000 (4.01.305) or CA Index Name.

The identity and purity of intermediates and final compounds was ascertained through NMR and TLC chromatography. All melting points were determined on a microscope hot stage Büchi apparatus and are uncorrected.

^{1}H NMR spectra were recorded with Avance 400 instruments (Bruker Biospin Version 002 with SGU). Chemical shifts (δ) are reported in ppm to the nearest 0.01 ppm, using the solvent as internal standard. Coupling constants (*J* values) are given in Hz and were calculated using 'TopSpin 1.3' software rounded to the nearest 0.1 Hz. Data are reported as follows: chemical shift, multiplicity [exch, exchange; br, broad; s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; sext, sextet; sept, septet; m, multiplet; or as a combination of these (e.g. dd, dt *etc.*)], integration, assignment and coupling constant(s). Diastereotopic protons are assigned as CH-*H*. Mass spectra (m/z) were recorded on a ESI-TOF mass spectrometer (Bruker Micro TOF) and reported mass values are within the error limits of ± 5 ppm mass units. IR spectra were measured as Nujol mulls for solids and neat for liquids with a PerkinElmer Spectrum (RX I FT-IR, Spectrum 1000) spectrometer. Absorption maxima (λ_{max}) are reported in wavenumbers (cm^{-1}).

Semi-preparative HPLC enantioseparations were performed using stainless-steel Chiralcel OD® (250 mm x 4.6 mm I.D., 10 μm particle size, Chiral Technologies Europe, Illkirch, France) and Lux Amylose-2® (250 mm x 4.6 mm I.D., 5 μm particle size, Phenomenex, Bologna, Italy) columns. The HPLC apparatus consisted of a Perkin-Elmer (Norwalk, CT) series 200 composed by quaternary pump, autosampler, Peltier column oven and UV-VIS detector coupled with a Biologic BioFrac fraction collector (from Bio-Rad, Milan, Italy). UV detector wavelength was fixed at 250 nm. HPLC-grade solvents were supplied by Sigma-Aldrich (Milan, Italy). The mobile phases were a mixtures of *n*-hexane/IPA and they were degassed by sonication just before use. The signal was acquired and processed by Totalchrom 6.3.1.0504

software. The values of retention time (t_R) are given in minutes. After semipreparative separation, the collected fractions were analyzed on the same HPLC instrument by chiral Lux Amylose-2® (50 mm x 4.6 mm I.D., 3 μm particle size, Phenomenex, Bologna, Italy) column to determine their enantiomeric excess (ee). The ee values were calculated from relative peak areas of enatiomeric pairs. The mobile phase was *n*-hexane/IPA 60:40. In analytical enantioseparations, a standard solution was prepared by diluting 0.1 mg of compounds into 1 mL of ethanol. The injection volume was 10 μL, the flow rate 1.0 mL/min, the temperature of column was 40 °C and UV detector wavelength was fixed at 250 nm.

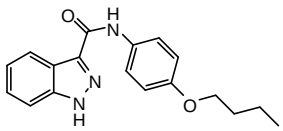
Specific rotations of enantiomers were measured at 589 nm with a Perkin-Elmer polarimeter model 241 equipped with a Na lamp. The volume of the cell was 2 mL, and the optical path was 10 cm. A standard solution was prepared by dissolving 20 mg of compounds into 2 mL of $CHCl_3$ (c = 1). The system was set at a temperature of 20 °C using a Neslab RTE 740 cryostat.

The circular dichroism (CD) spectra of enantiomers, dissolved in methanol (concentrations are about 0.25 mg/mL for analytes and 1 mg/mL for reference molecules) in a quartz cell (0.1 cm-path length) at 25 °C were measured using a Jasco model J-810 spectropolarimeter (Jasco, Ishikawa-cho, Hachioji City, Tokyo, Japan). The spectra were averaged over five instrumental scans from 350 to 215 nm at 50nm/min scanning speed, acquired and processed with Spectra Analysis software and the intensities are presented in terms of ellipticity values (mdeg).

5.2 Experimental

General Procedure for 1a,b. To a cooled (0 °C) and stirred suspension of commercially available indazole-3-carboxylic or indole-3-carboxylic acid (1.55 mmol) in $SOCl_2$ (3 mL), Et_3N (0.2 mL) was added. After 1 h at 60 °C, the mixture was cooled and the excess of $SOCl_2$ was removed in vacuo. The residue was then dissolved in anhydrous THF (1.5 mL) and cooled at 0 °C. A solution of 4-*n*.butoxyaniline (3.10 mmol) in anhydrous THF (1 mL) was added dropwise and the reaction was carried out at room temperature for 12 h. The mixture was concentrated in vacuo, diluted with ice-cold water (10 mL) and kept under stirring at 0 °C for 0.5 h; then the precipitate was filtered off and purified by crystallization from ethanol to afford intermediate compounds **1a,b**.

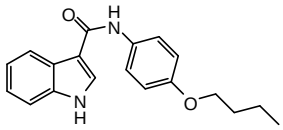
5.2.1 *N*-(4-Butoxyphenyl)-1*H*-indazole-3-carboxamide (1a)



Yield = 97 %; mp = 157-159 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, J = 6.7 Hz), 1.52 (sext, 2H, CH_2CH_3, J = 7.4 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, J = 6.9 Hz), 4.00 (t, 2H, OCH_2, J = 6.6 Hz), 6.94 (d, 2H, Ar, J = 8.3 Hz), 7.36 (t, 1H, Ar, J = 8.0 Hz), 7.50 (t, 1H, Ar, J = 8.4 Hz), 7.57 (d, 1H,

Ar, *J* = 8.4 Hz), 7.67 (d, 2H, Ar, *J* = 8.3 Hz), 8.49 (d, 1H, Ar, *J* = 8.2 Hz), 8.85 (exch br s, 1H, NH), 10.42 (exch br s, 1H, NH).

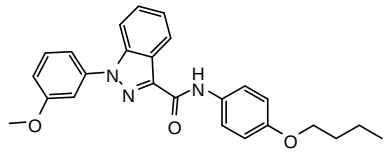
5.2.2 *N*-(4-Butoxyphenyl)-1*H*-indole-3-carboxamide (1b)



Yield = 94 %; mp = 181-182 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, *J* = 7.3 Hz), 1.52 (sext, 2H, CH_2CH_3, *J* = 7.5 Hz), 1.79 (quin, 2H, $CH_2CH_2CH_2$, *J* = 6.7 Hz), 3.97 (t, 2H, OCH_2, *J* = 6.4 Hz), 6.93 (d, 2H, Ar, *J* = 8.4 Hz), 7.31 (t, 2H, Ar, *J* = 3.2 Hz), 7.48 (d, 1H, Ar, *J* = 5.9 Hz), 7.56 (d, 2H, Ar, *J* = 8.5 Hz), 7.86 (s, 1H, Ar), 8.06 (d, 1H, Ar, *J* = 3.7 Hz), 8.78 (exch br s, 1H, NH), 9.21 (exch br s, 1H, NH).

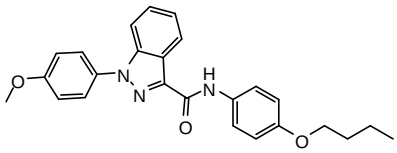
General Procedure for 2a-d. To the suspension of **1a** or **1b** (0.32 mmol), copper acetate (0.48 mmol) and 3- or 4-methoxyphenylboronic acid (0.64 mmol) in CH_2Cl_2 (2 mL), Et_3N (0.96 mmol) was added and the mixture was stirred at room temperature for 5-12 h. The suspension was extracted with 15% aqueous ammonia (10 mL), and the organic layer was washed with 10 mL of water and dried over Na_2SO_4. After removal of the solvent under reduced pressure, the residue was purified by flash column chromatography using as eluents CH_2Cl_2 for **2a,b** and CH_2Cl_2/MeOH 99:1 for **2c,d**.

5.2.3 *N*-(4-Butoxyphenyl)-1-(3-methoxyphenyl)-1*H*-indazole-3-carboxamide (2a)



Yield = 46 %; colorless-yellowish oil. ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, *J* = 7.4 Hz), 1.53 (sext, 2H, CH_2CH_3, *J* = 7.4 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, *J* = 8.1 Hz), 3.94 (s, 3H, OCH_3), 4.00 (t, 2H, OCH_2, *J* = 6.5 Hz), 6.95 (d, 2H, Ar, *J* = 7.9 Hz), 7.03 (dd, 1H, Ar, *J* = 6.1 Hz, *J* = 2.2 Hz), 7.36 (s, 1H, Ar), 7.38 (dd, 1H, Ar, *J* = 1.0 Hz, *J* = 1.0 Hz), 7.42 (d, 1H, Ar, *J* = 7.9 Hz) 7.52 (t, 2H, Ar, *J* = 8.2 Hz), 7.68 (d, 2H, Ar, *J* = 8.9 Hz), 7.77 (d, 1H, Ar, *J* = 8.6 Hz), 8.56 (d, 1H, Ar, *J* = 8.1 Hz), 8.86 (exch br s, 1H, NH).

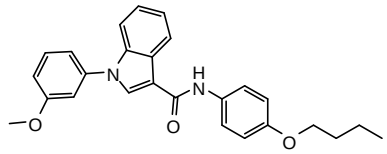
5.2.4 *N*-(4-Butoxyphenyl)-1-(4-methoxyphenyl)-1*H*-indazole-3-carboxamide (2b)



Yield = 60 %; colorless-yellowish oil. ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, *J* = 7.5 Hz), 1.53 (sext, 2H, CH_2CH_3, *J* = 7.4 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, *J* = 8.0 Hz), 3.94 (s, 3H, OCH_3), 4.00 (t, 2H,

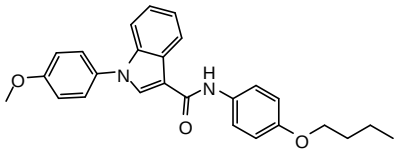
OCH_2, *J* = 6.6 Hz), 6.94 (d, 2H, Ar, *J* = 8.8 Hz), 7.13 (d, 2H, Ar, *J* = 9.0 Hz), 7.39 (t, 1H, Ar, *J* = 7.0 Hz), 7.49 (t, 1H, Ar, *J* = 8.4 Hz), 7.63-7.70 (m, 5H, Ar), 8.55 (d, 1H, Ar, *J* = 8.1 Hz), 8.85 (exch br s, 1H, NH).

5.2.5 *N*-(4-Butoxyphenyl)-1-(3-methoxyphenyl)-1*H*-indole-3-carboxamide (2c)



Yield = 23 %; colorless-yellowish oil. ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, *J* = 7.4 Hz), 1.53 (sext, 2H, CH_2CH_3, *J* = 7.4 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, *J* = 7.8 Hz), 3.90 (s, 3H, OCH_3), 4.00 (t, 2H, OCH_2, *J* = 6.6 Hz), 6.94 (d, 2H, Ar, *J* = 6.5 Hz), 7.01 (d, 1H, Ar, *J* = 8.2 Hz), 7.06 (s, 1H, Ar), 7.12 (d, 1H, Ar, *J* = 7.4 Hz), 7.36 (quin, 2H, Ar, *J* = 7.3 Hz), 7.48 (t, 1H, Ar, *J* = 8.0 Hz), 7.56-1.62 (m, 3H, Ar), 7.94 (br s, 1H, Ar), 8.15 (d, 2H, Ar, *J* = 7.2 Hz), 9.72 (exch br s, 1H, NH).

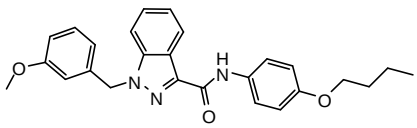
5.2.6 *N*-(4-Butoxyphenyl)-1-(4-methoxyphenyl)-1*H*-indole-3-carboxamide (2d)



Yield = 15 %; colorless-yellowish oil. ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, *J* = 7.4 Hz), 1.48 (sext, 2H, CH_2CH_3, *J* = 7.4 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, *J* = 7.8 Hz), 3.92 (s, 3H, OCH_3), 4.00 (t, 2H, OCH_2, *J* = 6.5 Hz), 6.94 (d, 2H, Ar, *J* = 8.9 Hz), 7.08 (d, 2H, Ar, *J* = 8.8 Hz), 7.33 (quin, 2H, Ar, *J* = 8.1 Hz), 7.42 (d, 2H, Ar, *J* = 8.7 Hz), 7.47 (d, 1H, Ar, *J* = 8.3 Hz), 7.57(d, 2H, Ar, *J* = 8.9 Hz), 7.88 (br s, 1H, Ar), 8.14 (d, 1H, Ar, *J* = 7.2 Hz).

General Procedure for 3a-h. Compounds **3a-h** were obtained starting from **1a** for **3a-d** and from **1b** for **3e-h** respectively. A mixture of **1a** or **1b** (0.32 mmol), K_2CO_3 (0.65 mmol), and the appropriate substituted benzyl halide (0.36 mmol) in anhydrous acetone (2 mL) was refluxed under stirring for 2-10 h. The mixture was then concentrated in vacuo, diluted with cold water, and extracted with CH_2Cl_2 (3 x 15 mL). The organic layer was dried with Na_2SO_4, evaporated in vacuo, and compounds **3a-h** were purified by column chromatography using alternatively CH_2Cl_2 (for **3a-d,g**) or CH_2Cl_2/MeOH 99:1 (for **3e,f,h**) as eluent.

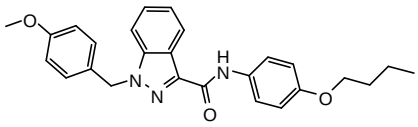
5.2.7 *N*-(4-Butoxyphenyl)-1-(3-methoxybenzyl)-1*H*-indazole-3-carboxamide (3a)



Yield = 65 %; colorless-yellowish oil. ^{1}H NMR ($CDCl_3$) δ 1.00 (t, 3H, CH_2CH_3, *J* = 7.4 Hz), 1.52 (sext, 2H, CH_2CH_3, *J* = 5.4 Hz), 1.79 (quin, 2H, $CH_2CH_2CH_2$, *J* = 6.4 Hz), 3.63 (s, 3H, OCH_3), 4.00 (t, 2H,

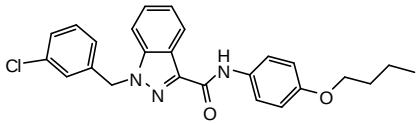
OCH_2, *J* = 6.5 Hz), 5.74 (s, 2H, CH_2N), 6.29 (s, 1H, Ar), 6.80 (d, 1H, Ar, *J* = 8.8 Hz), 6.94 (d, 2H, Ar, *J* = 9.0 Hz), 7.29 (s, 1H, Ar), 7.36 (d, 2H, Ar, *J* = 8.8 Hz), 7.44 (t, 2H, Ar, *J* = 8.2 Hz), 7.66 (d, 2H, Ar, *J* = 9.0 Hz), 8.49 (d, 1H, Ar, *J* = 8.2 Hz), 8.77 (exch br s, 1H, NH).

5.2.8 *N*-(4-Butoxyphenyl)-1-(4-methoxybenzyl)-1*H*-indazole-3-carboxamide (3b)



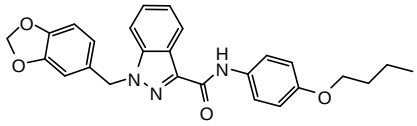
Yield = 73 %; mp = 115-16 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, *J* = 7.4 Hz), 1.53 (sext, 2H, CH_2CH_3, *J* = 7.4 Hz), 1.78 (quin, 2H, $CH_2CH_2CH_2$, *J* = 8.0 Hz), 3.79 (s, 3H, OCH_3), 3.99 (t, 2H, OCH_2, *J* = 6.5 Hz), 5.60 (s, 2H, CH_2N), 6.87 (d, 2H, Ar, *J* = 8.5 Hz), 6.94 (d, 2H, Ar, *J* = 8.9 Hz), 7.19 (d, 2H, Ar, *J* = 8.6 Hz), 7.30-7.34 (m, 1H, Ar), 7.40 (s, 2H, Ar), 7.67 (d, 2H, Ar, *J* = 8.9 Hz), 8.47 (d, 1H, Ar, *J* = 8.2 Hz), 8.80 (exch br s, 1H, NH).

5.2.9 *N*-(4-Butoxyphenyl)-1-(3-chlorobenzyl)-1*H*-indazole-3-carboxamide (3c)



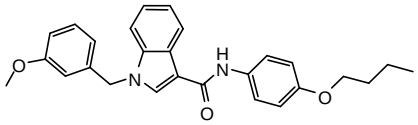
Yield = 86 %; mp = 119-20 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, *J* = 7.4 Hz), 1.52 (sext, 2H, CH_2CH_3, *J* = 7.6 Hz), 1.81 (quin, 2H, $CH_2CH_2CH_2$, *J* = 6.6 Hz), 4.00 (t, 2H, OCH_2, *J* = 6.5 Hz), 5.63 (s, 2H, CH_2N), 6.94 (d, 2H, Ar, *J* = 9.0 Hz), 7.08 (d, 2H, Ar, *J* = 6.7 Hz), 7.24 (s, 1H, Ar), 7.30-7.27 (m, 1H, Ar), 7.33-7.39 (m, 2H, Ar), 7.43-7.47 (m, 1H, Ar), 7.67 (d, 2H, Ar, *J* = 9.0 Hz), 8.49 (d, 1H, Ar, *J* = 8.1 Hz), 8.76 (exch br s, 1H, NH).

5.2.10 1-(1,3-Benzodioxol-5-ylmethyl)-*N*-(4-butoxyphenyl)-1*H*-indazole-3-carboxamide (3d)



Yield = 78 %; mp = 134-35 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, *J* = 7.3 Hz), 1.53 (sext, 2H, CH_2CH_3, *J* = 7.4 Hz), 1.76-1.83 (m, 2H, $CH_2CH_2CH_2$), 4.00 (t, 2H, OCH_2, *J* = 6.5 Hz), 5.56 (s, 2H, CH_2N), 5.95 (s, 2H, OCH_2O), 6.72 (s, 1H, Ar), 6.78 (s, 2H, Ar), 6.94 (d, 2H, Ar, *J* = 8.9 Hz), 7.30-7.34 (m, 1H, Ar), 7.42 (s, 2H, Ar), 7.67 (d, 2H, Ar, *J* = 8.9 Hz), 8.47 (d, 1H, Ar, *J* = 8.1 Hz), 8.78 (exch br s, 1H, NH).

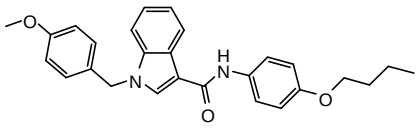
5.2.11 *N*-(4-Butoxyphenyl)-1-(3-methoxybenzyl)-1*H*-indole-3-carboxamide (3e)



Yield = 58 %; mp = 166-67 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, *J* = 7.3 Hz), 1.52 (sext, 2H, CH_2CH_3, *J* = 7.6 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, *J* = 7.0 Hz), 3.77 (s, 3H, OCH_3), 3.99 (t, 2H,

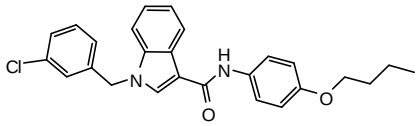
OCH_2, *J* = 6.5 Hz), 5.31 (s, 2H, CH_2N), 6.71 (s, 1H, Ar), 6.75 (d, 1H, Ar, *J* = 7.5 Hz), 6.85 (d, 1H, Ar, *J* = 8.3 Hz), 6.92 (d, 2H, Ar, *J* = 8.8 Hz) 7.25 (d, 1H, Ar, *J* = 7.8 Hz), 7.29-7.33 (m, 2H, Ar), 7.38 (d, 1H, Ar, *J* = 8.0 Hz), 7.53 (d, 2H, Ar, *J* = 8.3 Hz), 7.73 (br s, 1H, Ar), 8.10 (d, 1H, Ar, *J* = 7.1 Hz).

5.2.12 *N*-(4-Butoxyphenyl)-1-(4-methoxybenzyl)-1*H*-indole-3-carboxamide (3f)



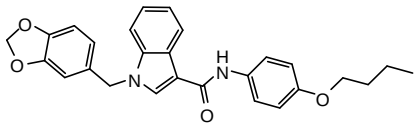
Yield = 29 %; mp = 160-62 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, *J* = 7.4 Hz), 1.52 (sext, 2H, CH_2CH_3, *J* = 7.6 Hz), 1.79 (quin, 2H, $CH_2CH_2CH_2$, *J* = 7.1 Hz), 3.81 (s, 3H, OCH_3), 3.98 (t, 2H, OCH_2, *J* = 6.6 Hz), 5.28 (s, 2H, CH_2N), 6.89 (q, 4H, Ar, *J* = 8.4 Hz), 7.13 (d, 2H, Ar, *J* = 8.7 Hz), 7.29-7.34 (m, 2H, Ar), 7.40 (d, 1H, Ar, *J* = 8.8 Hz), 7.53 (d, 2H, Ar, *J* = 8.9 Hz), 7.73 (br s, 1H, Ar), 8.10 (d, 1H, Ar, *J* = 12.1 Hz).

5.2.13 *N*-(4-Butoxyphenyl)-1-(3-chlorobenzyl)-1*H*-indole-3-carboxamide (3g)



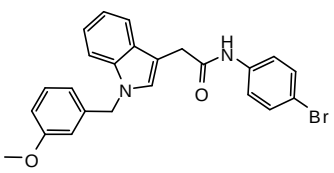
Yield = 51 %; mp = 136-37 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, *J* = 7.3 Hz), 1.52 (sext, 2H, CH_2CH_3, *J* = 7.6 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, *J* = 7.0 Hz), 3.99 (t, 2H, OCH_2, *J* = 6.5 Hz), 5.32 (s, 2H, CH_2N), 6.92 (d, 2H, Ar, *J* = 8.9 Hz), 7.02 (d, 1H, Ar, *J* = 7.1 Hz), 7.17 (s, 1H, Ar), 7.25-7.34 (m, 5H, Ar), 7.53 (d, 2H, Ar, *J* = 8.3 Hz), 7.75 (br s, 1H, Ar), 8.10 (d, 1H, Ar, *J* = 8.4 Hz).

5.2.14 1-(1,3-Benzodioxol-5-ylmethyl)-*N*-(4-butoxyphenyl)-1*H*-indole-3-carboxamide (3h)



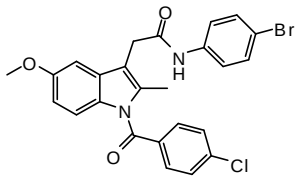
Yield = 42 %; mp = 165-67 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, *J* = 7.4 Hz), 1.52 (sext, 2H, CH_2CH_3, *J* = 7.4 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, *J* = 6.8 Hz), 3.99 (t, 2H, OCH_2, *J* = 6.6 Hz), 5.23 (s, 2H, CH_2N), 5.96 (s, 2H, OCH_2O), 6.64 (s, 1H, Ar, *J* = 8.4 Hz), 6.71 (d, 1H, Ar, *J* = 7.9 Hz), 6.78 (d, 1H, Ar, *J* = 7.9 Hz), 6.92 (d, 2H, Ar, J = 8.9 Hz), 7.31-7.33 (m, 2H, Ar), 7.40 (m, 1H, Ar), 7.53 (d, 2H, Ar, *J* = 8.2 Hz), 7.71 (br s, 1H, Ar), 8.09 (d, 1H, Ar, *J* = 7.7 Hz).

5.2.15 *N*-(4-Bromophenyl)-2-(1-(3-methoxybenzyl)-1*H*-indol-3-yl)acetamide (5)



A mixture of **4** (0.46 mmol), K_2CO_3 (0.92 mmol) and 3-methoxybenzyl chloride (0.69 mmol) in anhydrous acetone (3 mL) was refluxed under stirring for 4 h and then concentrated in vacuo. After dilution with cold water, it was extracted with CH_2Cl_2 (3 x 15 mL) and the solvent was evaporated in vacuo to afford the crude **5**, which was purified by column chromatography using toluene/ethyl acetate 7:3 as eluent. Yield = 29 %; mp = 153-55 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3,77 (s, 3H, OCH_3); 3,90 (s, 2H, $COCH_2$); 5,33 (s, 2H, CH_2N); 6,68 (s, 1H, CH); 6,76 (d, 1H, Ar, J = 7.1 Hz); 7,84 (dd, 1H, Ar, J = 8.5 Hz, J = 1.9 Hz); 7,17-7,30 (m, 5H, Ar); 7,36-7,41 (m, 4H, Ar); 7,63 (d, 1H, Ar, J = 8.2 Hz).

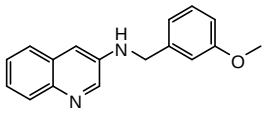
5.2.16 *N*-(4-Bromophenyl)-2-[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetamide (6)



To a solution of indomethacin (0.28 mmol) in anhydrous CH_2Cl_2 (5 ml) were added DCC (0.28 mmol) and 4-Bromoaniline (0.28 mmol). The reaction mixture was stirred at room temperature for 24 h. The solid residue was filtered off in vacuo and the solution was washed with HCl 2 N (2 x 10 mL) and with H_2O (10 mL). The organic phase was dried over $NaSO_4$ and concentrated under reduce pressure. The resulting residue was purified by flash column chromatography using CH_2Cl_2/MeOH 99:1 as eluent to furnish **6** as a white solid. Yield = 35 %; mp = 200-01 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2,48 (s, 3H, CH_3); 3,83 (s, 5H, OCH_3 + $COCH_2$); 6,74 (dd, 1H, Ar, J = 6.6 Hz, J = 2.5 Hz); 6,88 (d, 1H, Ar, J = 9.1 Hz); 6,94 (d, 1H, Ar, J = 2.3 Hz); 7,25 (exch, br, s, 1H, NH); 7,31 (d, 2H, Ar, J = 8.8 Hz); 7,41 (d, 2H, Ar, J = 8.7 Hz); 7,52 (d, 2H, Ar, J = 8.4 Hz); 7,71 (d, 2H, Ar, J = 8.4 Hz).

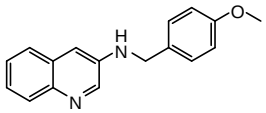
General Procedure for 7a-d. A mixture of 3-aminoquinoline (0.69 mmol), K_2CO_3 (1.38 mmol) and the appropriate substituted benzyl halide (0.69 mmol) in anhydrous acetone (3 mL) was refluxed under stirring for 1-3 h. Extra benzyl halide (0.69 mmol) was added and the reaction was kept refluxing for additional 2-4 h. The mixture was then concentrated in vacuo, diluted with cold water and extracted with CH_2Cl_2 (3 x 15 mL). The organic layer was dried over Na_2SO_4 and evaporated in vacuo to afford the crude compounds **7a-d** which were purified by column chromatography using cyclohexane/ethyl acetate 1:2 (for **7a,b,d**) or 2:1 (for **7c**) respectively as eluent.

5.2.17 *N*-(3-Methoxybenzyl)quinolin-3-amine (7a)



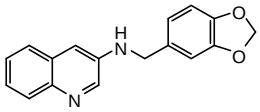
Yield = 49 %; clear oil. ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H, OCH_3), 4.44 (s, 2H, CH_2N), 4.67 (exch br s, 1H, NH), 6.86 (dd, 1H, Ar, J = 8.2 Hz, J = 2.5 Hz), 6.98 (s, 1H, Ar), 7.02 (d, 1H, Ar, J = 7.6 Hz), 7.07 (d, 1H, Ar, J = 2.7 Hz), 7.31 (t, 1H, Ar, J = 7.9 Hz), 7.43-7.45 (m, 2H, Ar), 7.59-7.61 (m, 2H, Ar), 7.98-8.01 (m, 1H, Ar), 8.59 (d, 1H, Ar, J = 2.8 Hz).

5.2.18 *N*-(4-Methoxybenzyl)quinolin-3-amine (7b)



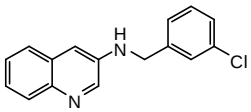
Yield = 60 %; mp = 80-82 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.83 (s, 3H, OCH_3), 4.37 (s, 2H, CH_2N), 4.43 (exch br s, 1H, NH), 6.92 (d, 2H, Ar, J = 8.7 Hz), 7.05 (d, 1H, Ar, J = 2.7 Hz), 7.34 (d, 2H, Ar, J = 8.6 Hz), 7.42-7.44 (m, 2H, Ar), 7.59-7.63 (m, 1H, Ar), 7.95-7.98 (m, 1H, Ar), 8.50 (d, 1H, Ar, J = 2.8 Hz).

5.2.19 *N*-(1,3-Benzodioxol-5-ylmethyl)quinolin-3-amine (7c)



Yield = 26 %; brown oil. ^{1}H NMR ($CDCl_3$) δ 4.40 (s, 2H, CH_2N), 5.21 (exch br s, 1H, NH), 5.97 (s, 3H, OCH_2O), 6.81 (d, 1H, Ar, J = 7.8 Hz), 6.91 (d, 1H, Ar, J = 11.2 Hz), 7.13 (s, 1H, Ar), 7.46-7.49 (m, 2H, Ar), 7.62-7.65 (m, 1H, Ar), 8.06-8.09 (m, 1H, Ar), 8.78 (s, 1H, Ar).

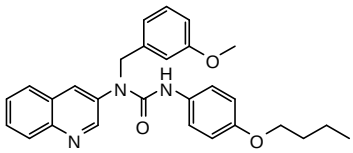
5.2.20 *N*-(3-Chlorobenzyl)quinolin-3-amine (7d)



Yield = 22 %; clear oil. ^{1}H NMR ($CDCl_3$) δ 4.51 (d, 2H, CH_2N, J = 4.5 Hz), 5.73 (exch br s, 1H, NH), 7.13 (d, 1H, Ar, J = 2.6 Hz), 7.29-7.34 (m, 3H, Ar), 7.44 (s, 1H, Ar), 7.49-7.52 (m, 2H, Ar), 7.63-7.65 (m, 1H, Ar), 8.13 (t, 1H, Ar, J = 6.3 Hz), 8.94 (s, 1H, Ar).

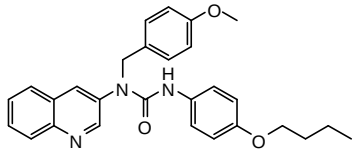
General Procedure for 8a-d. The appropriate amine of type **7** (**7a-d**) (0.34 mmol) was dissolved in 2 mL of anhydrous CH_2Cl_2 and 4-*n*.butoxyphenyl isocyanate (0.37 mmol) was added under stirring. The reaction was carried out at room temperature for 3-8 h, then the solid residue was filtered off and the solution was evaporated in vacuo to afford compounds **8a-d**, which were purified by flash column chromatography using cyclohexane/ethyl acetate 1:1 (for **7a,b**) or 2:1 (for **7c,d**) respectively as eluent.

5.2.21 3-(4-Butoxyphenyl)-1-(3-methoxybenzyl)-1-(quinolin-3-yl)urea (8a)



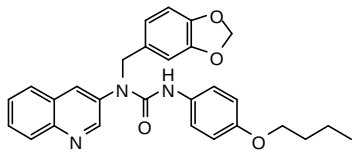
Yield = 58 %; clear oil. ^{1}H NMR ($CDCl_3$) δ 0.98 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.48 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.75 (quin, 2H, $CH_2CH_2CH_2$, J = 7.0 Hz), 3.77 (s, 3H, OCH_3), 3.93 (t, 2H, OCH_2, J = 6.5 Hz), 5.04 (s, 2H, CH_2N), 6.10 (exch br s, 1H, NH), 6.81-6.84 (m, 3H, Ar), 6.88 (d, 2H, Ar, J = 7.4 Hz), 7.19-7.25 (m, 3H, Ar), 7.63 (t, 1H, Ar, J = 8.2 Hz), 7.78-7.82 (m, 2H, Ar), 8.01 (s, 1H, Ar), 8.18 (d, 1H, Ar, J = 8.7 Hz), 8.79 (d, 1H, Ar, J = 2.4 Hz).

5.2.22 3-(4-Butoxyphenyl)-1-(4-methoxybenzyl)-1-(quinolin-3-yl)urea (8b)



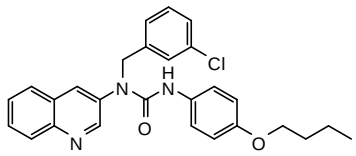
Yield = 47 %; clear oil. ^{1}H NMR ($CDCl_3$) δ 0.97 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.48 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.74 (quin, 2H, $CH_2CH_2CH_2$, J = 7.0 Hz), 3.79 (s, 3H, OCH_3), 3.92 (t, 2H, OCH_2, J = 6.5 Hz), 4.97 (s, 2H, CH_2N), 6.18 (exch br s, 1H, NH), 6.81 (dd, 4H, Ar, J = 2.0 Hz, J = 6.6 Hz), 7.21 (dd, 4H, Ar, J = 6.4 Hz, J = 2.1 Hz), 7.62 (t, 1H, Ar, J = 7.0 Hz), 7.78 (t, 2H, Ar, J = 7.2 Hz), 7.97 (s, 1H, Ar), 8.14 (d, 1H, Ar, J = 8.0 Hz), 8.71 (d, 1H, Ar, J = 2.3 Hz).

5.2.23 1-(1,3-Benzodioxol-5-ylmethyl)-3-(4-butoxyphenyl)-1-(quinolin-3-yl)urea (8c)



Yield = 36 %; brown oil. ^{1}H NMR ($CDCl_3$) δ 0.97 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.48 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.75 (quin, 2H, $CH_2CH_2CH_2$, J = 7.0 Hz), 3.92 (t, 2H, OCH_2, J = 6.6 Hz), 4.96 (s, 2H, CH_2N), 5.96 (s, 3H, OCH_3), 6.20 (exch br s, 1H, NH), 6.65-6-71 (m, 2H, Ar), 6.81 (d, 2H, Ar, J = 9.0 Hz), 6.91 (s, 1H, Ar), 7.21 (d, 2H, Ar, J = 9.0 Hz), 7.66 (t, 1H, Ar, J = 7.2 Hz), 7.82 (q, 2H, Ar, J = 6.7 Hz), 8.06 (s, 1H, Ar), 8.21 (d, 1H, Ar, J = 8.5 Hz), 8.75 (d, 1H, Ar, J = 2.4 Hz).

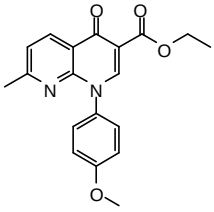
5.2.24 3-(4-Butoxyphenyl)-1-(3-chlorobenzyl)-1-(quinolin-3-yl)urea (8d)



Yield = 44 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.98 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.48 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.75 (quin, 2H, $CH_2CH_2CH_2$, J = 7.1 Hz), 3.93 (t, 2H, OCH_2, J = 6.6 Hz), 5.03 (s, 2H, CH_2N), 6.33 (exch br s, 1H, NH), 6.82 (d, 2H, Ar, J = 9.0 Hz), 7.17-7.26 (m, 5H, Ar), 7.35 (s, 1H, Ar), 7.67 (t, 1H, Ar, J = 7.8 Hz), 7.79-7.85 (m, 2H, Ar), 8.06 (s, 1H, Ar), 8.19 (d, 1H, Ar, J = 8.4 Hz), 8.77 (d, 1H, Ar, J = 2.4 Hz).

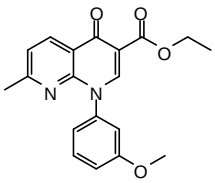
General Procedure for 11a,b. To a suspension of **10** (0.86 mmol), copper acetate (1.29 mmol) and the appropriate butoxyphenylboronic acid (1.72 mmol) in CH_2Cl_2 (2 mL), Et_3N (1.72 mmol) was added. The mixture was stirred at room temperature for 15-20 h. The suspension was extracted with 33% aqueous ammonia/saturated aqueous EDTA 1:1 solution (3 x 10 mL) and the organic layer was washed with 10 mL of water and dried over Na_2SO_4. After removal of the solvent, the residue was purified by flash column chromatography using as eluent cyclohexane/ethyl acetate 1:3.

5.2.25 Ethyl-1-(4-methoxyphenyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (11a)



Yield = 34 %; mp = 146-47 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.42 (t, 3H, CH_2CH_3, J = 7.1 Hz), 2.52 (s, 3H, CH_3C=N), 3.92 (s, 3H, OCH_3), 4.41 (q, 2H, OCH_2, J = 7.1 Hz), 7.06 (d, 2H, Ar, J = 9.0 Hz), 7.25 (d, 1H, Ar, J = 8.1 Hz), 7.35 (d, 2H, Ar, J = 8.9 Hz), 8.67 (s, 1H, Ar), 8.69 (d, 1H, Ar, J = 8.1 Hz).

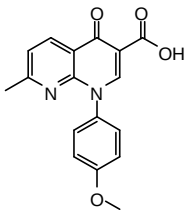
5.2.26 Ethyl-1-(3-methoxyphenyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (11b)



Yield = 14 %; mp = 165-66 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.43 (t, 3H, CH_2CH_3, J = 7.1 Hz), 2.54 (s, 3H, CH_3C=N), 3.89 (s, 3H, OCH_3), 4.42 (q, 2H, OCH_2, J = 7.1 Hz), 6.99 (t, 1H, Ar, J = 2.2 Hz), 7.03 (d, 1H, Ar, J = 7.8 Hz), 7.08 (d, 1H, Ar, J = 7.7 Hz), 7.27 (d, 1H, Ar, J = 8.5 Hz), 7.48 (t, 1H, Ar, J = 8.1 Hz), 8.69 (s, 1H, Ar), 8.70 (d, 1H, Ar, J = 8.1 Hz).

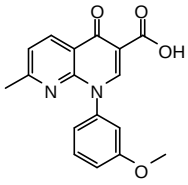
General Procedure for 12a,b. A suspension of the appropriate derivative **11a** or **11b** respectively (0.29 mmol), and 6 N NaOH (3 mL) in ethanol (3 mL) was stirred at rt for 0.5-1 h. The mixture was then concentrated in vacuo, diluted with cold water and acidified with 6 N HCl. The final product was filtered off by suction and recrystallized from ethanol.

5.2.27 1-(4-Methoxyphenyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (12a)



Yield = ~ 100 %; mp = 268-70 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.61 (s, 3H, CH_3C=N), 3.94 (s, 3H, OCH_3), 7.08 (d, 2H, Ar, J = 9.0 Hz), 7.35 (d, 2H, Ar, J = 9.0 Hz), 7.41 (d, 1H, Ar, J = 8.2 Hz), 8.73 (d, 1H, Ar, J = 8.2 Hz), 8.97 (s, 1H, Ar), 14.60 (exch br s, 1H, COOH).

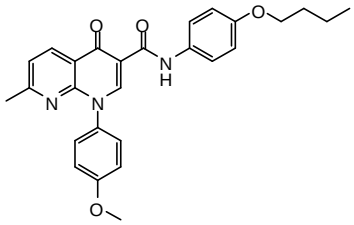
5.2.28 1-(3-Methoxyphenyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (12b)



Yield = 80 %; mp = 217-19 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.61 (s, 3H, CH_3C=N), 3.90 (s, 3H, OCH_3), 6.97 (t, 1H, Ar, J = 2.2 Hz), 7.01 (d, 1H, Ar, J = 7.8 Hz), 7.12 (d, 1H, Ar, J = 6.0 Hz), 7.42 (d, 1H, Ar, J = 8.2 Hz), 7.50 (t, 1H, Ar, J = 8.1 Hz), 8.74 (d, 1H, Ar, J = 8.2 Hz), 8.98 (s, 1H, Ar), 14.58 (exch br s, 1H, COOH).

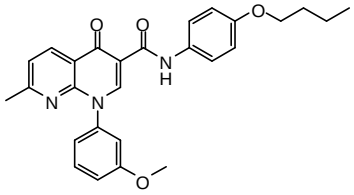
General Procedure for 14a,b. To cooled (0 °C) and stirred suspension of **12a** or **12b** (0.19 mmol) in $SOCl_2$ (1.5 mL), Et_3N (0.2 mL) was added. After 2-3 h at room temperature the excess of $SOCl_2$ was removed in vacuo. Due to their instability, the intermediates **13a,b** obtained were not isolated and characterized. Thus, the residue was dissolved in anhydrous THF (2 mL) and cooled to 0 °C. A solution of 4-*n*.butoxyaniline (0.38 mmol) in anhydrous THF (1 mL) was added dropwise to the mixture and the reaction was carried out at room temperature for 2 h. The mixture was concentrated under reduced pressure and, after dilution with ice-cold water (10 mL), the precipitate was then filtered and purified by flash column chromatography using cyclohexane/ethyl acetate 2:1 (for **14a**) or cyclohexane/ethyl acetate 1:1 (for **14b**) as eluent.

5.2.29 *N*-(4-Butoxyphenyl)-1-(4-methoxyphenyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (14a)



Yield = 35 %; mp = 154-55 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.00 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.52 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.79 (quin, 2H, $CH_2CH_2CH_2$, J = 7.9 Hz), 2.58 (s, 3H, CH_3C=N), 3.94 (s, 3H, OCH_3), 3.99 (t, 2H, OCH_2, J = 6.5 Hz), 6.92 (d, 2H, Ar, J = 9.0 Hz), 7.07 (d, 2H, Ar, J = 8.9 Hz), 7.35 (d, 1H, Ar, J = 8.2 Hz), 7.38 (d, 2H, Ar, J = 8.9 Hz), 7.70 (d, 2H, Ar, J = 9.0 Hz), 8.74 (d, 1H, Ar, J = 8.2 Hz), 9.08 (s, 1H, Ar), 11.97 (exch br s, 1H, NH).

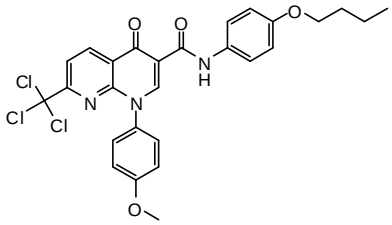
5.2.30 *N*-(4-Butoxyphenyl)-1-(3-methoxyphenyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (14b)



Yield = 44 %; mp = 203-05 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.00 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.52 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.79 (quin, 2H, $CH_2CH_2CH_2$, J = 7.1 Hz), 2.59 (s, 3H, CH_3C=N), 3.89 (s, 3H, OCH_3), 3.99 (t, 2H, OCH_2, J = 6.5 Hz), 6.92 (d, 2H, Ar, J = 9.0 Hz), 7.01 (s, 1H, Ar), 7.05 (d, 1H, Ar, J = 7.8 Hz), 7.10 (d, 1H, Ar, J = 7.7 Hz), 7.35 (d, 1H, Ar, J = 8.2 Hz), 7.48 (t, 1H, Ar, J = 8.1 Hz), 7.70 (d, 2H, Ar, J = 9.0 Hz), 8.74 (d, 1H, Ar, J = 8.2 Hz), 9.09 (s, 1H, Ar), 11.95 (exch br s, 1H, NH).

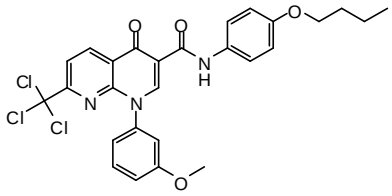
General Procedure for 16a,b. To cooled (0 °C) and stirred suspensions of **12a** or **12b** (0.29 mmol) in $SOCl_2$ (1.5 mL), Et_3N (0.2 mL) was added. After 2-3 h at 60 °C, the mixture was allowed to cool down and the excess of $SOCl_2$ was removed in vacuo. Due to their instability, the intermediates **15a,b** obtained were not isolated and characterized. Thus, the residue was dissolved in anhydrous THF (1 mL) and cooled to 0 °C. A solution of 4-*n*.Butoxyaniline (0.58 mmol) in anhydrous THF (1 mL) was added dropwise to the mixture and the reaction was carried out at room temperature for 2 h. The mixture was then concentrated in vacuo and diluted with ice-cold water (10 mL). The precipitate was filtered off and purified by flash column chromatography using CH_2Cl_2 (for **15a**) or CH_2Cl_2/MeOH 99:1 (for **15b**) as eluent.

5.2.31 *N*-(4-Butoxyphenyl)-1-(4-methoxyphenyl)-4-oxo-7-trichloromethyl-1,4-dihydro-1,8-naphthyridine-3-carboxamide (16a)



Yield = 49 %; mp = 289-91 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.52 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.81 (quin, 2H, $CH_2CH_2CH_2$, J = 6.6 Hz), 3.94 (s, 3H, OCH_3), 4.00 (t, 2H, OCH_2, J = 6.5 Hz), 6.93 (d, 2H, Ar, J = 9.0 Hz), 7.07 (d, 2H, Ar, J = 8.9 Hz), 7.44 (d, 2H, Ar, J = 8.9 Hz), 7.70 (d, 2H, Ar, J = 9.00 Hz), 8.15 (d, 1H, Ar, J = 8.4 Hz), 9.02 (d, 1H, Ar, J = 8.4 Hz), 9.20 (s, 1H, Ar), 11.75 (exch br s, 1H, NH). MS (ESI), calcd. For $C_{27}H_{24}Cl_3N_3O_4$, 560,86. Found: *m/z* 560.36 $[M]^+$, 562.36 $[M + H]^+$.

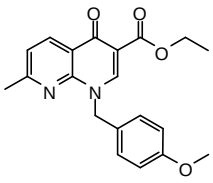
5.2.32 *N*-(4-Butoxyphenyl)-1-(3-methoxyphenyl)-4-oxo-7-trichloromethyl-1,4-dihydro-1,8-naphthyridine-3-carboxamide (16b)



Yield = 54 %; mp = 148-50 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.52 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, J = 6.8 Hz), 3.88 (s, 3H, OCH_3), 3.99 (t, 2H, OCH_2, J = 6.5 Hz), 6.93 (d, 2H, Ar, J = 8.9 Hz), 7.08 (s, 1H, Ar), 7.10 (d, 2H, Ar, J = 8.1 Hz), 7.49 (t, 1H, Ar, J = 8.1 Hz), 7.70 (d, 2H, Ar, J = 8.9 Hz), 8.15 (d, 1H, Ar, J = 8.4 Hz), 9.02 (d, 1H, Ar, J = 8.4 Hz), 9.23 (s, 1H, Ar), 11.73 (exch br s, 1H, NH). MS (ESI), calcd. For $C_{27}H_{24}Cl_3N_3O_4$, 560,86. Found: *m/z* 560.36 $[M]^+$, 562.36 $[M + H]^+$, 584.18 $[M + Na]^+$.

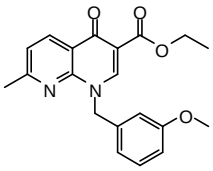
General Procedure for 17a-c. A mixture of **10** (0.65 mmol), K_2CO_3 (1.30 mmol), and the appropriate substituted benzyl halide (0.71 mmol) in anhydrous acetone (3 mL) was refluxed under stirring for 5-7 h. The mixture was then concentrated in vacuo, diluted with cold water and extracted with CH_2Cl_2 (3 x 15 mL). The organic layer was dried over Na_2SO_4, evaporated in vacuo and compounds **17a-c** were purified by column chromatography using alternatively cyclohexane/ethyl acetate 1:6 (for **17a,b**) or cyclohexane/ethyl acetate 1:9 (for **17c**) as eluent.

5.2.33 Ethyl-1-(4-methoxybenzyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (17a)



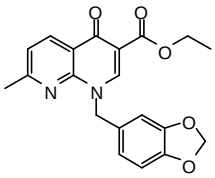
Yield = 35 %; mp = 107-09 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.41 (t, 3H, CH_2CH_3, J = 7.1 Hz), 2.69 (s, 3H, CH_3C=N), 3.80 (s, 3H, OCH_3), 4.40 (q, 2H, OCH_2, J = 7.1 Hz), 5.59 (s, 2H, NCH_2), 6.88 (d, 2H, Ar, J = 8.6 Hz), 7.26 (d, 1H, Ar, J = 8.1 Hz), 7.32 (d, 2H, Ar, J = 8.1 Hz), 8.66 (d, 1H, Ar, J = 8.1 Hz), 8.71 (s, 1H, Ar).

5.2.34 Ethyl-1-(3-methoxybenzyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (17b)



Yield = 18 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.43 (t, 3H, CH_2CH_3, J = 7.0 Hz), 2.69 (s, 3H, CH_3C=N), 3.80 (s, 3H, OCH_3), 4.43 (q, 2H, OCH_2, J = 7.1 Hz), 5.69 (s, 2H, NCH_2), 6.87 (d, 1H, Ar, J = 8.5 Hz), 6.92 (s, 1H, Ar), 6.93 (d, 1H, Ar, J = 8.2 Hz), 7.26-7.31 (m, 2H, Ar), 8.68 (d, 1H, Ar, J = 8.0 Hz), 8.71 (s, 1H, Ar).

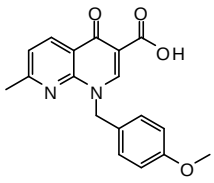
5.2.35 Ethyl-1-(1,3-benzodioxol-5-ylmethyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (17c)



Yield = 70 %; mp = 152-54 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.42 (t, 3H, CH_2CH_3, J = 7.1 Hz), 2.69 (s, 3H, CH_3C=N), 4.40 (q, 2H, OCH_2, J = 7.1 Hz), 5.54 (s, 2H, NCH_2), 5.96 (s, 2H, OCH_2O), 6.78 (d, 1H, Ar, J = 7.7 Hz), 6.87 (d, 2H, Ar, J = 8.2 Hz), 7.28 (d, 1H, Ar, J = 3.0 Hz), 8.66 (d, 2H, Ar, J = 7.3 Hz).

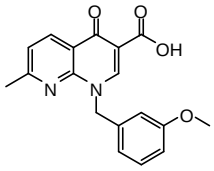
General Procedure for 18a-c. A suspension of the appropriate derivative **17a-c** (0.65 mmol), and 6 N NaOH (6 mL) in ethanol (5 mL) was stirred at rt 2 h. The mixture was then concentrated in vacuo, diluted with cold water and acidified with 6 N HCl. The pure final compound of type **18** was filtered off by suction and recrystallized from ethanol.

5.2.36 1-(4-Methoxybenzyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (18a)



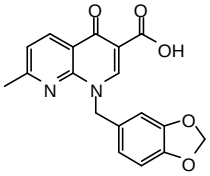
Yield = 67 %; mp = 215-17 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.79 (s, 3H, CH_3C=N), 3.81 (s, 3H, OCH_3), 5.69 (s, 2H, NCH_2), 6.89 (d, 2H, Ar, J = 8.5 Hz), 7.36 (d, 2H, Ar, J = 8.5 Hz), 7.42 (d, 1H, Ar, J = 8.2 Hz), 8.70 (d, 1H, Ar, J = 8.2 Hz), 8.97 (s, 1H, Ar), 14.63 (exch br s, 1H, OH).

5.2.37 1-(3-Methoxybenzyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (18b)



Yield = 77 %; mp = 244-46 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.77 (s, 3H, CH_3C=N), 3.80 (s, 3H, OCH_3), 5.73 (s, 2H, NCH_2), 6.87-6.91 (m, 2H, Ar), 6.93 (d, 1H, Ar, J = 7.7 Hz), 7.29 (t, 2H, Ar, J = 8.1 Hz), 7.42 (d, 1H, Ar, J = 8.2 Hz), 8.71 (d, 1H, Ar, J = 8.2 Hz), 8.97 (s, 1H, Ar), 14.60 (exch br s, 1H, OH).

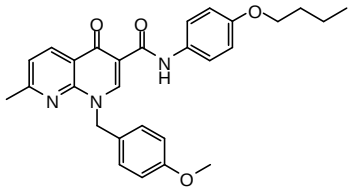
5.2.38 1-(1,3-Benzodioxol-5-ylmethyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (18c)



Yield = 68 %; mp = 237-39 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.79 (s, 3H, CH_3C=N), 5.66 (s, 2H, NCH_3), 5.98 (s, 2H, OCH_2O), 6.80 (d, 1H, Ar, J = 7.9 Hz), 6.88 (s, 1H, Ar), 6.90 (d, 1H, Ar, J = 7.9 Hz), 7.43 (d, 1H, Ar, J = 8.2 Hz), 8.70 (d, 1H, Ar, J = 8.2 Hz), 8.95 (s, 1H, Ar).

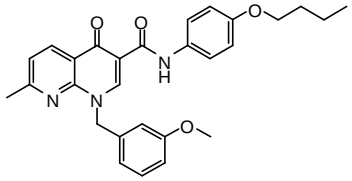
General Procedure for 19a-c. To a cooled (0 °C) and stirred solution of compound of type **18** (**18a-c**) (0.17 mmol) in anhydrous DMF (1 mL), Et_3N (5 drops), ***diethyl cyanophosphonate (0.68 mmol) and*** 4-*n*.Butoxyaniline (0.17 mmol) were added. After 0.5 h at 0 °C, the reaction was keept at room temperature for 15 h. The mixture was then diluted with ice-cold water (10 mL) and kept under stirring for 0.5 h at 0 °C. The precipitate was filtered off and purified by flash column chromatography using cyclohexane/ethyl acetate 2:1 as eluent.

5.2.39 *N*-(4-Butoxyphenyl)-1-(4-methoxybenzyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (19a)



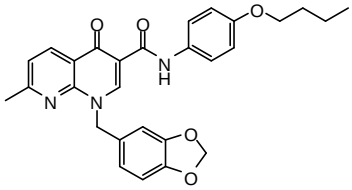
Yield = 75 %; mp = 190-91 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.00 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.51 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.79 (quin, 2H, $CH_2CH_2CH_2$, J = 7.0 Hz), 2.75 (s, 3H, CH_3C=N), 3.80 (s, 3H, OCH_3), 3.98 (t, 2H, OCH_2, J = 6.5 Hz), 5.68 (s, 2H, NCH_2), 6.89 (qd, 4H, Ar, J = 3.5 Hz, J = 9.0 Hz), 7.34-7.37 (m, 3H, Ar), 7.68 (d, 2H, Ar, J = 7.9 Hz), 8.69 (d, 1H, Ar, J = 8.1 Hz), 9.08 (s, 1H, Ar), 11.97 (exch br s, 1H, NH).

5.2.40 *N*-(4-Butoxyphenyl)-1-(3-methoxybenzyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (19b)



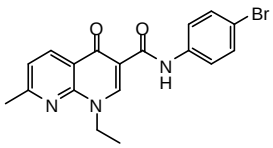
Yield = 71 %; mp = 145-47 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.00 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.51 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.79 (quin, 2H, $CH_2CH_2CH_2$, J = 6.9 Hz), 2.74 (s, 3H, CH_3C=N), 3.80 (s, 3H, OCH_3), 3.98 (t, 2H, OCH_2, J = 6.5 Hz), 5.72 (s, 2H, NCH_2), 6.86 (d, 1H, Ar, J = 8.5 Hz), 6.91 (d, 2H, Ar, J = 8.9 Hz), 6.95-7.02 (m, 2H, Ar), 7.27 (t, 1H, Ar, J = 7.9 Hz), 7.37 (d, 1H, Ar, J = 7.0 Hz), 7.69 (d, 2H, Ar, J = 8.2 Hz), 8.71 (d, 1H, Ar, J = 8.2 Hz), 9.08 (s, 1H, Ar), 11.97 (exch br s, 1H, NH).

5.2.41 1-(1,3-Benzodioxol-5-ylmethyl)-*N*-(4-butoxyphenyl)-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (19c)



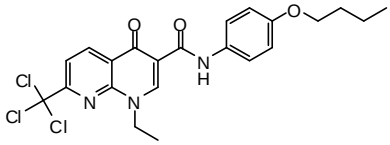
Yield = 62 %; mp = 181-83 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.00 (t, 3H, CH_2CH_3, J = 7.3 Hz), 1.51 (sext, 2H, CH_2CH_3, J = 7.8 Hz), 1.79 (quin, 2H, $CH_2CH_2CH_2$, J = 6.8 Hz), 2.77 (s, 3H, CH_3C=N), 3.98 (t, 2H, OCH_2, J = 6.4 Hz), 5.65 (s, 2H, NCH_2), 5.96 (s, 2H, OCH_2O), 6.79 (d, 1H, Ar, J = 7.8 Hz), 6.91 (d, 4H, Ar, J = 8.5 Hz), 7.38 (d, 1H, Ar, J = 6.7 Hz), 7.69 (d, 2H, Ar, J = 5.6 Hz), 8.71 (d, 1H, Ar, J = 8.0 Hz), 9.06 (s, 1H, Ar), 11.97 (exch br s, 1H, NH).

5.2.42 *N*-(4-Bromophenyl)-1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (20)



To a cooled (-5 °C) and stirred solution of nalidixic acid (0.43 mmol) in anhydrous tetrahydrofuran (3 mL), Et_3N (1.50 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C, and ethyl chloroformate (0.47 mmol) was added. After 1 h 4-bromo aniline was added and the reaction was carried out at room temperature for 12 h. The mixture was then concentrated in vacuo, diluted with cold water (20 mL) and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated to afford final compound **20**, which was purified by two consecutive flash chromatography using firstly cyclohexane/ethyl acetate 2:1 and then CH_2Cl_2 as eluents. Yield = 18 %; mp = 290-92 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.56 (t, 3H, CH_2CH_3, J = 7.2 Hz), 2.74 (s, 3H, CH_3C=N), 4.62 (q, 2H, NCH_2, J = 7.2 Hz), 7.97 (d, 1H, Ar, J = 8.2 Hz), 7.48 (d, 2H, Ar, J = 8.7 Hz), 7.70 (d, 2H, Ar, J = 8.8 Hz), 8.70 (d, 1H, Ar, J = 8.1 Hz), 9.00 (s, 1H, Ar), 12.24 (exch br s, 1H, NH).

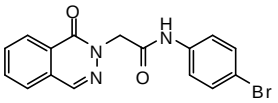
5.2.43 *N*-(4-Butoxyphenyl)-1-ethyl-4-oxo-7-trichloromethyl-1,4-dihydro-1,8-naphthyridine-3-carboxamide (21)



To a cooled (0 °C) and stirred suspension of nalidixic acid (0.43 mmol) in $SOCl_2$ (1.5 mL), Et_3N (0.2 mL) was added. After 2 h at 60 °C, the mixture was allowed to cool down and the excess of $SOCl_2$ was removed in vacuo. The residue was dissolved in anhydrous THF (3 mL) and cooled again to 0 °C. A solution of 4-*n*.butoxyaniline (0.86 mmol) in anhydrous THF (2 mL) was then added dropwise to the mixture. Finally, the reaction was carried out at room temperature for 2 h. The mixture was concentrated under reduced pressure and diluted with ice-cold water (10 mL). The precipitate was then filtered off and purified by flash column chromatography using CH_2Cl_2/MeOH/CH_3COOH 99:1:0.1 as eluent.

Yield = 15 %; mp = 162-63 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, $CH_2CH_2CH_3$, J = 7.4 Hz), 1.52 (sext, 2H, $CH_2CH_2CH_3$, J = 7.6 Hz), 1.62 (t, 3H, NCH_2CH_3, J = 7.2 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, J = 7.1 Hz), 4.00 (t, 2H, OCH_2, J = 6.6 Hz), 4.64 (q, 2H, NCH_2, J = 7.2 Hz), 6.93 (d, 2H, Ar, J = 9.0 Hz), 7.69 (d, 2H, Ar, J = 9.0 Hz), 8.15 (d, 1H, Ar, J = 8.4 Hz), 8.99 (d, 1H, Ar, J = 8.4 Hz), 9.12 (s, 1H, Ar), 11.76 (exch br s, 1H, NH).

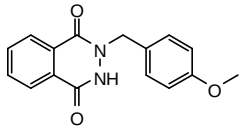
5.2.44 *N*-(4-Bromophenyl)-2-(1-oxophthalazin-2(1*H*)-yl)acetamide (23)



A solution of **22** (1.54 mmol) in anhydrous acetonitrile (1 ml) was added dropwise to a stirred solution of commercially available 1-(*2H*)-phthalazinone (1.03 mmol) in anhydrous acetonitrile (3 mL). K_2CO_3 (2.06 mmol) was added and the reaction was carried out for 3 h at reflux. Removal of the solvent gave a residue which was poured into ice-cold water and after 1 h stirring in ice-bath the final product was filtered off by suction and recrystallized from ethanol. Yield = 45 %; mp = 245-46 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 4.97 (s, 2H, NCH_2CO), 7.53 (qd, 4H, Ar, *J* = 13.9 Hz, *J* = 8.3 Hz), 7.91 (m, 1H, Ar), 7.99 (d, 2H, Ar, *J* = 5.8 Hz), 8.26 (d, 1H, Ar, *J* = 8.0 Hz), 8.48 (s, 1H, Ar), 10.46 (exch br s, 1H, NH).

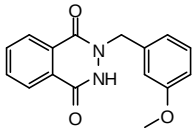
General Procedure for 24a-c. Commercially available 2,3-diidrophthalazin-1,4-dione (4.93 mmol), K_2CO_3 (9.80 mmol) and the suitable benzyl halide (5.43 mmol) were stirred in anhydrous DMF (5 mL) for 2-4 h at 80 °C. The mixture was then diluted with ice-cold water and extracted with CH_2Cl_2 (3 x 15 mL). The organic layer was dried over Na_2SO_4 and evaporated in vacuo. Compounds **24a-c** were then purified by flash column chromatography using cyclohexane/ethyl acetate 1:1 as eluent.

5.2.45 2-(4-Methoxybenzyl)-2,3-dihydrophthalazine-1,4-dione (24a)



Yield = 23 %; mp = 150-52 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H, OCH_3), 5.31 (s, 2H, NCH_2), 6.96 (d, 2H, Ar, *J* = 8.6 Hz), 7.44 (d, 2H, Ar, *J* = 8.5 Hz), 7.83 (quin, 2H, Ar, *J* = 3.7 Hz), 8.07 (dd, 1H, Ar, *J* = 3.1 Hz, *J* = 3.5 Hz), 8.41 (dd, 1H, Ar, *J* = 2.4 Hz, *J* = 2.8 Hz), 9.98 (exch br s, 1H, NH).

5.2.46 2-(3-Methoxybenzyl)-2,3-dihydrophthalazine-1,4-dione (24b)

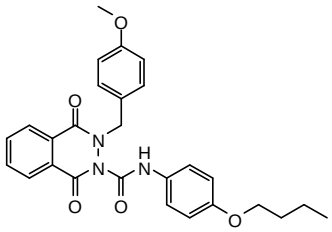


Yield = 18 %; mp = 184-86 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.87 (s, 3H, OCH_3), 5.36 (s, 2H, NCH_2), 6.93 (dd, 1H, Ar, *J* = 5.8 Hz, *J* = 2.4 Hz), 7.06 (s, 1H, Ar), 7.09 (d, 1H, Ar, *J* = 7.6 Hz), 7.36 (t, 1H, Ar, *J* = 7.9 Hz), 7.82-7.89 (m, 2H, Ar), 8.09 (dd, 1H, Ar, *J* = 6.9 Hz, *J* = 1.9 Hz), 8.44 (dd, 1H, Ar, *J* = 4.0 Hz, *J* = 2.1 Hz), 9.94 (exch br s, 1H, NH).

General Procedure for 25a-c. The appropriate derivative of type **24** (**24a-c**) (1.10 mmol) was dissolved in 3 mL of anhydrous CH_2Cl_2 and 4-butoxyphenyl isocyanate (2.20 mmol) was added under

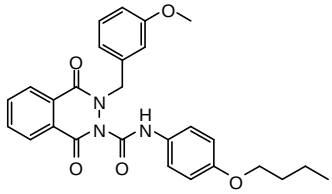
stirring at 0 °C. The reaction was carried out at 0 °C for 2 h and subsequently at room temperature for 12 h. The solid residue was filtered off and the solution was evaporated in vacuo to afford compounds **25a-c**, which were purified by flash column chromatography using CH_2Cl_2 as eluent.

5.2.47 *N*-(4-Butoxyphenyl)-3-(4-methoxybenzyl)-1,4-dioxo-3,4-dihydro-phthalazine-2(1*H*)-carboxamide (25a)



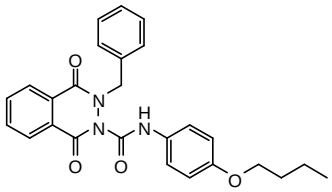
Yield = 53 %; mp = 117-19 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.52 (sext, 2H, CH_2CH_3, J = 7.5 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, J = 6.8 Hz), 3.84 (s, 3H, OCH_3), 3.99 (t, 2H, OCH_2, J = 6.5 Hz), 5.51 (s, 2H, NCH_2), 6.95 (t, 4H, Ar, J = 8.7 Hz), 7.56 (d, 2H, Ar, J = 8.5 Hz), 7.62 (d, 2H, Ar, J = 8.9 Hz), 7.86 (quin, 2H, Ar, J = 7.8 Hz), 8.06 (d, 1H, Ar, J = 7.8 Hz), 8.47 (d, 1H, Ar, J = 7.6 Hz), 11.86 (exch br s, 1H, NH).

5.2.48 *N*-(4-Butoxyphenyl)-3-(3-methoxybenzyl)-1,4-dioxo-3,4-dihydro-phthalazine-2(1*H*)-carboxamide (25b)



Yield = 75 %; mp = 104-06 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.52 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, J = 7.0 Hz), 3.87 (s, 3H, OCH_3), 3.99 (t, 2H, OCH_2, J = 6.5 Hz), 5.56 (s, 2H, NCH_2), 6.93 (dd, 3H, Ar, J = 4.6 Hz, J = 3.2 Hz), 7.18 (d, 1H, Ar, J = 7.0 Hz), 7.19 (s, 1H, Ar), 7.35 (t, 1H, Ar, J = 7.7 Hz), 7.61 (d, 2H, Ar, J = 9.0 Hz), 7.84-7.92 (m, 2H, Ar), 8.10 (d, 1H, Ar, J = 7.1 Hz), 8.49 (d, 1H, Ar, J = 7.2 Hz), 11.84 (exch br s, 1H, NH).

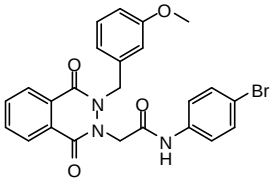
5.2.49 3-Benzyl-*N*-(4-butoxyphenyl)-1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carboxamide (25c)



Yield = 86 %; mp = 117-19 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.52 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.80 (quin, 2H, $CH_2CH_2CH_2$, J = 6.9 Hz), 3.99 (t, 2H, OCH_2, J = 6.5 Hz), 5.59 (s, 2H, NCH_2), 6.94 (d, 2H, Ar, J = 8.9 Hz), 7.37-7.46 (m, 3H, Ar), 7.62 (dd, 4H, Ar, J = 1.3 Hz, J = 5.3

Hz), 7.84-7.92 (m, 2H, Ar), 8.09 (d, 1H, Ar, *J* = 7.8 Hz), 8.49 (d, 1H, Ar, *J* = 7.5 Hz), 11.85 (exch br s, 1H, NH).

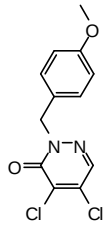
5.2.50 *N*-(4-Bromophenyl)-2-(3-(3-methoxybenzyl)-1,4-dioxo-3,4-dihydro-phthalazin-2(1*H*)-yl)acetamide (26)



A suspension of the intermediate **24b** (0.18 mmol), K_2CO_3 (0.36 mmol) and amide **22** (0.27 mmol) in anhydrous acetonitrile (1 mL) was stirred at reflux for 3 h. The solvent was then evaporated in vacuo and the mixture was puored into ice-cold water. After 1 h stirring in ice-bath, the precipitate was filtered off and purified through two consecutive crystallization from ethanol. Yield = 45 %; mp = 178-80 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3H, OCH_3), 4.95 (s, 2H, NCH_2-C_6H_3), 5.39 (s, 2H, NCH_2CO), 6.91 (d, 1H, Ar, *J* = 8.5 Hz), 7.06 (s, 1H, Ar), 7.09 (d, 1H, Ar, *J* = 6.7 Hz), 7.34 (t, 1H, Ar, *J* = 7.6 Hz), 7.43 (s, 4H, Ar), 7.78 (t, 2H, Ar, *J* = 4.8 Hz), 8.09-8.12 (m, 1H, Ar), 8.45-8.48 (m, 1H, Ar), 8.80 (exch br s, 1H, NH).

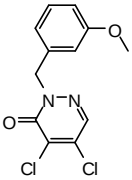
General Procedure for 27a,b. K_2CO_3 (6.06 mmol) and tetrabutylammonium bromide (0.30 mmol) were added to a srirred solution of ***4,5-dichloro***-3*(2H)*-***pyridazinone*** (3.03 mmol) in anhydrous acetonitrile (3 mL). The appropriate benzyl chloride (3- or 4-methoxybenzyl chloride) (4.54 mmol) was added and the reaction was carried out at reflux for 5-7 h. The mixture was then allowed to cool down and the solvent was evaporated in vacuo. Ice-cold water was added to the residue and after 1 h stirring in ice-bath, compounds **27a,b** were filtered off and recrystallized from ethanol.

5.2.51 4,5-Dichloro-2-(4-methoxybenzyl)pyridazin-3(2*H*)-one (27a)



Yield = 81 %; mp = 116-17 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.81 (s, 3H, OCH_3), 5.28 (s, 2H, NCH_2), 6.88 (d, 2H, Ar, *J* = 8.6 Hz), 7.42 (d, 2H, Ar, *J* = 8.5 Hz), 7.79 (s, 1H, Ar).

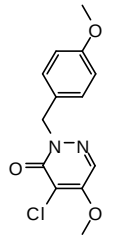
5.2.52 4,5-Dichloro-2-(3-methoxybenzyl)pyridazin-3(2*H*)-one (27b)



Yield = 60 %; mp = 80-82 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H, OCH_3), 5.31 (s, 2H, NCH_2), 6.87 (dd, 1H, Ar, *J* = 5.6 Hz, *J* = 2.6 Hz), 6.99-7.05 (m, 2H, Ar), 7.27 (t, 1H, Ar, *J* = 3.6 Hz), 7.80 (s, 1H, Ar).

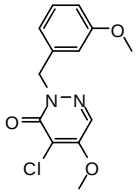
General Procedure for 28a,b. Compound **27a** or **27b** (0.88 mmol) was added to a stirred solution of Na° (1.76 mmol) in 3 mL of anhydrous methanol. The reaction mixture was stirred for 1 h at room temperature. After removal of the solvent in vacuo, ice-cold water was added to the residue and the precipitate was filtered off by suction and purified by crystallization from ethanol.

5.2.53 4-Chloro-5-methoxy-2-(4-methoxybenzyl)pyridazin-3(2*H*)-one (28a)



Yield = 53 %; mp = 135-37 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.80 (s, 3H, C_6H_4-O*C*H_3), 4.06 (s, 3H, OCH_3 pyridaz.), 5.31 (s, 2H, NCH_2), 6.87 (d, 2H, Ar, *J* = 8.6 Hz), 7.42 (d, 2H, Ar, *J* = 8.6 Hz), 7.80 (s, 1H, Ar).

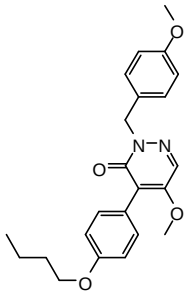
5.2.54 4-Chloro-5-methoxy-2-(3-methoxybenzyl)pyridazin-3(2*H*)-one (28b)



Yield = 60 %; mp = 80-82 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.81 (s, 3H, C_6H_4-O*C*H_3), 4.07 (s, 3H, OCH_3 pyridaz.), 5.34 (s, 2H, NCH_2), 6.85 (dd, 1H, Ar, *J* = 6.2 Hz, *J* = 1.89 Hz), 6.99 (s, 1H, Ar), 7.03 (d, 1H, Ar, *J* = 7.3 Hz), 7.26 (t, 1H, Ar, *J* = 7.9 Hz), 7.83 (s, 1H, Ar).

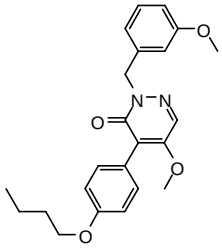
General Procedure for 29a,b. To the suspension of **28a** or **28b** (0.71 mmol), $Pd(PPh_3)_4$ [tetrakis(triphenylphosphine)palladium(0), 0.02 mmol] and 4-butoxyphenylboronic acid (1.07 mmol) in toluene (2 mL), Na_2CO_3 (1.42 mmol, 2 M in H_2O) was added and the mixture was stirred at reflux for 2 h. Extra 4-butoxyphenylboronic acid (1.07 mmol) was added and the reaction was refluxed for additional 3-6 h. The solvent was evaporated under vacuo, then the suspension was diluted with ice-cold water and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and the residue was purified by flash column chromatography using cyclohexane/ethyl acetate 3:1 as eluent.

5.2.55 4-(4-Butoxyphenyl)-5-methoxy-2-(4-methoxybenzyl)pyridazin-3(2*H*)-one (29a)



Yield = 82 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.99 (t, 2H, CH_2CH_3, J = 7.4 Hz), 1.51 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.79 (quin, 2H, $CH_2CH_2CH_2$, J = 6.9 Hz), 3.80 (s, 3H, C_6H_4-OCH_3), 3.89 (s, 3H, OCH_3 pyridaz.), 4.01 (t, 2H, OCH_2, J = 6.5 Hz), 5.31 (s, 2H, NCH_2), 6.87 (d, 2H, Ar, J = 8.6 Hz), 6.94 (d, 2H, Ar, J = 8.8 Hz), 7.48 (q, 4H, Ar, J = 8.0 Hz), 7.89 (s, 1H, Ar).

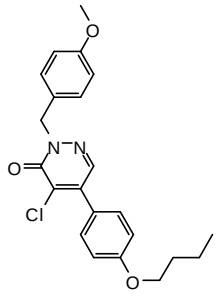
5.2.56 4-(4-Butoxyphenyl)-5-methoxy-2-(3-methoxybenzyl)pyridazin-3(2*H*)-one (29b)



Yield = 28 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.99 (t, 2H, CH_2CH_3, J = 7.4 Hz), 1.51 (sext, 2H, CH_2CH_3, J = 7.4 Hz), 1.79 (quin, 2H, $CH_2CH_2CH_2$, J = 6.9 Hz), 3.81 (s, 3H, C_6H_4-OCH_3), 3.90 (s, 3H, OCH_3 pyridaz.), 4.00 (t, 2H, OCH_2, J = 6.5 Hz), 5.35 (s, 2H, NCH_2), 6.84 (dd, 1H, Ar, J = 6.0 Hz, J = 2.3 Hz), 6.94 (d, 2H, Ar, J = 8.7 Hz), 7.02 (s, 1H, Ar), 7.06 (d, 1H, Ar, J = 7.6 Hz), 7.26 (t, 1H, Ar, J = 8.0 Hz), 7.51 (d, 2H, Ar, J = 8.7 Hz), 7.91 (s, 1H, Ar).

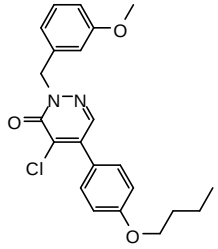
General Procedure for 30a,b. To a suspension of **27a** or **27b** (0.53 mmol), $PdCl_2[(C_2H_5)_3P]_2$ [trans-dichlorobis(triethylphosphine)palladium(II), 0.05 mmol] and 4-butoxyphenylboronic acid (0.26 mmol) in DMF (2 mL), Na_2CO_3 (0.53 mmol, 1 M in H_2O) was added and the mixture was stirred at room temperature for 6-12 h. The suspension was diluted with ice-cold water and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and the residue was purified by flash column chromatography using CH_2Cl_2 (for **30a**) and CH_2Cl_2/MeOH/NH_4OH 99:1:0.1 (for **30b**) as eluents.

5.2.57 5-(4-Butoxyphenyl)-4-chloro-2-(4-methoxybenzyl)pyridazin-3(2*H*)-one (30a)



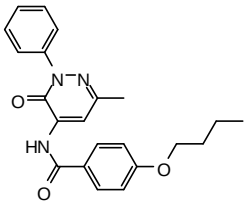
Yield = 22 %; mp = 84-85 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 2H, CH_2CH_3, J = 7.4 Hz), 1.53 (sext, 2H, CH_2CH_3, J = 7.6 Hz), 1.82 (quin, 2H, $CH_2CH_2CH_2$, J = 6.8 Hz), 3.81 (s, 3H, OCH_3), 4.03 (t, 2H, OCH_2, J = 6.5 Hz), 5.34 (s, 2H, NCH_2), 6.90 (d, 2H, Ar, J = 8.4 Hz), 7.01 (d, 2H, Ar, J = 8.6 Hz), 7.48 (dd, 4H, Ar, J = 5.9 Hz, J = 8.6 Hz), 7.78 (s, 1H, Ar).

5.2.58 5-(4-Butoxyphenyl)-4-chloro-2-(3-methoxybenzyl)pyridazin-3(2*H*)-one (30b)



Yield = 14 %; mp = 73-75 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 2H, CH_2CH_3, J = 7.4 Hz), 1.53 (sext, 2H, CH_2CH_3, J = 7.5 Hz), 1.82 (quin, 2H, $CH_2CH_2CH_2$, J = 7.0 Hz), 3.83 (s, 3H, OCH_3), 4.04 (t, 2H, OCH_2, J = 6.5 Hz), 5.38 (s, 2H, NCH_2), 6.88 (dd, 1H, Ar, J = 5.7 Hz, J = 2.6 Hz), 7.02 (d, 2H, Ar, J = 8.8 Hz), 7.07 (s, 1H,Ar), 7.10 (d, 1H, Ar, J = 7.6 Hz), 7.29 (t, 1H, Ar, J = 8.0 Hz), 7.47 (d, 2H, Ar, J = 8.7 Hz), 7.79 (s, 1H, Ar).

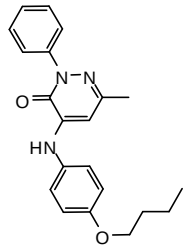
5.2.59 4-Butoxy-*N*-(6-methyl-3-oxo-2-phenyl-2,3-dihydropyridazin-4-yl)benzamide (35)



4-Butoxybenzoic acid (0.36 mmol) was converted into the corresponding chloride as follows: $SOCl_2$ (20.7 mmol) was added and the stirred suspension was cooled to 0 °C and treated with Et_3N (0.99 mmol). After 4 h of reflux, the mixture was evaporated in vacuo and the residue was washed with cyclohexane (3 x 5 mL). The residue was dissolved in anhydrous toluene (5 mL), then added of compound **34** (0.40 mmol) dissolved in anhydrous toluene (1 mL) and Et_3N (1.48 mmol). After 4 h of reflux the solvent was removed in vacuo, cold water was added and the aqueous layer was extracted with CH_2Cl_2 (3 x 20 mL).

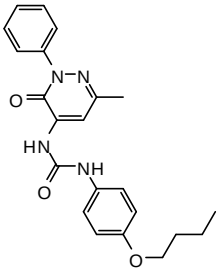
Evaporation of the organic solvent in vacuo gave a residue that was purified by column chromatography (eluent: cyclohexane/ethyl acetate 3:1). Yield = 37 %; mp = 95-97 °C (EtOH). IR (cm^{-1}) 3269 (NH), 1682 (CO), 1644 (CO). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H,CH_2CH_3, J = 7.4 Hz), 1.53 (sext, 2H, CH_2CH_3, J = 7.5 Hz), 1.82 (quin, 2H, $CH_2CH_2CH_2$, J = 7.0 Hz), 2.45 (s, 3H, 6-CH_3), 4.06 (t, 2H, CH_2O, J = 6.5 Hz), 6.99 (d, 2H, Ar, J = 8.8 Hz), 7.43 (t, 1H, Ar, J = 7.4 Hz), 7.52 (t, 2H, Ar, J = 7.8 Hz), 7.65 (d, 2H, Ar, J = 7.7 Hz), 7.91 (d, 2H, Ar, J = 8.8 Hz), 8.24 (s, 1H, Ar), 9.46 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{22}H_{23}N_3O_3$, 377,44. Found: *m/z* 378.18 $[M + H]^+$.

5.2.60 4-(4-Butoxyphenylamino)-6-methyl-2-phenylpyridazin-3(2*H*)-one (36)



To a suspension of **34** (0.55 mmol), copper acetate (0.82 mmol) and 4-butoxyphenylboronic acid (1.08 mmol) in CH_2Cl_2 (2 mL), Et_3N (1.08 mmol) was added and the mixture was stirred at room temperature for 12 h. The suspension was extracted with 15% aqueous ammonia (10 mL), and the organic layer was washed with 10 mL of water and dried over Na_2SO_4. After removal of the solvent, the residue was purified by column chromatography using as eluent cyclohexane/ethyl acetate 3:1. The analytical sample was obtained through a further purification performed on silica gel preparative TLC (eluent: cyclohexane/ethyl acetate 3:1). Yield = 16 %; mp = 137-139 °C (EtOH). IR (cm^{-1}) 3275 (NH), 1633 (CO). ^{1}H NMR ($CDCl_3$) δ 1.02 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.54 (sext, 2H, CH_2CH_3, J = 7.0 Hz), 1.81 (quin, 2H, $CH_2CH_2CH_2$), 2.31 (s, 3H, 6-CH_3), 4.01 (t, 2H, CH_2O, J = 6.5 Hz), 6.40 (s, 1H, Ar), 6.97 (d, 2H, Ar, J = 8.9 Hz), 7.20 (d, 2H, Ar, J = 8.8 Hz), 7.41 (t, 1H, Ar, J = 7.4 Hz), 7.51 (t, 3H, Ar, J = 7.7 Hz), 7.64 (d, 2H, Ar, J = 8.0 Hz). MS (ESI) calcd. For $C_{21}H_{23}N_3O_2$, 349.43. Found: *m/z* 350.19 $[M + H]^+$.

5.2.61 1-(4-Butoxyphenyl)-3-(6-methyl-3-oxo-2-phenyl-2,3-dihydro pyridazin-4-yl)urea (37)

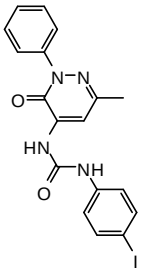


To a cooled and stirred suspension of **34** (0.50 mmol) and anhydrous sodium acetate (1.20 mmol) in anhydrous THF (5 mL), triphosgene (1.75 mmol) was added. The mixture was stirred for additional 10

min at 0 °C and refluxed for 2 h. The solvent was removed in vacuo, and the residue was dissolved in anhydrous THF (2 mL). 4-Butoxyaniline (1.20 mmol) was added and the mixture was stirred at room temperature for 12 h. After dilution with ice-cold water, the suspension was extracted with CH_2Cl_2 (3 x 20 mL). Removal of the solvent in vacuo afforded a crude residue which was purified by column chromatography using absolute EtOH/CH_2Cl_2/petroleum ether/toluene/33% ammonia 3.3:19.7:6.5:70:0.5 as eluent. Yield = 31 %; mp =189-191 °C (EtOH). IR (cm^{-1}) 3270 (NH), 3255 (NH), 1705 (CO), 1625 (CO). ^{1}H NMR ($CDCl_3$) δ 1.01 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.52 (sext, 2H, CH_2CH_3, J = 7.4 Hz), 1.79 (quin, 2H, $CH_2CH_2CH_2$, J = 7.0 Hz), 2.40 (s, 3H, 6-CH_3), 3.95 (t, 2H, OCH_2, J = 6.6 Hz), 6.70 (s, 4H, Ar), 7.17 (t, 1H, Ar, J = 7.5 Hz), 7.32 (t, 2H, Ar, J = 7.8 Hz), 7.61 (d, 2H, Ar, J = 7.9 Hz), 8.11 (exch br s, 1H, NH), 8.14 (s, 1H, Ar), 9.33 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{22}H_{24}N_4O_3$, 392.45. Found: *m/z* 393.19 $[M + H]^+$.

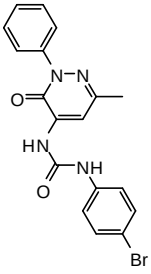
General Procedure for 38a-c. To a stirred solution of compound **34** (0.35 mmol) in anhydrous toluene (2 mL), the proper aryl isocyanate (0.40 mmol) was added. The mixture was refluxed for 4-7 h. After cooling, the precipitate was collected by suction and purified by crystallization from toluene (**38b** and **38c**). In the case of **38a**, after cooling, cold water was added and the mixture was extracted with CH_2Cl_2 (3 x 20 mL). Removal of the solvent gave a residue that was purified by column chromatography using CH_2Cl_2/CH_3OH 9.9:0.1 as eluent.

5.2.62 1-(4-Iodophenyl)-3-(6-methyl-3-oxo-2-phenyl-2,3-dihydropyridazin-4-yl)urea (38a)



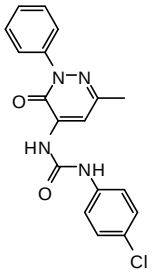
Yield = 45 %; mp = 264-265 °C (EtOH/toluene). IR (cm^{-1}) 3270 (NH), 3256 (NH), 1708 (CO), 1627 (CO). ^{1}H NMR ($CDCl_3$) δ 2.44 (s, 3H,CH_3), 6.50 (d, 2H, Ar, J = 8.7 Hz), 7.21 (t, 1H, Ar, J = 7.5 Hz), 7.36-7.41 (m, 4H, Ar), 7.68 (d, 2H, Ar, J = 7.7 Hz), 8.15 (s, 1H, Ar), 8.41 (exch br s, 1H, NH), 9.38 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{18}H_{15}IN_4O_2$, 446.24. Found: *m/z* 447.03 $[M + H]^+$.

5.2.63 1-(4-Bromophenyl)-3-(6-methyl-3-oxo-2-phenyl-2,3-dihydropyridazin-4-yl)urea (38b)



Yield = 50 %; mp = 263-265 °C (toluene). IR (cm^{-1}) 3271 (NH), 3250 (NH), 1704 (CO), 1625 (CO). ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 3H,CH_3), 6.60 (d, 2H, Ar, J = 8.6 Hz), 7.19-7.22 (m, 3H, Ar), 7.38 (t, 2H, Ar, J = 7.8 Hz), 7.69 (d, 2H, Ar, J = 7.9 Hz), 8.16 (s, 1H, Ar), 8.44 (exch, br, s, 1H, NH), 9.40 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{18}H_{15}BrN_4O_2$, 399.24. Found: *m/z* 399.05 $[M]^+$.

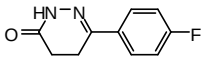
5.2.64 1-(4-Chlorophenyl)-3-(6-methyl-3-oxo-2-phenyl-2,3-dihydropyridazin-4-yl)urea (38c)



Yield = 85 %; mp = 274-276 °C (toluene). IR (cm^{-1}) 3269 (NH), 3255 (NH), 1705 (CO), 1626 (CO). ^{1}H NMR ($CDCl_3$) δ 2.44 (s, 3H, CH_3), 6.65 (d, 2H, Ar, J = 8.8 Hz), 7.07 (d, 2H, Ar, J = 8.8 Hz), 7.21 (t, 1H, Ar, J = 7.5 Hz), 7.38 (t, 2H, Ar, J = 7.9 Hz), 7.69 (d, 2H, Ar, J = 7.6 Hz), 8.16 (s, 1H, Ar), 8.43 (exch br s, 1H, NH), 9.40 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{18}H_{15}ClN_4O_2$, 354.79. Found: *m/z* 355.10 $[M + H]^+$.

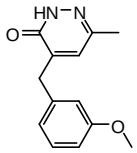
General Procedure for 42a-m. To a stirred solution of the suitable commercially available or synthesized γ-keto acid (1.00 mmol) in EtOH (2 mL), hydrazine hydrate (1.00 mmol) was added dropwise. The mixture was heated at 60 °C for 1-3 h. After cooling, the precipitate was collected by suction and purified by recrystallization alternatively from toluene or ethanol.

5.2.65 6-(4-Fluorophenyl)-4,5-dihydropyridazin-3(2*H*)-one (42j)



Yield = 95 %; mp = 192-93 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.64 (dd, 2H, CO$CH_2$$CH_2$, J = 1.2 Hz, J = 7.9 Hz), 3.00 (dd, 2H, $COCH_2$$CH_2$, J = 1.2 Hz, J = 7.3 Hz), 7.13 (qd, 2H, Ar, J = 1.8 Hz, J = 4.7 Hz), 7.72-7.76 (qd, 2H, Ar, J = 0.4 Hz, J = 3.1 Hz), 8.51 (exch br s, 1H, NH).

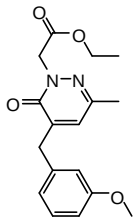
5.2.66 4-(3-Methoxybenzyl)-6-methylpyridazin-3(2*H*)-one (43)



To 7 mL of KOH in absolute EtOH (5%, w/v), **42d** (1.79 mmol) and 3-methoxybenzaldehyde (1.79 mmol) were added. The reaction was carried out under stirring for 3 h. After cooling, the mixture was concentrated in vacuo, diluted with cold water (10-15 mL) and acidified with 2 N HCl. The suspension

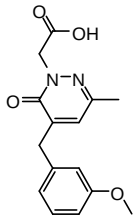
was extracted with CH_2Cl_2 (3 x 15 mL). The organic phase was dried (Na_2SO_4) and evaporated in vacuo to afford compound **43**, which was purified by flash column chromatography using cyclohexane/ethyl acetate 1:1 as eluent. Yield = 61 %; mp =133-134 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H, 6-CH_3), 3.82 (s, 3H, OCH_3), 3.90 (s, 2H, *CH_2*-Ar), 6.73 (s, 1H, Ar), 6.82-6.86 (m, 3H, Ar), 7.276-7.28 (m, 1H, Ar).

5.2.67 [5-(3-Methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetic acid ethyl ester (44)



A mixture of **43** (1.13 mmol), K_2CO_3 (2.26 mmol), and ethyl bromoacetate (3.40 mmol) in CH_3CN (3 mL) was refluxed under stirring for 6 h. The mixture was then concentrated in vacuo, diluted with cold water, and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated in vacuo and compound **44** was purified by column chromatography using cyclohexane/ethyl acetate 1:1 as eluent. Yield = 98 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 3H, CH_2*CH_3*, *J* = 7.1 Hz), 2.22 (s, 3H, 3-CH_3), 3.80 (s, 3H, OCH_3), 3.86 (s, 2H, *CH_2*-Ar), 4.22-4.25 (m, 2H, O*CH_2*CH_3), 4.85 (s, 2H, NCH_2CO), 6.67 (s, 1H, Ar), 6.78-6.82 (m, 3H, Ar), 7.26 (t, 1H, Ar, *J* =7.8 Hz).

5.2.68 [5-(3-Methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetic Acid (45)

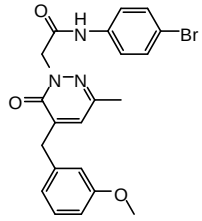


A suspension of **44** (0.5 mmol), 6 N NaOH (6 mL) and ethanol (2 mL) was stirred at rt to 80 °C for 1 h. The mixture was then concentrated in vacuo, diluted with cold water and acidified with 6 N HCl. The final product **45** was filtered off with suction and recrystallized from ethanol. Yield = 97 %; mp =194-195 °C (EtOH). ^{1}H NMR (DMSO-d_6) δ 2.21 (s, 3H, 3-CH_3), 3.72 (s, 3H, OCH_3), 3.76 (s, 2H, *CH_2*-Ar), 4.69 (s, 2H, NCH_2CO), 6.79-6.85 (m, 3H, Ar), 7.07 (s, 1H, Ar), 7.22 (t, 1H, Ar, *J* = 7.8 Hz).

General Procedure for 46a-s. To a cooled (-5 °C) and stirred solution of compound **45** (0.35 mmol) in anhydrous tetrahydrofuran (3-5 mL), Et_3N (1.22 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C and ethyl chloroformate (0.38 mmol) was added. After 1 h, the appropriate substituted aryl(cycloalkyl)amine (0.7 mmol), was added and the reaction was carried out at room temperature for 12 h. The mixture was then concentrated in vacuo, diluted with cold water (20-30

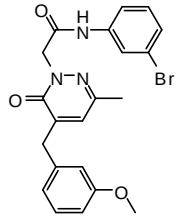
mL) and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated to afford final compounds **46a-s**, which were purified by column chromatography using cyclohexane/ethyl acetate 1:1 as eluent for compounds **46a,d,e,g-m,p,q**, toluene/NH_4OH/EtOH/CH_2Cl_2/petroleum ether 7:0.05:0.30:2: 0.65 for compounds **46b,c**, cyclohexane/ethyl acetate 1:2 for compounds **46f,n,o,r**, CH_2Cl_2/ CH_3OH 9.5:0.5 for compound **46s**.

5.2.69 *N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetamide (46a)



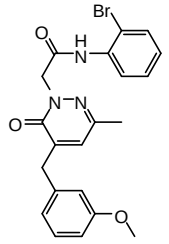
Yield = 86 %; mp = 171-172 °C (EtOH). IR (cm^{-1}) 3300 (NH), 1708 (CO), 1644 (CO). ^{1}H NMR ($CDCl_3$) δ 2.20 (s, 3H, 3-CH_3), 3.70 (s, 3H, OCH_3), 3.73 (s, 2H, *CH_2*-Ar), 4.80 (s, 2H,NCH_2CO), 6.77-6.82 (m, 3H, Ar), 7.13 (s, 1H, Ar), 7.20 (t, 1H, Ar, *J* = 8.0 Hz), 7.47 (s, 4H, Ar). MS (ESI) calcd. For $C_{21}H_{20}BrN_3O_3$, 442.31. Found: *m/z* 442.08 $[M]^+$.

5.2.70 *N*-(3-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetamide (46b)



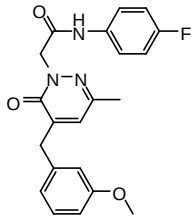
Yield = 85 %; mp = 73-75 °C (EtOH). IR (cm^{-1}) 3295 (NH), 1708 (CO), 1642 (CO). ^{1}H NMR ($CDCl_3$) δ 2.30 (s, 3H, 3-CH_3), 3.80 (s, 3H, OCH_3), 3.92 (s, 2H, *CH_2*-Ar), 4.95 (s, 2H, NCH_2CO), 6.80-6.86 (m, 4H, Ar), 7.14 (t, 1H, Ar, *J* = 8.0 Hz), 7.22 (m, 1H, Ar, *J* = 8.2 Hz), 7.27- 7.31 (m, 1H, Ar), 7.39 (d, 1H, Ar, *J* = 8.0 Hz), 7.77 (s, 1H, Ar), 9.05 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{21}H_{20}BrN_3O_3$, 442.31. Found: *m/z* 441.11. $[M - H]^+$.

5.2.71 *N*-(2-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetamide (46c)



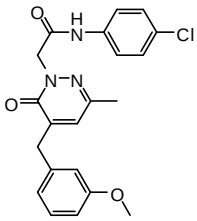
Yield = 80 %; oil. IR (cm^{-1}) 3300 (NH), 1708 (CO), 1644 (CO). ^1H NMR ($CDCl_3$) δ 2.30 (s, 3H, 3-CH_3), 3.82 (s, 3H, OCH_3), 3.92 (s, 2H, *CH_2*-Ar), 5.00 (s, 2H, NCH_2CO), 6.76 (s, 1H, Ar), 6.80-6.86 (m, 3H, Ar), 7.00 (t, 1H, Ar, *J* = 7.7 Hz), 7.27-7.34 (m, 2H, Ar), 7.52 (d, 1H, Ar, *J* = 8.0 Hz), 8.36 (d, 1H, Ar, *J* = 8.2 Hz), 8.54 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{21}H_{20}BrN_3O_3$, 442.31. Found: *m/z* 443.08. $[M + H]^+$.

5.2.72 *N*-(4-Fluorophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetamide (46d)



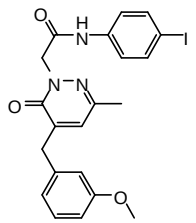
Yield = 97 %; mp = 145-147 °C (EtOH). IR (cm^{-1}) 3300 (NH), 1707 (CO), 1643 (CO). ^1H NMR ($CDCl_3$) δ 2.30 (s, 3H, 3-CH_3), 3.80 (s, 3H, OCH_3), 3.90 (s, 2H, *CH_2*-Ar), 4.95 (s, 2H, NCH_2CO), 6.80-6.84 (m, 4H, Ar), 6.90 (t, 2H, Ar, *J* = 8.7 Hz), 7.25-7.29 (m, 1H, Ar), 7.39-7.42 (m, 2H, Ar), 9.21 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{21}H_{20}FN_3O_3$, 381.40. Found: *m/z* 382.16 $[M + H]^+$.

5.2.73 *N*-(4-Chlorophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetamide (46e)



Yield = 86 %; mp = 170-171 °C (EtOH). IR (cm^{-1}) 3296 (NH), 1705 (CO), 1644 (CO). ^1H NMR ($CDCl_3$) δ 2.30 (s, 3H, 3-CH_3), 3.80 (s, 3H, OCH_3), 3.90 (s, 2H, *CH_2*-Ar), 4.95 (s, 2H, NCH_2CO), 6.79 (s, 1H, Ar), 6.80-6.85 (m, 3H, Ar), 7.16 (d, 2H, Ar, *J* = 8.8 Hz), 7.25-7.29 (m, 1H, Ar), 7.38 (d, 2H, Ar, *J* = 8.8 Hz), 9.28 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{21}H_{20}ClN_3O_3$, 397.85. Found: *m/z* 398.13 $[M + H]^+$.

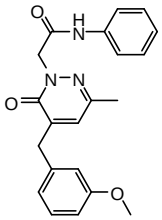
5.2.74 *N*-(4-Iodophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetamide (46f)



Yield = 99 %; mp = 179-180 °C (EtOH). IR (cm^{-1}) 3300 (NH), 1707 (CO), 1645 (CO). ^1H NMR ($CDCl_3$) δ 2.30 (s, 3H, 3-CH_3), 3.79 (s, 3H, OCH_3), 3.88 (s, 2H, *CH_2*-Ar), 4.95 (s, 2H, NCH_2CO), 6.78-6.84 (m,

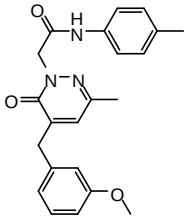
4H, Ar), 7.17 (d, 2H, Ar, *J* = 8.7 Hz), 7.24-7.28 (m, 1H, Ar), 7.45 (d, 2H, Ar, *J* = 8.7 Hz), 9.41 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{21}H_{20}IN_3O_3$, 489.31. Found: *m/z* 490.06 $[M + H]^+$.

5.2.75 2-[5-(3-Methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-*N*-phenyl-acetamide (46g)



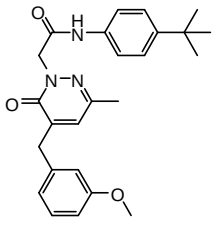
Yield = 97 %; oil. IR (cm^{-1}) 3295 (NH), 1708 (CO), 1644 (CO). ^1H NMR ($CDCl_3$) δ 2.28 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.91 (s, 2H, *CH_2*-Ar), 4.96 (s, 2H, NCH_2CO), 6.78-6.86 (m, 4H, Ar), 7.08 (t, 1H, Ar, *J* = 7.4 Hz), 7.26-7.30 (m, 3H, Ar), 7.50 (d, 2H, Ar, *J* = 8.0Hz), 8.93 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{21}H_{21}N_3O_3$, 363.41. Found: *m/z* 364.16 $[M + H]^+$.

5.2.76 2-[5-(3-Methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-*N*-4-tolyl-acetamide (46h)



Yield = 78 %; mp =142-144 °C (EtOH). IR (cm^{-1}) 3300 (NH), 1708 (CO), 1644 (CO). ^1H NMR ($CDCl_3$) δ 2.28 (s, 3H, 3-CH_3), 2.30 (s, 3H, *CH_3*-Ar), 3.81 (s, 3H, OCH_3), 3.90 (s, 2H, *CH_2*-Ar), 4.94 (s, 2H, NCH_2CO), 6.76 (s, 1H, Ar), 6.80-6.86 (m, 3H, Ar), 7.08 (d, 2H, Ar, *J* = 8.3 Hz), 7.26-7.30 (m, 1H, Ar), 7.38 (d, 2H, Ar, *J* = 8.4 Hz), 8.82 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{22}H_{23}N_3O_3$, 377.44. Found: *m/z* 378.18 $[M + H]^+$.

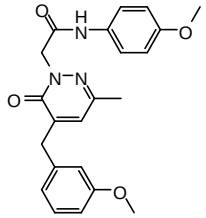
5.2.77 *N*-(4-tert-Butylphenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetamide (46i)



Yield = 95 %; mp = 54-56 °C (EtOH). IR (cm^{-1}) 3296 (NH), 1705 (CO), 1644 (CO). ^1H NMR ($CDCl_3$) δ 1.30 (s, 9H, C-*(CH_3)$_3$*), 2.27 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.90 (s, 2H, *CH_2*-Ar), 4.96 (s, 2H, NCH_2CO), 6.76 (s, 1H, Ar), 6.80 (s, 1H, Ar), 6.81-6.86 (m, 2H, Ar), 7.26-7.30 (m, 3H, Ar), 7.43 (d, 2H,

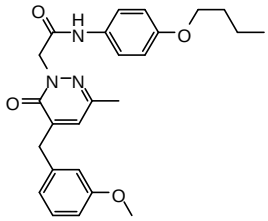
Ar, *J* = 8.6 Hz), 8.88 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{25}H_{29}N_3O_3$, 419.52. Found: *m/z* 420.22 $[M + H]^+$.

5.2.78 2-[5-(3-Methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-*N*-(4-methoxyphenyl)-acetamide (46j)



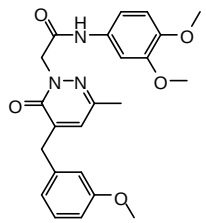
Yield = 75 %; mp = 65-67 °C (EtOH). IR (cm^{-1}) 3300 (NH), 1705 (CO), 1644 (CO). ^1H NMR ($CDCl_3$) δ 2.28 (s, 3H, 3-CH_3), 3.80 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.91 (s, 2H, *CH_2*-Ar), 4.94 (s, 2H, NCH_2CO), 6.77 (s, 1H, Ar), 6.80 (s, 1H, Ar), 6.81-6.86 (m, 4H, Ar), 7.27-7.30 (m, 1H, Ar), 7.42 (d, 2H, Ar, *J* = 8.9 Hz), 8.71 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{22}H_{23}N_3O_4$, 393.44. Found: *m/z* 394.18 $[M + H]^+$.

5.2.79 *N*-(4-Butoxyphenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetamide (46k)



Yield = 73 %; oil. IR (cm^{-1}) 3300 (NH), 1708 (CO), 1645 (CO). ^1H NMR ($CDCl_3$) δ 0.99 (t, 3H, $O(CH_2)_3$*CH_3*, *J* = 7.5 Hz), 1.50 (sext, 2H, OCH_2CH_2*CH_2*, *J* = 7.5 Hz), 1.76 (quin, 2H, OCH_2*CH_2*CH_2, *J* = 7.0 Hz), 2.28 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.90 (s, 2H, *CH_2*-Ar), 3.93 (t, 2H, O*CH_2*CH_2CH_2, *J* = 6.5 Hz), 4.94 (s, 2H, NCH_2CO), 6.76 (s, 1H, Ar), 6.80-6.86 (m, 5H, Ar), 7.26-7.30 (m, 1H, Ar), 7.40 (d, 2H, Ar, *J* = 8.9 Hz), 8.76 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{25}H_{29}N_3O_4$, 435.52. Found: *m/z* 436.23 $[M + H]^+$.

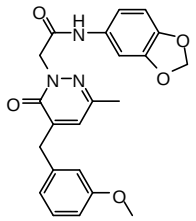
5.2.80 *N*-(3,4-Dimethoxyphenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetamide (46 l)



Yield = 74 %; mp = 57-59 °C (EtOH). IR (cm^{-1}) 3298 (NH), 1708 (CO), 1640 (CO). ^1H NMR ($CDCl_3$) δ 2.29 (s, 3H, 3-CH_3), 3.80 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.90 (s, 2H, *CH_2*-Ar),

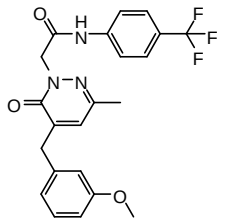
4.95 (s, 2H, NCH_2CO), 6.72 (d, 1H, Ar, *J* = 8.6 Hz), 6.78 (s, 2H, Ar), 6.82-6.88 (m, 3H, Ar), 7.25-7.29 (m, 1H, Ar), 7.32 (d, 1H, Ar, *J* = 2.2 Hz), 8.93 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{23}H_{25}N_3O_5$, 423.46. Found: *m/z* 424.19 $[M + H]^+$.

5.2.81 *N*-Benzo-1,3-dioxol-5-yl-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetamide (46m)



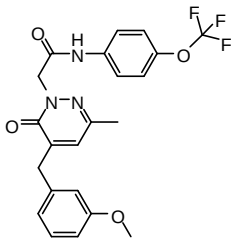
Yield = 98 %; oil. IR (cm^{-1}) 3300 (NH), 1707 (CO), 1643 (CO). ^1H NMR ($CDCl_3$) δ 2.28 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.89 (s, 2H, *CH_2*-Ar), 4.93 (s, 2H, NCH_2CO), 5.92 (s, 2H, O-CH_2-O), 6.65 (d, 1H, Ar, *J* = 8.3 Hz), 6.77-6.85 (m, 5H, Ar), 7.21 (d, 1H, Ar, *J* = 2.0 Hz), 7.25-7.29 (m, 1H, Ar), 9.04 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{22}H_{21}N_3O_5$, 407.42. Found: *m/z* 408.16 $[M + H]^+$.

5.2.82 2-[5-(3-Methoxybenzyl)-3-methyl-6-oxo-6H-pyridazin-1-yl]-*N*-(4-trifluoro methylphenyl)-acetamide (46n)



Yield = 80 %; mp = 175-176 °C (EtOH). IR (cm^{-1}) 3297 (NH), 1708 (CO), 1646 (CO). ^1H NMR ($CDCl_3$) δ 2.33 (s, 3H, 3-CH_3), 3.79 (s, 3H, OCH_3), 3.91 (s, 2H, *CH_2*-Ar), 5.00 (s, 2H, NCH_2CO), 6.81-6.85 (m, 3H, Ar), 6.89 (s, 1H, Ar), 7.25-7.29 (m, 1H, Ar), 7.38 (d, 2H, Ar, *J* = 8.7 Hz), 7.47 (d, 2H, Ar, *J* = 8.7 Hz), 9.62 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{22}H_{20}F_3N_3O_3$, 431.41. Found: *m/z* 432.16 $[M + H]^+$.

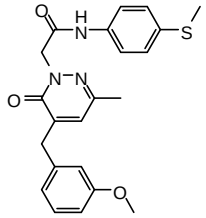
5.2.83 2-[5-(3-Methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-*N*-(4-trifluoro methoxyphenyl)-acetamide (46o)



Yield = 87 %; mp = 168-169 °C (EtOH). IR (cm^{-1}) 3300 (NH), 1708 (CO), 1644 (CO). ^1H NMR ($CDCl_3$) δ 2.31 (s, 3H, 3-CH_3), 3.78 (s, 3H, OCH_3), 3.89 (s, 2H, *CH_2*-Ar), 5.00 (s, 2H, NCH_2CO), 6.79-6.83 (m,

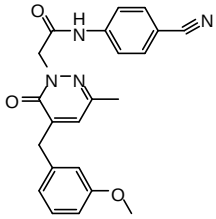
3H, Ar), 6.87 (s, 1H, Ar), 7.00 (d, 2H, Ar, *J* = 8.6 Hz), 7.24- 7.28 (m, 1H, Ar), 7.41 (d, 2H, Ar, *J* = 9.0 Hz), 9.54 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{22}H_{20}F_3N_3O_4$, 447.41. Found: *m/z* 448.15 $[M + H]^+$.

5.2.84 2-[5-(3-Methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]-*N*-[4-(methylthio) phenyl]acetamide (46p)



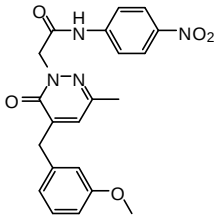
Yield = ~ 100 %; mp = 166-167 °C (EtOH). IR (cm^{-1}) 3285 (NH), 1720 (CO), 1634 (CO). ^{1}H NMR ($CDCl_3$) δ 2.28 (s, 3H, 3-CH_3), 2.45 (s, 3H, SCH_3), 3.80 (s, 3H, OCH_3), 3.89 (s, 2H, *CH_2*-Ar), 4.958 (s, 2H, NCH_2CO), 6.79-6.85 (m, 4H, Ar), 7.12-7.15 (m, 1H, Ar), 7.27 (t, 1H, Ar, *J* = 7.8 Hz), 7.39 (dd, 2H, Ar, *J* = 5.0 Hz), 9.13 (exch br s, 1H, NH).

5.2.85 *N*-(4-Cyanophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-acetamide (46q)



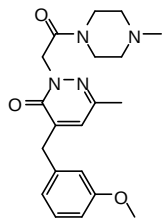
Yield = 55 %; mp = 156-158 °C (EtOH). IR (cm^{-1}) 3285 (NH), 2221 (CN), 1716 (CO), 1644 (CO). ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H, 3-CH_3), 3.80 (s, 3H, OCH_3), 3.91 (s, 2H, *CH_2*-Ar), 4.98 (s, 2H, N*CH_2*CO), 6.80-6.88 (m, 4H, Ar), 7.26-7.30 (m, 1H, Ar), 7.50-7.57 (m, 4H, Ar), 9.60 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{22}H_{20}N_4O_3$, 388.42. Found: *m/z* 389.16 $[M + H]^+$.

5.2.86 2-[5-(3-Methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-*N*-(4-nitrophenyl)-acetamide (46r)



Yield = 49 %; mp = 165-166 °C (EtOH). IR (cm^{-1}) 3298 (NH), 1708 (CO), 1644 (CO). ^{1}H NMR ($CDCl_3$) δ 2.34 (s, 3H, 3-CH_3), 3.79 (s, 3H, OCH_3), 3.92 (s, 2H, *CH_2*-Ar), 5.01 (s, 2H, NCH_2CO), 6.80-6.84 (m, 3H, Ar), 6.94 (s, 1H, Ar), 7.25-7.28 (m, 1H, Ar), 7.49 (d, 2H, Ar, *J* = 9.2 Hz), 7.99 (d, 2H, Ar, *J* = 9.2 Hz), 9.92 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{21}H_{20}N_4O_5$, 408.41. Found: *m/z* 409.15 $[M + H]^+$.

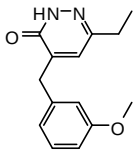
5.2.87 4-(3-Methoxybenzyl)-6-methyl-2-[2-(4-methylpiperazin-1-yl)-2-oxo-ethyl]-pyridazin-3(2*H*)-one (46s)



Yield = 62 %; oil. IR (cm^{-1}) 1673 (CO), 1644 (CO). ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H, 3-CH_3), 2.55 (s, 3H, CH_3N), 2.74-2.81 (m, 4H, Ar), 3.77-3.82 (m, 7H (4H, Ar; 3H, OCH_3)), 3.87 (s, 2H, *CH_2*-Ar), 4.96 (s, 2H, NCH_2CO), 6.69 (s, 1H, Ar), 6.80 (s, 1H, Ar), 6.82-6.85 (m, 2H, Ar), 7.26-7.30 (m, 1H, Ar). MS (ESI) calcd. For $C_{20}H_{26}N_4O_3$, 370.45. Found: *m/z* 371.21 $[M + H]^+$.

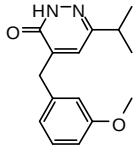
General procedure for 47a-o. To 8 mL of KOH in absolute EtOH (5%, w/v), the appropriate compound of type **42** (**42a-c,e-j,l**) (1.39 mmol) and 3- or 4-methoxybenzaldehyde (1.39 mmol) were added. The mixture was refluxed under stirring for 1-3 h. After cooling, the mixture was concentrated in vacuo, diluted with cold water (10-15 mL) and acidified with 2 N HCl. For compounds **47a-e** the suspension was extracted with CH_2Cl_2 (3 x 15 mL). Removal of the solvent afforded the final compounds, which were purified by column chromatography using respectively cyclohexane/ethyl acetate 1:1 (for **47a,e**), 2:1 (for **47b,c**) and 1:3 (for **47d**) as eluent. On the contrary, compounds **47f-o**, after 1 h stirring in ice-bath, were filtered off by suction from the acidic solutions and recrystallized from ethanol.

5.2.88 6-Ethyl-4-(3-methoxybenzyl)pyridazin-3(2*H*)-one (47a)



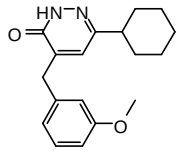
Yield = 20 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.18 (t, 3H, CH_2*CH_3*, *J* = 7.5 Hz), 2.55 (q, 2H, *CH_2*CH_3, *J* = 7.6 Hz), 3.83 (s, 3H, OCH_3), 3.90 (s, 2H, C_6H_4-*CH_2*), 6.75 (s, 1H, Ar), 6.82-6.86 (m, 3H, Ar), 7.29 (t, 1H, Ar, *J* = 7.8 Hz), 11.12 (exch br s, 1H, NH).

5.2.89 6-Isopropyl-4-(3-methoxybenzyl)pyridazin-3(2*H*)-one (47b)



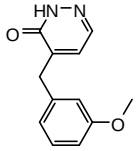
Yield = 44 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.19 (d, 6H, CH*(CH_3)$_2$*, *J* = 8.3 Hz), 2.83 (sept, 1H, *CH*(CH_3)$_2$, *J* = 8.3 Hz), 3.83 (s, 3H, OCH_3), 3.91 (s, 2H, C_6H_4-*CH_2*), 6.83-6.87 (m, 4H, Ar), 7.29 (t, 1H, Ar, *J* = 8.0 Hz).

5.2.90 6-Cyclohexyl-4-(3-methoxybenzyl)pyridazin-3(2*H*)-one (47c)



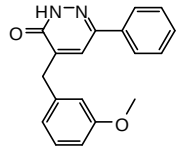
Yield = 96 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.28-1.37 (m, 4H, 2 x CH_2 cyclohexyl), 1.62-1.83 (m, 6H, 3 x CH_2 cyclohexyl), 2.35-2.45 (m, 1H, CH cyclohexyl), 3.81 (s, 3H, OCH_3), 3.88 (s, 2H, C_6H_4-CH_2), 6.34-6.88 (m, 2H, Ar), 6.97 (d, 2H, Ar, J = 7.3 Hz), 7.30 (t, 1H, Ar, J = 8.0 Hz).

5.2.91 4-(3-Methoxybenzyl)pyridazin-3(2*H*)-one (47d)



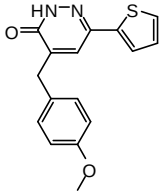
Yield = 27 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 3.83 (s, 3H, OCH_3), 3.92 (s, 2H, C_6H_4-CH_2), 6.81-6.89 (m, 4H, Ar), 7.29 (t, 2H, Ar, J = 8.1 Hz), 7.77 (d, 1H, Ar, J = 4.0 Hz).

5.2.92 4-(3-Methoxybenzyl)-6-phenylpyridazin-3(2*H*)-one (47e)



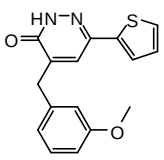
Yield = 45 %; mp = 103-05 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.84 (s, 3H, OCH_3), 4.00 (s, 2H, C_6H_4-CH_2), 6.85-6.92 (m, 3H, Ar), 6.96 (d, 2H, Ar, J = 8.5 Hz), 7.28-7.38 (m, 2H, Ar), 7.43-7.45 (m, 2H, Ar), 7.69-7.71 (m, 1H, Ar).

5.2.93 4-(4-Methoxybenzyl)-6-(thiophen-2-yl)pyridazin-3(2*H*)-one (47f)



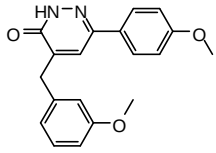
Yield = 97 %; mp = 125-26 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.84 (s, 3H, OCH_3), 3.94 (s, 2H, C_6H_4-CH_2), 6.91-6.98 (m, 3H, Ar), 7.03-7.08 (m, 1H, Ar), 7.22-7.25 (m, 2H, Ar), 7.31-7.33 (m, 1H, Ar), 7.36 (d, 2H, Ar, J = 6.5 Hz).

5.2.94 4-(3-Methoxybenzyl)-6-(thiophen-2-yl)pyridazin-3(2*H*)-one (47g)



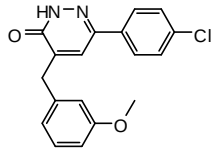
Yield = 97 %; mp = 132-34 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.84 (s, 3H, OCH_3), 3.97 (s, 2H, C_6H_4-CH_2), 6.86-6.90 (m, 3H, Ar), 7.05 (dd, 1H, Ar, J = 1.1 Hz, J = 3.8 Hz), 7.24-7.33 (m, 3H, Ar), 7.37 (d, 2H, Ar, J = 5.1 Hz).

5.2.95 4-(3-Methoxybenzyl)-6-(4-methoxyphenyl)pyridazin-3(2*H*)-one (47i)



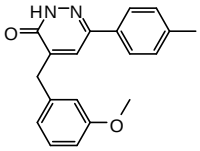
Yield = 99 %; mp = 150-152 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.83 (s, 3H, C_6H_4-OCH_3), 3.86 (s, 3H, CH_2-C_6H_4-OCH_3), 3.98 (s, 2H, C_6H_4-CH_2), 6.86 (d, 1H, Ar, J = 7.0 Hz), 6.95 (d, 2H, Ar, J = 7.6 Hz), 7.30-7.32 (m, 2H, Ar), 7.62 (d, 2H, Ar, J = 8.8 Hz).

5.2.96 6-(4-Chlorophenyl)-4-(3-methoxybenzyl)pyridazin-3(2*H*)-one (47k)



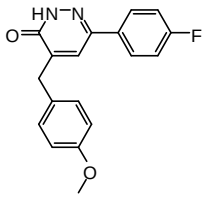
Yield ~ 100 %; mp = 164-166 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.83 (s, 3H, OCH_3), 3.98 (s, 2H, C_6H_4-CH_2), 6.86-6.90 (m, 3H, Ar), 7.29-7.33 (m, 2H, Ar), 7.40 (d, 2H, Ar, J = 8.6 Hz), 7.62 (d, 2H, Ar, J = 8.8 Hz).

5.2.97 4-(3-Methoxybenzyl)-6-*p*-tolylpyridazin-3(2*H*)-one (47m)



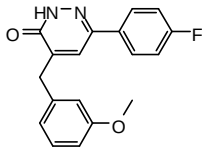
Yield = 98 %; mp = 138-140 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3H, C_6H_4-CH_3), 3.83 (s, 3H, OCH_3), 3.98 (s, 2H, C_6H_4-CH_2), 6.86 (d, 2H, Ar, J = 6.7 Hz), 6.91 (s, 1H, Ar), 7.24 (d, 2H, Ar, J = 8.0 Hz), 7.30-7.33 (m, 1H, Ar), 7.35 (s, 1H, Ar), 7.58 (d, 2H, Ar, J = 8.3 Hz).

5.2.98 6-(4-Fluorophenyl)-4-(4-methoxybenzyl)pyridazin-3(2*H*)-one (47n)



Yield = 70 %; mp = 106-108 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.84 (s, 3H, OCH_3), 3.94 (s, 2H, C_6H_4-CH_2), 6.92 (d, 2H, Ar, J = 5.0 Hz), 7.12 (d, 2H, Ar, J = 8.4 Hz), 7.22 (d, 2H, Ar, J = 8.4 Hz), 7.65-7.69 (m, 2H, Ar).

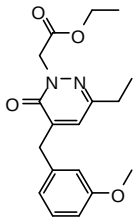
5.2.99 6-(4-Fluorophenyl)-4-(3-methoxybenzyl)pyridazin-3(2*H*)-one (47o)



Yield = 70 %; mp = 106-108 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.83 (s, 3H, OCH_3), 3.98 (s, 2H, C_6H_4-CH_2), 6.86-6.91 (m, 3H, Ar), 7.12 (t, 2H, Ar, J = 8.5 Hz), 7.29-7.33 (m, 2H, Ar), 7.66-7.69 (m, 2H, Ar).

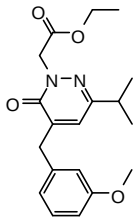
General procedure for 48a-o. A mixture of the appropriate intermediate **47** (**47a-o**) (1.34 mmol), K_2CO_3 (2.68 mmol), and ethyl bromoacetate (2.01 mmol) in CH_3CN (8 mL) was refluxed under stirring for 1-3 h. The mixture was then concentrated in vacuo, diluted with cold water and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated in vacuo and compounds **48a-o** were purified by column chromatography using cyclohexane/ethyl acetate 2:1 as eluent.

5.2.100 Ethyl-2-[3-ethyl-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetate (48a)



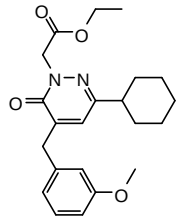
Yield = 98 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.15 (t, 3H, CH_2CH_3, J = 4.1 Hz), 1.30 (t, 3H, OCH_2CH_3, J = 6.9 Hz), 2.53 (q, 2H, CH_2CH_3, J = 7.6 Hz), 3.82 (s, 3H, OCH_3), 3.88 (s, 2H, CH_2-Ar), 4.26 (q, 2H, OCH_2CH_3, J = 7.1 Hz), 4.87 (s, 2H, NCH_2CO), 6.72 (s, 1H, Ar), 6.80-6.85 (m, 3H, Ar), 7.28 (t, 1H, Ar, J = 6.2 Hz).

5.2.101 Ethyl-2-[3-isopropyl-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetate (48b)



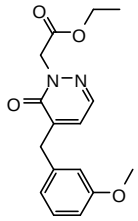
Yield = 93 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.15 (d, 6H, $CH(CH_3)_2$, J = 6.9 Hz), 1.28-1.32 (m, 3H, OCH_2CH_3), 2.73-2.83 (m, 1H, $CH(CH_3)_2$), 3.81 (s, 3H, OCH_3), 3.89 (s, 2H, CH_2-Ar), 4.23-4.28 (m, 2H, OCH_2CH_3), 4.86 (s, 2H, NCH_2CO), 6.77 (s, 1H, Ar), 6.80-6.84 (m, 3H, Ar), 7.27 (t, 1H, Ar, J = 7.8 Hz).

5.2.102 Ethyl-2-[3-cyclohexyl-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetate (48c)



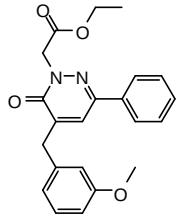
Yield = 58 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.28-1.34 (m, 9H, 3 x CH_2 cyclohexyl + $OCH_2\mathit{CH_3}$), 1.70-1.82 (m, 4H, 2 x CH_2 cyclohexyl), 2.42-2.51 (m, 1H, CH cyclohexyl), 3.83 (s, 3H, OCH_3), 3.89 (s, 2H, $\mathit{CH_2}$-Ar), 4.23-4.29 (m, 2H, $O\mathit{CH_2}CH_3$), 4.87 (s, 2H, NCH_2CO), 6.76 (s, 1H, Ar), 6.80-6.95 (m, 3H, Ar), 7.27 (dt, 1H, Ar, J = 11.7 Hz, J = 2.0 Hz).

5.2.103 Ethyl-2-[5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetate (48d)



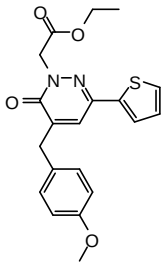
Yield = 47 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, $CH_2\mathit{CH_3}$, J = 7.1 Hz), 3.82 (s, 3H, OCH_3), 3.91 (s, 2H, $\mathit{CH_2}$-Ar), 4.27 (q, 2H, $O\mathit{CH_2}CH_3$, J = 7.2 Hz), 4.92 (s, 2H, NCH_2CO), 6.79-6.80 (m, 2H, Ar), 6.83 (d, 2H, Ar, J = 7.8 Hz), 7.28 (t, 1H, Ar, J = 8.0 Hz), 7.67 (d, 1H, Ar, J = 4.1 Hz).

5.2.104 Ethyl-2-[5-(3-methoxybenzyl)-6-oxo-3-phenylpyridazin-1(6*H*)-yl]acetate (48e)



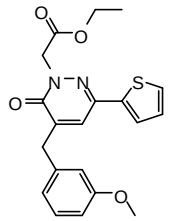
Yield = 47 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.29-1.33 (td, 3H, $CH_2\mathit{CH_3}$, J = 6.8 Hz, J = 1.9 Hz), 3.80 (s, 3H, OCH_3), 3.95 (s, 2H, $\mathit{CH_2}$-Ar), 4.27 (q, 2H, $O\mathit{CH_2}CH_3$, J = 7.1 Hz), 4.99 (s, 2H, NCH_2CO), 6.81-6.89 (m, 3H, Ar), 6.92-94 (m, 2H, Ar), 7.24-7.31 (m, 2H, Ar), 7.40-7.42 (m, 2H, Ar), 7.66-7.68 (m, 1H, Ar).

5.2.105 Ethyl-2-[5-(4-methoxybenzyl)-6-oxo-3-(thiophen-2-yl)pyridazin-1(6*H*)-yl]acetate (48f)



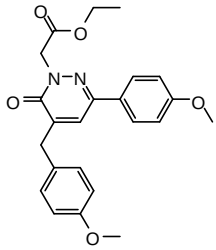
Yield = 91 %; mp = 127-29 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.32 (t, 3H, CH_2CH_3, J = 6.9 Hz), 3.84 (s, 3H, OCH_3), 3.93 (s, 2H, CH_2-Ar), 4.28 (q, 2H, OCH_2CH_3, J = 7.1 Hz), 4.94 (s, 2H, NCH_2CO), 6.92 (d, 2H, Ar, J = 8.0 Hz), 7.03 (t, 1H, Ar, J = 4.0 Hz), 7.20-7.28 (m, 4H, Ar), 7.35 (d, 1H, Ar, J = 4.4 Hz).

5.2.106 Ethyl-2-[5-(3-methoxybenzyl)-6-oxo-3-(thiophen-2-yl)pyridazin-1(6*H*)-yl]acetate (48g)



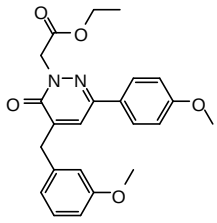
Yield ~ 100 %; mp = 149-51 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.32 (t, 3H, CH_2CH_3, J = 7.0 Hz), 3.84 (s, 3H, OCH_3), 3.96 (s, 2H, CH_2-Ar), 4.28 (q, 2H, OCH_2CH_3, J = 7.1 Hz), 4.94 (s, 2H, NCH_2CO), 6.87 (t, 3H, Ar, J = 8.4 Hz), 7.03-7.05 (m, 1H, Ar), 7.23-7.40 (m, 4H, Ar).

5.2.107 Ethyl-2-[5-(4-methoxybenzyl)-3-(4-methoxyphenyl)-6-oxopyridazin-1(6*H*)-yl]acetate (48h)



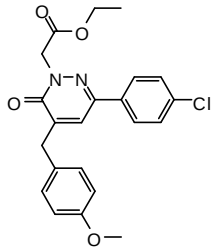
Yield ~ 100 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, CH_2CH_3, J = 3.9 Hz), 3.82 (s, 3H, CH_2-C_6H_4-OCH_3), 3.84 (s, 3H, C_6H_4-OCH_3), 3.88 (s, 2H, CH_2-Ar), 4.26 (q, 2H, OCH_2CH_3, J = 7.1 Hz), 4.97 (s, 2H, NCH_2CO), 6.88-6.99 (m, 5H, Ar), 7.21 (q, 2H, Ar, J = 6.7 Hz), 7.60 (d, 2H, Ar, J = 8.8 Hz).

5.2.108 Ethyl-2-[5-(3-methoxybenzyl)-3-(4-methoxyphenyl)-6-oxopyridazin-1(6*H*)-yl]acetate (48i)



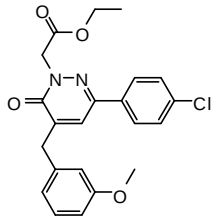
Yield ~ 100 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.32 (t, 3H, CH_2CH_3, J = 7.2 Hz), 3.82 (s, 3H, CH_2-C_6H_4-OCH_3), 3.84 (s, 3H, C_6H_4-OCH_3), 3.97 (s, 2H, CH_2-Ar), 4.28 (q, 2H, OCH_2CH_3, J = 7.1 Hz), 4.98 (s, 2H, NCH_2CO), 6.84-6.95 (m, 6H, Ar), 7.27-7.31 (m, 1H, Ar), 7.61 (dd, 2H, Ar, J = 4.8 Hz, J = 2.0 Hz).

5.2.109 Ethyl-2-[3-(4-chlorophenyl)-5-(4-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetate (48j)



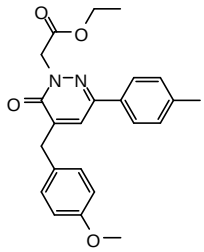
Yield ~ 100 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, CH_2CH_3, J = 7.1 Hz), 3.82 (s, 3H, CH_2-C_6H_4-OCH_3), 3.87 (s, 2H, CH_2-Ar), 4.24 (q, 2H, OCH_2CH_3, J = 6.2 Hz), 4.98 (s, 2H, NCH_2CO), 6.90 (d, 2H, Ar, J = 8.5 Hz), 7.21 (t, 2H, Ar, J = 7.4 Hz), 7.35-7.39 (m, 3H, Ar), 7.60 (d, 2H, Ar, J = 8.6 Hz).

5.2.110 Ethyl-2-[3-(4-chlorophenyl)-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetate (48k)



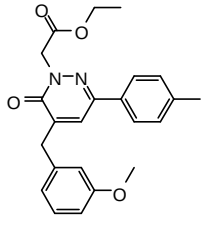
Yield ~ 100 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.32 (t, 3H, CH_2CH_3, J = 7.2 Hz), 3.82 (s, 3H, OCH_3), 3.97 (s, 2H, CH_2-Ar), 4.27 (q, 2H, OCH_2CH_3, J = 4.0 Hz), 4.99 (s, 2H, NCH_2CO), 6.84-6.86 (m, 3H, Ar), 7.26-7.32 (m, 2H, Ar), 7.38 (d, 2H, Ar, J = 8.6 Hz), 7.61 (d, 2H, Ar, J = 8.6 Hz).

5.2.111 Ethyl-2-[5-(4-methoxybenzyl)-6-oxo-3-(*p*-tolyl)pyridazin-1(6*H*)-yl]acetate (48l)



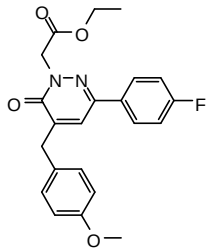
Yield = 98 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, CH_2CH_3, J = 7.1 Hz), 2.38 (s, 3H, C_6H_4-CH_3), 3.83 (s, 3H, OCH_3), 3.93 (s, 2H, CH_2-Ar), 4.27 (q, 2H, OCH_2CH_3, J = 7.1 Hz), 4.83 (s, 2H, NCH_2CO), 6.88-6.97 (m, 5H, Ar), 7.27 (d, 2H, Ar, J = 8.5 Hz), 8.07 (d, 2H, Ar, J = 9.0 Hz).

5.2.112 Ethyl-2-[5-(3-methoxybenzyl)-6-oxo-3-(*p*-tolyl)pyridazin-1(6*H*)-yl]acetate (48m)



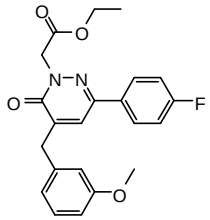
Yield = 97 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, CH_2CH_3, J = 7.1 Hz), 2.39 (s, 3H, C_6H_4-CH_3), 3.82 (s, 3H, CH_2-C_6H_4-OCH_3), 3.97 (s, 2H, CH_2-Ar), 4.27 (q, 2H, OCH_2CH_3, J = 7.2 Hz), 4.99 (s, 2H, NCH_2CO), 6.84-6.89 (m, 3H, Ar), 6.95-6.97 (m, 1H, Ar), 7.22 (d, 2H, Ar, J = 7.9 Hz), 7.27-7.31 (m, 1H, Ar), 8.07 (d, 2H, Ar, J = 6.6 Hz).

5.2.113 Ethyl-2-[3-(4-fluorophenyl)-5-(4-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetate (48n)



Yield = 92 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.32 (t, 3H, CH_2CH_3, J = 7.1 Hz), 3.84 (s, 3H, OCH_3), 3.94 (s, 2H, CH_2-Ar), 4.27 (q, 2H, OCH_2CH_3, J = 7.1 Hz), 4.99 (s, 2H, NCH_2CO), 6.92 (d, 3H, Ar, J = 8.6 Hz), 7.10 (t, 2H, Ar, J = 8.5 Hz), 7.21 (d, 2H, Ar, J = 9.9 Hz), 7.63-7.67 (m, 3H, Ar).

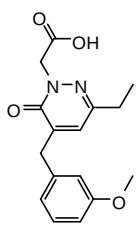
5.2.114 Ethyl-2-[3-(4-fluorophenyl)-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetate (48o)



Yield = 88 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.32 (td, 3H, CH_2CH_3, J = 5.9 Hz, J = 1.3 Hz), 3.82 (s, 3H, OCH_3), 3.98 (s, 2H, CH_2-Ar), 4.27 (q, 2H, OCH_2CH_3, J = 7.1 Hz), 4.99 (s, 2H, NCH_2CO), 6.84-6.89 (m, 3H, Ar), 7.10 (t, 2H, Ar, J = 8.6 Hz), 7.25-7.30 (m, 2H, Ar), 7.63-7.67 (m, 3H, Ar).

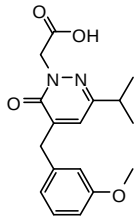
General procedure for 49a-o. A suspension of the appropriate compound type **48** (**48a-o**) (1.33 mmol) in 6 N NaOH (10 mL) was stirred at rt to 80 °C for 1-2 h. The mixture was diluted with cold water and acidified with 6 N HCl. After 1 h stirring in ice-bath, the products **49a-o** were filtered off by suction and recrystallized from ethanol.

5.2.115 2-[3-Ethyl-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetic acid (49a)



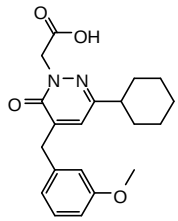
Yield ~ 100 %; mp = 117-19 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.17 (t, 3H, CH_2CH_3, J = 7.5 Hz), 2.55 (q, 2H, CH_2CH_3, J = 7.5 Hz), 3.82 (s, 3H, OCH_3), 3.91 (s, 2H, CH_2-Ar), 4.94 (s, 2H, NCH_2CO), 6.75 (s, 1H, Ar), 6.79 (s, 1H, Ar), 6.82-6.85 (m, 2H, Ar), 7.28 (t, 1H, Ar, J = 7.2 Hz).

5.2.116 2-[3-Isopropyl-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetic acid (49b)



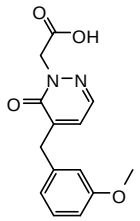
Yield = 69 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.16 (d, 6H, $CH(CH_3)_2$, J = 6.9 Hz), 2.75-2.85 (m, 1H, $CH(CH_3)_2$), 3.81 (s, 3H, OCH_3), 3.91 (s, 2H, CH_2-Ar), 4.93 (s, 2H, NCH_2CO), 6.70 (exch br s, 1H, OH), 6.82 (t, 4H, Ar, J = 8.0 Hz), 7.27 (t, 1H, Ar, J = 4.2 Hz).

5.2.117 2-[3-Cyclohexyl-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetic acid (49c)



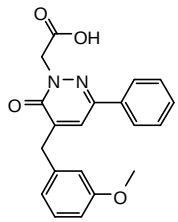
Yield = 70 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.21-1.40 (m, 6H, 3 x CH_2 cyclohexyl), 1.70-1.82 (m, 4H, 2 x CH_2 cyclohexyl), 2.41-2.50 (m, 1H, CH cyclohexyl), 3.83 (s, 3H, OCH_3), 3.88 (s, 2H, CH_2-Ar), 4.65 (s, 2H, NCH_2CO), 6.79 (s, 1H, Ar), 6.81-6.98 (m, 3H, Ar), 7.30 (t, 1H, Ar, J = 7.1 Hz).

5.2.118 2-[5-(3-Methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetic acid (49d)



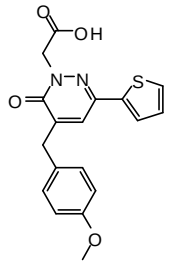
Yield ~ 100 %; oil. ^{1}H NMR ($CDCl_3$) δ 3.81 (s, 3H, OCH_3), 3.91 (s, 2H, CH_2-Ar), 4.98 (s, 2H, NCH_2CO), 6.49 (exch br s, 1H, OH), 6.78 (s, 1H, Ar), 6.81-6.85 (m, 3H, Ar), 7.28 (t, 1H, Ar, J = 4.6 Hz), 7.72 (d, 1H, Ar, J = 4.1 Hz).

5.2.119 2-[5-(3-Methoxybenzyl)-6-oxo-3-phenylpyridazin-1(6*H*)-yl]acetic acid (49e)



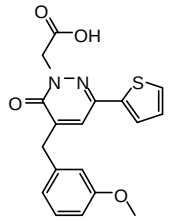
Yield = 60 %; mp = 173-75 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.79 (s, 3H, OCH_3), 3.93 (s, 2H, *CH_2*-Ar), 5.03 (s, 2H, NCH_2CO), 5.57 (exch br s, 1H, OH), 6.82-6.84 (m, 3H, Ar), 6.93 (t, 2H, Ar, *J* = 4.16 Hz), 7.24-7.31 (m, 2H, Ar), 7.41 (t, 2H, Ar, *J* = 3.84 Hz), 7.66-7.68 (m, 1H, Ar).

5.2.120 2-[5-(4-Methoxybenzyl)-6-oxo-3-(thiophen-2-yl)pyridazin-1(6*H*)-yl]acetic acid (49f)



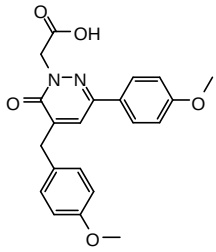
Yield = 89 %; mp = 159-61 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.84 (s, 3H, OCH_3), 3.94 (s, 2H, *CH_2*-Ar), 5.01 (s, 2H, NCH_2CO), 6.92 (dd, 2H, Ar, *J* = 9.2 Hz, *J* = 1.4 Hz), 7.04 (dd, 1H, Ar, *J* = 1.4 Hz, *J* = 6.0 Hz), 7.20-7.23 (m, 3H, Ar), 7.25 (dd, 1H, *J* = 6.4 Hz, *J* = 1.0 Hz), 7.37 (dd, 1H, Ar, *J* = 6.4 Hz, *J* = 1.1 Hz).

5.2.121 2-[5-(3-Methoxybenzyl)-6-oxo-3-(thiophen-2-yl)pyridazin-1(6*H*)-yl]acetic acid (49g)



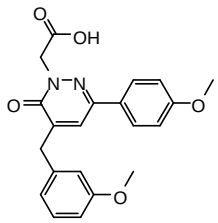
Yield ~ 100 %; mp = 185-87 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H, OCH_3), 3.97 (s, 2H, *CH_2*-Ar), 5.01 (s, 2H, NCH_2CO), 6.84-6.88 (m, 3H, Ar), 7.03 (t, 1H, Ar, *J* = 4.8 Hz), 7.23 (t, 2H, Ar, *J* = 3.2 Hz), 7.28 (t, 1H, Ar, *J* = 4.4 Hz), 7.36 (d, 1H, Ar, *J* = 5.0 Hz).

5.2.122 2-[5-(4-Methoxybenzyl)-3-(4-methoxyphenyl)-6-oxopyridazin-1(6*H*)-yl]acetic acid (49h)



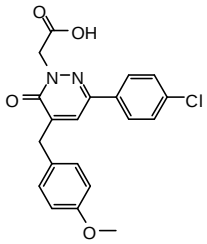
Yield = 99 %; mp = 115-17 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.64 (s, 3H, CH_2-C_6H_4-O*CH_3*), 3.66 (s, 3H, C_6H_4-O*CH_3*), 3.79 (s, 2H, *CH_2*-Ar), 4.88 (s, 2H, NCH_2CO), 6.65-6.75 (m, 4H, Ar), 6.90-7.01 (m, 1H, Ar), 7.05-7.15 (m, 1H, Ar), 7.28-7.37 (m, 3H, Ar).

5.2.123 2-[5-(3-Methoxybenzyl)-3-(4-methoxyphenyl)-6-oxopyridazin-1(6*H*)-yl]acetic acid (49i)



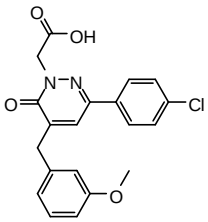
Yield = 98 %; 110-12 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H, CH_2-C_6H_4-OCH_3), 3.84 (s, 3H, C_6H_4-OCH_3), 3.88 (s, 2H, CH_2-Ar), 5.03 (s, 2H, NCH_2CO), 6.89-6.94 (m, 4H, Ar), 7.19 (d, 2H, Ar, J = 8.6 Hz), 7.24 (s, 1H, Ar), 7.60 (d, 2H, Ar, J = 8.8 Hz).

5.2.124 2-[3-(4-Chlorophenyl)-5-(4-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetic acid (49j)



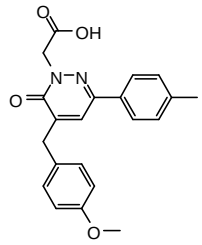
Yield ~ 100 %; mp = 147-49 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.83 (s, 3H, CH_2-C_6H_4-OCH_3), 3.92 (s, 2H, CH_2-Ar), 5.05 (s, 2H, NCH_2CO), 6.92 (d, 2H, Ar, J = 8.5 Hz), 7.20 (d, 2H, Ar, J = 8.5 Hz), 7.25 (s, 1H, Ar), 7.39 (d, 2H, Ar, J = 8.5 Hz), 7.61 (d, 2H, Ar, J = 8.6 Hz).

5.2.125 2-[3-(4-Chlorophenyl)-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetic acid (49k)



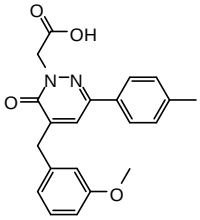
Yield ~ 100 %; mp = 142-44 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.81 (s, 3H, OCH_3), 3.97 (s, 2H, CH_2-Ar), 5.04 (s, 2H, NCH_2CO), 6.83-6.87 (m, 3H, Ar), 7.28 (d, 2H, Ar, J = 7.8 Hz), 7.38 (d, 2H, Ar, J = 8.7 Hz), 7.60 (d, 2H, Ar, J = 8.7 Hz).

5.2.126 2-[5-(4-Methoxybenzyl)-6-oxo-3-(*p*-tolyl)pyridazin-1(6*H*)-yl]acetic acid (49l)



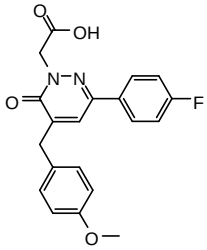
Yield = 98 %; mp = 158-60 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.39 (s, 3H, C_6H_4-CH_3), 3.83 (s, 3H, OCH_3), 3.95 (s, 2H, CH_2-Ar), 5.05 (s, 2H, NCH_2CO), 6.89-6.97 (m, 4H, Ar), 7.21 (dd, 2H, Ar, J = 1.9 Hz, J = 6.3 Hz), 7.29 (s, 1H, Ar), 7.56 (d, 2H, Ar, J = 8.1 Hz).

5.2.127 2-[5-(3-Methoxybenzyl)-6-oxo-3-(*p*-tolyl)pyridazin-1(6*H*)-yl]acetic acid (49m)



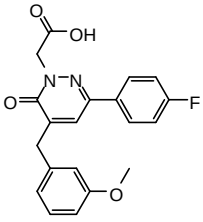
Yield = 97 %; mp = 150-52 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H, C_6H_4-CH_3), 3.66 (s, 3H, CH_2-C_6H_4-OCH_3), 3.82 (s, 2H, CH_2-Ar), 4.85 (s, 2H, NCH_2CO), 6.70 (d, 2H, Ar, J = 5.3 Hz), 6.95 (s, 1H, Ar), 7.03-7.12 (m, 3H, Ar), 7.28 (t, 1H, Ar, J = 7.9 Hz), 7.40 (d, 2H, Ar, J = 7.8 Hz).

5.2.128 2-[3-(4-Fluorophenyl)-5-(4-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetic acid (49n)



Yield = 57 %; mp = 82-84 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.83 (s, 3H, OCH_3), 3.93 (s, 2H, CH_2-Ar), 5.03 (s, 2H, NCH_2CO), 6.91 (d, 2H, Ar, J = 7.8 Hz), 7.10 (t, 2H, Ar, J = 8.4 Hz), 7.14-7.23 (m, 3H, Ar), 7.65 (t, 2H, Ar, J = 5.2 Hz).

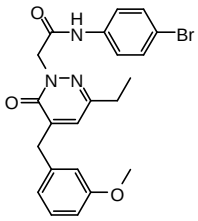
5.2.129 2-[3-(4-Fluorophenyl)-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetic acid (49o)



Yield = 75 %; mp = 103-05 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.81 (s, 3H, OCH_3), 3.97 (s, 2H, *CH_2*-Ar), 5.03 (s, 2H, CH_2CO), 6.83-6.87 (m, 3H, Ar), 7.10 (t, 2H, Ar, *J* = 6.6 Hz), 7.27-7.31 (m, 2H, Ar), 7.63-7.67 (m, 3H, Ar).

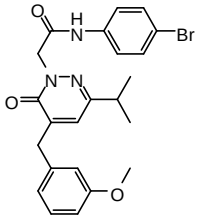
General procedure for 50a-v. To a cooled (-5 °C) and stirred solution of compound type **49** (**49a-o**) (0.60 mmol) in anhydrous tetrahydrofuran (6 mL), Et_3N (2.10 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C and ethyl chloroformate (0.66 mmol) was added. After 1 h, the commercially available substituted arylamine (1.20 mmol) was added. The reaction was carried out at room temperature for 12 h, then the mixture was concentrated in vacuo, diluted with cold water (20-30 mL) and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated to afford final compounds **46a-s**, which were purified by column chromatography using cyclohexane/ethyl acetate 2:1 for compounds **50a,g**, cyclohexane/ethyl acetate 3:1 for compounds **50b,e**, CH_2Cl_2/ CH_3OH/NH_4OH 96:4:0.4 for compound **50c**, cyclohexane/ethyl acetate 1:1 for compounds **50d,f,i,j** and n-hexane/ethyl acetate 3:2 for compound **50h,k-v** as eluents.

5.2.130 *N*-(4-Bromophenyl)-2-[3-ethyl-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50a)



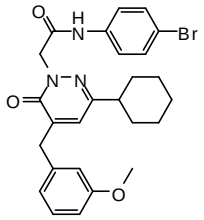
Yield = 61 %; mp = 150-52 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.20 (t, 3H, CH_2*CH_3*, *J* = 7.6 Hz), 2.59 (q, 2H, *CH_2*CH_3, *J* = 7.6 Hz), 3.81 (s, 3H, OCH_3), 3.91 (s, 2H, *CH_2*-Ar), 4.95 (s, 2H, NCH_2CO), 6.80-6.86 (m, 4H, Ar), 7.24-7.30 (m, 3H, Ar), 7.55 (d, 2H, Ar, *J* = 8.7 Hz), 9.18 (exch br s, 1H, NH).

5.2.131 *N*-(4-Bromophenyl)-2-[3-isopropyl-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50b)



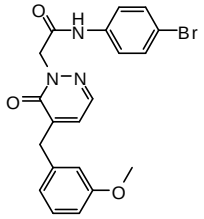
Yield = 67 %; mp = 60-62 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.20 (d, 6H, CH(*CH_3*)*$_2$*, *J* = 6.9 Hz), 2.82-2.90 (m, 1H, *CH*(CH_3)$_2$), 3.81 (s, 3H, OCH_3), 3.92 (s, 2H, *CH_2*-Ar), 4.95 (s, 2H, NCH_2CO), 6.82-6.85 (m, 3H, Ar), 6.92 (s, 1H Ar), 7.28 (t, 1H, Ar, *J* = 7.8 Hz), 7.37 (s, 4H, Ar), 9.23 (exch br s, 1H, NH).

5.2.132 *N*-(4-Bromophenyl)-2-[3-cyclohexyl-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50c)



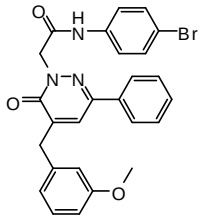
Yield = 36 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.30-1.35 (m, 6H, 3 x CH_2 cyclohexyl), 1.70-1.82 (m, 4H, 2 x CH_2 cyclohexyl), 2.45-2.55 (m, 1H, CH cyclohexyl), 3.82 (s, 3H, OCH_3), 3.92 (s, 2H, *CH_2*-Ar), 4.95 (s, 2H, NCH_2CO), 6.81-6.88 (m, 4H, Ar), 7.28 (t, 1H, Ar, *J* = 9.4 Hz), 7.41 (s, 4H, Ar), 9.15 (exch br s, 1H, NH).

5.2.133 *N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50d)



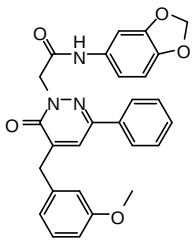
Yield = 70 %; mp = 138-40 °C (EtOH). ^{1}H NMR (DMSO-d_6) δ 3.73 (s, 3H, OCH_3), 3.79 (s, 2H, *CH_2*-Ar), 4.90 (s, 2H, NCH_2CO), 6.83 (t, 3H, Ar, *J* = 9.5 Hz), 7.14 (d, 1H, Ar, *J* = 3.8 Hz), 7.23 (t, 1H, Ar, *J* = 7.7 Hz), 7.53 (q, 4H, Ar, *J* = 8.2 Hz), 7.85 (d, 1H, Ar, *J* = 3.9 Hz), 10.47 (exch br s, 1H, NH).

5.2.134 *N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-6-oxo-3-phenyl-pyridazin-1(6*H*)-yl]acetamide (50e)



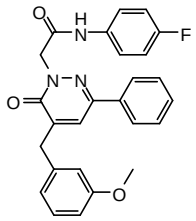
Yield = 56 %; mp = 85-87 °C (EtOH). ^{1}H NMR (DMSO-d_6) δ 3.73 (s, 3H, OCH_3), 3.89 (s, 2H, *CH_2*-Ar), 4.99 (s, 2H, NCH_2CO), 6.80 (d, 1H, Ar, *J* = 8.0 Hz), 7.93 (d, 2H, Ar, *J* = 9.1 Hz), 7.23 (t, 1H, Ar, *J* = 7.9 Hz), 7.45-7.51 (m, 5H, Ar), 7.56 (d, 2H, Ar, *J* = 8.9 Hz), 7.83 (d, 2H, Ar, *J* = 7.1 Hz), 7.86 (s, 1H, Ar), 10.51 (exch br s, 1H, NH).

5.2.135 *N*-(1,3-Benzodioxol-5-yl)-2-[5-(3-methoxybenzyl)-6-oxo-3-phenylpyridazin-1(6*H*)-yl]acetamide (50f)



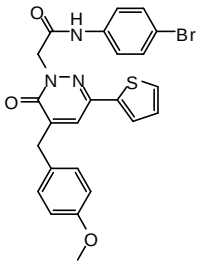
Yield = 41 %; mp = 201-03 °C (EtOH). ^{1}H NMR (DMSO-d_6) δ 3.73 (s, 3H, OCH_3), 3.88 (s, 2H, *CH_2*-Ar), 4.95 (s, 2H, NCH_2CO), 5.99 (s, 2H, OCH_2O), 6.80 (d, 1H, Ar, *J* = 6.0 Hz), 6.87 (d, 1H, Ar, *J* = 8.3 Hz), 6.92-6.97 (m, 3H, Ar), 7.23 (t, 1H, Ar, *J* = 7.8 Hz), 7.28 (s, 1H, Ar), 7.44-7.52 (m, 3H, Ar), 7.83 (d, 2H, Ar, *J* = 6.8 Hz), 7.95 (s, 1H, Ar), 10.27 (exch br s, 1H, NH).

5.2.136 *N*-(4-Fluorophenyl)-2-[5-(3-methoxybenzyl)-6-oxo-3-phenyl-pyridazin-1(6*H*)-yl]acetamide (50g)



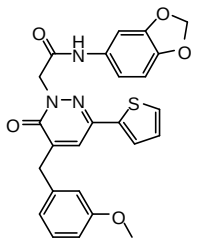
Yield = 78 %; mp = colorless oil. ^{1}H NMR ($CDCl_3$) δ 3.81 (s, 3H, OCH_3), 4.01 (s, 2H, *CH_2*-Ar), 5.10 (s, 2H, NCH_2CO), 6.85-6.90 (m, 3H, Ar), 6.96-7.04 (m, 2H, Ar), 7.29-7.34 (m, 1H, Ar), 7.43-7.49 (m, 6H, Ar), 7.71-7.74 (m, 2H, Ar), 8.95 (exch br s, 1H, NH).

5.2.137 *N*-(4-Bromophenyl)-2-[5-(4-methoxybenzyl)-6-oxo-3-(thiophen-2-yl)pyridazin-1(6*H*)-yl]acetamide (50h)



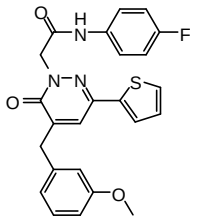
Yield = 77 %; mp = 193-94 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.83 (s, 3H, OCH_3), 3.95 (s, 2H, *CH_2*-Ar), 5.02 (s, 2H, NCH_2CO), 6.91 (dd, 2H, Ar, *J* = 4.6 Hz, *J* = 2.0 Hz), 7.06 (dd, 1H, Ar, *J* = 1.4 Hz, *J* = 3.9 Hz), 7.21 (d, 2H, Ar, *J* = 6.7 Hz), 7.30 (dd, 1H, Ar, *J* = 2.6 Hz, *J* = 1.0 Hz), 7.32-7.38 (m, 5H, Ar), 7.83 (dd, 1H, Ar, *J* = 4.0 Hz, *J* = 1.1 Hz), 9.00 (exch br s, 1H, NH).

5.2.138 *N*-(1,3-Benzodioxol-5-yl)-2-[5-(3-methoxybenzyl)-6-oxo-3-(thiophen-2-yl)pyridazin-1(6*H*)-yl]acetamide (50i)



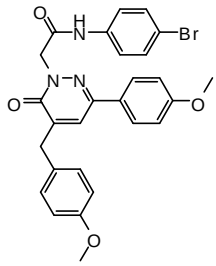
Yield = 41 %; mp = 192-94 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H, OCH_3), 3.98 (s, 2H, *CH_2*-Ar), 5.02 (s, 2H, NCH_2CO), 5.94 (s, 2H, OCH_2O), 6.71 (d, 1H, Ar, *J* = 8.3 Hz), 6.81-6.89 (m, 4H, Ar), 7.05 (dd, 1H, Ar, *J* = 1.3 Hz, *J* = 3.7 Hz), 7.23 (d, 1H, Ar, *J* = 1.9 Hz), 7.28-7.32 (m, 3H, Ar), 7.38 (dd, 1H, Ar, *J* = 4.2 Hz, *J* = 0.9 Hz), 8.71 (exch br s, 1H, NH).

5.2.139 *N*-(4-Fluorophenyl)-2-[5-(3-methoxybenzyl)-6-oxo-3-(thiophen-2-yl)pyridazin-1(6*H*)-yl]acetamide (50j)



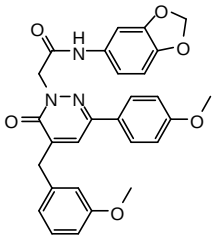
Yield = 37 %; mp = 187-89 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.81 (s, 3H, OCH_3), 3.99 (s, 2H, *CH_2*-Ar), 5.03 (s, 2H, NCH_2CO), 6.85-6.89 (m, 3H, Ar), 6.96 (t, 2H, Ar, *J* = 8.6 Hz), 7.05 (dd, 1H, Ar, *J* = 1.4 Hz, *J* = 3.7 Hz), 7.27-7.30 (m, 2H, Ar), 7.32 (s, 1H, Ar), 7.39 (dd, 1H, Ar, *J* = 4.0 Hz, *J* = 1.1 Hz), 7.43 (m, 2H, Ar), 8.89 (exch br s, 1H, NH).

5.2.140 *N*-(4-Bromophenyl)-2-[5-(4-methoxybenzyl)-3-(4-methoxyphenyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50k)



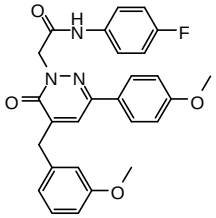
Yield = 32 %; mp = 203-04 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.83 (s, 3H, $CH_2C_6H_4$-OCH_3), 3.86 (s, 3H, C_6H_4-OCH_3), 3.97 (s, 2H, *CH_2*-Ar), 5.07 (s, 2H, NCH_2CO), 6.90-6.96 (m, 4H, Ar), 7.22 (d, 2H, Ar, *J* = 8.6 Hz), 7.36-7.38 (m, 5H, Ar), 7.67 (d, 1H, Ar, *J* = 8.8 Hz), 9.14 (exch br s, 1H, NH).

5.2.141 *N*-(1,3-Benzodioxol-5-yl)-2-[5-(3-methoxybenzyl)-3-(4-methoxy-phenyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50l)



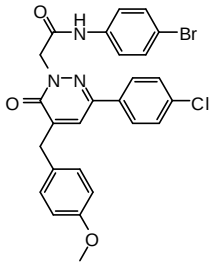
Yield = 89 %; mp = 184-85 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.81 (s, 3H, $CH_2C_6H_4$-OCH_3), 3.85 (s, 3H, C_6H_4-OCH_3), 3.99 (s, 2H, *CH_2*-Ar), 5.05 (s, 2H, NCH_2CO), 5.93 (s, 2H, OCH_2O), 6.68 (d, 1H, Ar, *J* = 8.3 Hz), 6.79 (dd, 1H, Ar, *J* = 6.2 Hz, *J* = 2.1 Hz), 6.84-6.90 (m, 3H, Ar), 6.94 (dd, 2H, Ar, *J* = 4.9 Hz, *J* = 2.0 Hz), 7.22 (d, 1H, Ar, *J* = 2.0 Hz), 7.27-7.31 (m, 1H, Ar), 7.38 (s, 1H, Ar), 7.66 (d, 2H, Ar, *J* = 1.9 Hz), 8.95 (exch br s, 1H, NH).

5.2.142 *N*-(4-Fluorophenyl)-2-[5-(3-methoxybenzyl)-3-(4-methoxyphenyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50m)



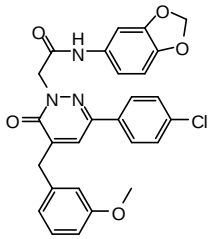
Yield = 84 %; mp = 164-65 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.80 (s, 3H, $CH_2C_6H_4$-OCH_3), 3.86 (s, 3H, C_6H_4-OCH_3), 3.99 (s, 2H, *CH_2*-Ar), 5.07 (s, 2H, NCH_2CO), 6.83-6.96 (m, 7H, Ar), 7.29 (t, 1H, Ar, *J* = 1.8 Hz), 7.40-7.45 (m, 3H, Ar), 7.67 (d, 2H, Ar, *J* = 7.5 Hz), 9.17 (exch br s, 1H, NH).

5.2.143 *N*-(4-Bromophenyl)-2-[3-(4-chlorophenyl)-5-(4-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50n)



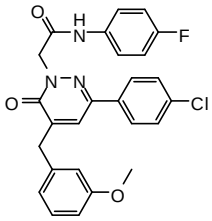
Yield = 27 %; mp = 219-21 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H, OCH_3), 3.97 (s, 2H, *CH_2*-Ar), 5.08 (s, 2H, NCH_2CO), 6.91 (d, 2H, Ar, *J* = 8.7 Hz), 7.21 (d, 2H, Ar, *J* = 8.7 Hz), 7.32-7.37 (m, 5H, Ar), 7.41 (d, 2H, Ar, *J* = 8.8 Hz), 7.66 (d, 2H, Ar, *J* = 8.7 Hz), 9.12 (exch br s, 1H, NH).

5.2.144 *N*-(1,3-Benzodioxol-5-yl)-2-[3-(4-chlorophenyl)-5-(3-methoxy-benzyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50o)



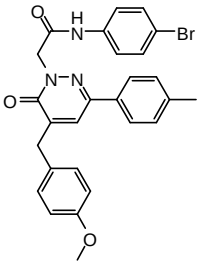
Yield = 99 %; mp = 202-203 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H, OCH_3), 4.01 (s, 2H, *CH_2*-Ar), 5.05 (s, 2H, NCH_2CO), 5.95 (s, 2H, OCH_2O), 6.73 (d, 1H, Ar, *J* = 8.4 Hz), 6.80-6.90 (m, 4H, Ar), 7.23 (d, 1H, Ar, *J* = 1.8 Hz), 7.30 (t, 1H, Ar, *J* = 8.2 Hz), 7.36 (s, 1H, Ar), 7.41 (d, 2H, Ar, *J* = 8.5 Hz), 7.66 (d, 2H, Ar, *J* = 8.5 Hz), 8.70 (exch br s, 1H, NH).

5.2.145 *N*-(4-Fluorophenyl)-2-[3-(4-chlorophenyl)-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50p)



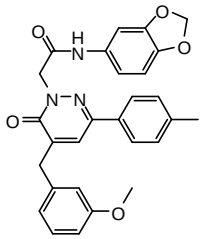
Yield = 63 %; mp = 143-45 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.80 (s, 3H, OCH_3), 4.00 (s, 2H, *CH_2*-Ar), 5.08 (s, 2H, NCH_2CO), 6.84-6.95 (m, 5H, Ar), 7.27-7.31 (m, 1H, Ar), 7.39-7.44 (m, 5H, Ar), 7.66 (d, 2H, Ar, *J* = 8.5 Hz), 9.06 (exch br s, 1H, NH).

5.2.146 *N*-(4-Bromophenyl)-2-[5-(4-methoxybenzyl)-6-oxo-3-(*p*-tolyl) pyridazin-1(6*H*)-yl]acetamide (50q)



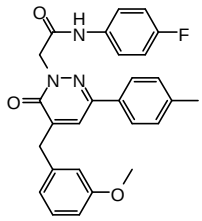
Yield = 58 %; mp = 273-274 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3H, C_6H_4-*CH_3*), 3.81 (s, 3H, OCH_3), 3.95 (s, 2H, *CH_2*-Ar), 5.09 (s, 2H, NCH_2CO), 6.89 (d, 2H, Ar, *J* = 8.1 Hz), 7.20-7.33 (m, 8H, Ar), 7.41 (s, 1H, Ar), 7.62 (d, 2H, Ar, *J* = 7.7 Hz), 9.38 (exch br s, 1H, NH).

5.2.147 *N*-(1,3-Benzodioxol-5-yl)-2-[5-(3-methoxybenzyl)-6-oxo-3-(*p*-tolyl) pyridazin-1(6*H*)-yl]acetamide (50r)



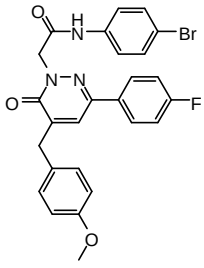
Yield = 66 %; mp = 198-99 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3H, C_6H_4-CH_3), 3.81 (s, 3H, OCH_3), 4.00 (s, 2H, CH_2-Ar), 5.06 (s, 2H, NCH_2CO), 5.94 (s, 2H, OCH_2O), 6.70 (d, 1H, Ar, *J* = 8.4 Hz), 6.79-6.90 (m, 4H, Ar), 7.24 (d, 3H, Ar, *J* = 8.0 Hz), 7.29 (t, 1H, Ar, *J* = 7.8 Hz), 7.40 (s, 1H, Ar), 7.61 (d, 2H, Ar, *J* = 8.1 Hz), 8.84 (exch br s, 1H, NH).

5.2.148 *N*-(4-Fluorophenyl)-2-[5-(3-methoxybenzyl)-6-oxo-3-(*p*-tolyl) pyridazin-1(6*H*)-yl]acetamide (50s)



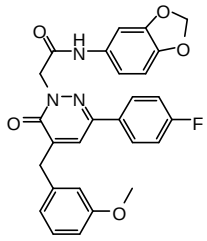
Yield = 54 %; mp = 173-74 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3H, C_6H_4-CH_3), 3.80 (s, 3H, OCH_3), 3.99 (s, 2H, CH_2-Ar), 5.08 (s, 2H, NCH_2CO), 6.83-6.95 (m, 5H, Ar), 7.23-7.26 (m, 2H, Ar), 7.28 (t, 1H, Ar, *J* = 5.3 Hz), 7.42-7.45 (m, 3H, Ar), 7.61 (d, 2H, Ar, *J* = 8.2 Hz), 9.11 (exch br s, 1H, NH).

5.2.149 *N*-(4-Bromophenyl)-2-[3-(4-fluorophenyl)-5-(4-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50t)



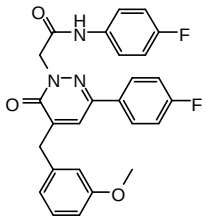
Yield = 75 %; mp = 188-90 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H, OCH_3), 3.97 (s, 2H, CH_2-Ar), 5.07 (s, 2H, NCH_2CO), 6.91 (dd, 2H, Ar, *J* = 4.6 Hz, *J* = 2.0 Hz), 7.12 (t, 2H, Ar, *J* = 6.7 Hz), 7.21 (d, 2H, Ar, *J* = 8.6 Hz), 7.30-7-40 (m, 5H, Ar), 7.69-7.73 (m, 2H, Ar), 9.15 (exch br s, 1H, NH).

5.2.150 *N*-(1,3-Benzodioxol-5-yl)-2-[3-(4-fluorophenyl)-5-(3-methoxy-benzyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50u)



Yield = 75 %; mp = 188-90 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H, OCH_3), 4.00 (s, 2H, *CH_2*-Ar), 5.05 (s, 2H, NCH_2CO), 5.95 (s, 2H, OCH_2O), 6.72 (d, 1H, Ar, *J* = 8.3 Hz), 6.80-6.90 (m, 4H, Ar), 7.12 (t, 2H, Ar, *J* = 8.8 Hz), 7.23 (s, 1H, Ar), 7.30 (t, 1H, Ar, *J* = 8.8 Hz), 7.36 (s, 1H, Ar), 7.71 (dd, 2H, Ar, *J* = 2.4 Hz, *J* = 5.3 Hz), 8.74 (exch br s, 1H, NH).

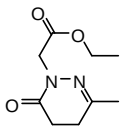
5.2.151 *N*-(4-Fluorophenyl)-2-[3-(4-fluorophenyl)-5-(3-methoxybenzyl)-6-oxopyridazin-1(6*H*)-yl]acetamide (50v)



Yield = 58 %; mp = 152-53 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.81 (s, 3H, OCH_3), 4.00 (s, 2H, *CH_2*-Ar), 5.08 (s, 2H, NCH_2CO), 6.85-6.89 (m, 3H, Ar), 6.92-6.96 (m, 2H, Ar), 7.10-7.15 (m, 2H, Ar), 7.28-7.32 (m, 1H, Ar), 7.39 (s, 1H, Ar), 7.42-7.46 (m, 2H, Ar), 7.69-7.73 (m, 2H, Ar), 9.02 (exch br s, 1H, NH).

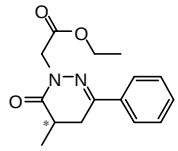
General procedure for 51a,(±)-51b. A mixture of **42d** or **(±)-42m** (7.41 mmol), K_2CO_3 (14.82 mmol) and ethyl bromoacetate (11.12 mmol) in CH_3CN (5 mL) was refluxed under stirring for 2-3 h. The mixture was then concentrated in vacuo, diluted with cold water, and extracted with CH_2Cl_2 (3 x 15 mL). The organic layer was evaporated in vacuo, and the final compounds **51a,(±)-b** were purified by column chromatography using cyclohexane/ethyl acetate 1:1 as eluent.

5.2.152 Ethyl-2-[3-Methyl-6-oxo-5,6-dihydropyridazin-1(4*H*)-yl]acetate (51a)



Yield = 98 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.22 (t, 3H, CH_2*CH_3*, *J* = 7.1 Hz), 2.01 (s, 3H, 3-CH_3), 2.48 (qd, 2H, CH_2CH_2 pyridaz., *J* = 7.9 Hz, *J* = 5.0 Hz), 4.15 (q, 2H, *CH_2*CH_3, *J* = 7.1 Hz), 4.40 (s, 2H, NCH_2CO).

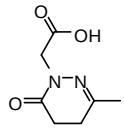
5.2.153 (±)-Ethyl-2-[5-methyl-6-oxo-3-phenyl-5,6-dihydropyridazin-1(4*H*)-yl]acetate [(±)-51b]



Yield ~ 100 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.28 (m, 6H, $CH_2CH\mathit{CH_3}$ + $CH_2\mathit{CH_3}$), 2.67-2.79 (m, 2H, $\mathit{CH_2}CHCH_3$), 3.07-3.14 (m, 1H, $CH_2\mathit{CH}CH_3$), 4.24 (q, 2H, $\mathit{CH_2}CH_3$, J = 6.9 Hz), 4.59 (d, 2H, NCH_2CO, J = 8.0 Hz), 7.40-7.43 (m, 3H, Ar), 7.72-7.75 (m, 2H, Ar).

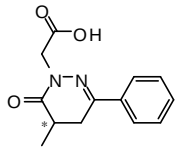
General procedure for 52a,(±)-52b. A suspension of **51a** or **(±)-52b** (7.29 mmol) in 6 N NaOH (10 mL) was stirred at rt to 80 °C for 3-5 h. The mixture was then diluted with cold water and acidified with 6 N HCl. Products **52a** and **(±)-52b** were filtered off by suction and recrystallized from cyclohexane (**52a**) or ethanol [**(±)-52b**].

5.2.154 2-[3-Methyl-6-oxo-5,6-dihydropyridazin-1(4H)-yl]acetic acid (52a)



Yield = 86 %; mp = 171-73 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.96 (s, 3H, 3-CH_3), 2.35-2.39 (m, 2H, CH_2 pyridaz.), 2.40-2.49 (m, 2H, CH_2 pyridaz.), 4.23 (s, 2H, NCH_2CO).

5.2.155 (±)-2-[5-Methyl-6-oxo-3-phenyl-5,6-dihydropyridazin-1(4*H*)-yl]acetic acid [(±)-52b]

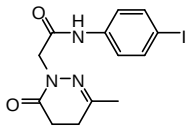


Yield ~ 100 %; mp = 87-89 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.31 (d, 3H, $CH_2CH\mathit{CH_3}$, J = 6.7 Hz), 2.68-2.78 (m, 2H, $\mathit{CH_2}CHCH_3$), 3.04-3.16 (m, 1H, $CH_2\mathit{CH}CH_3$), 4.64 (s, 2H, NCH_2CO), 6.98 (exch br s, 1H, OH), 7.41-7.43 (m, 3H, Ar), 7.73-7.75 (m, 2H, Ar).

General procedure for 53a,(±)-53b-d. To a cooled (-5 °C) and stirred solution of the appropriate derivative **52** [**52a,(±)-52b**] (2.06 mmol) in anhydrous tetrahydrofuran (6 mL), Et_3N (7.21 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C and ethyl chloroformate (2.27 mmol) was added. After 1 h, the appropriately substituted arylamine (4.12 mmol) was added. The reaction was carried out at room temperature for 12 h. The mixture was then concentrated in vacuo, diluted with cold water (20-30 mL) and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated to afford final

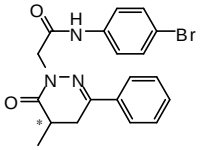
compounds **53a,(±)-53b-d**, which were purified by column chromatography using cyclohexane/ethyl acetate 1:3 (for compound **53a**), cyclohexane/ethyl acetate 2:1 (for compound **(±)-53b**) and *n*-hexane/ethyl acetate 3:2 (for compounds **(±)-53c,d**) as eluents.

5.2.156 *N*-(4-Iodophenyl)-2-[3-methyl-6-oxo-5,6-dihydropyridazin-1(4*H*)-yl]acetamide (53a)



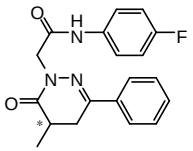
Yield = 98 %; mp = 174-76 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.11 (s, 3H, 3-CH_3), 2.55-2.60 (m, 4H, CH_2CH_2 pyridaz.), 4.52 (s, 2H, NCH_2CO), 7.31 (d, 2H, Ar, J = 8.8 Hz), 7.62 (d, 2H, Ar, J = 8.8 Hz), 8.11 (exch br s, 1H, NH).

5.2.157 (±)-*N*-(4-Bromophenyl)-2-[5-methyl-6-oxo-3-phenyl-5,6-dihydropyridazin-1(4*H*)-yl]acetamide [(±)-53b]



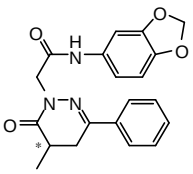
Yield = 23 %; mp = 148-149 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.37 (d, 3H, $CH_2CH\mathit{CH_3}$, J = 6.3 Hz), 2.74-2.81 (m, 2H, $\mathit{CH_2}CHCH_3$), 3.14-3.22 (quin, 1H, $CH_2\mathit{CH}CH_3$, J = 11.6 Hz), 4.68 (s, 2H, NCH_2CO), 7.28 (d, 2H, Ar, J = 10.1 Hz), 7.44 (t, 4H, Ar, J = 7.0 Hz), 7.60 (d, 1H, Ar, J = 3.9 Hz), 7.77-7.79 (m, 2H, Ar), 8.15 (exch, br, s, 1H, NH).

5.2.158 (±)-*N*-(4-Fluorophenyl)-2-[5-methyl-6-oxo-3-phenyl-5,6-dihydropyridazin-1(4*H*)-yl]acetamide [(±)-53c]



Yield = 61 %; mp = 164-165 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1,35 (d, 3H, $CH_2CH\mathit{CH_3}$, J = 6.4 Hz), 2,69-2.80 (m, 2H, $\mathit{CH_2}CHCH_3$), 3,11-3,20 (m, 1H, $CH_2\mathit{CH}CH_3$), 4,68 (s, 2H, NCH_2CO), 6.98 (t, 2H, Ar, J = 8.8 Hz), 7,42-7.49 (m, 5H, Ar), 7,76-7.78 (m, 2H, Ar), 8.25 (exch, br, s, 1H, NH).

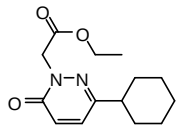
5.2.159 (±)-*N*-(1,3-Benzodioxol-5-yl)-2-[5-methyl-6-oxo-3-phenyl-5,6-dihydro pyridazin-1(4*H*)-yl]acetamide [(±)-53d]



Yield = 88 %; mp = 170-171 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.35 (d, 3H, CH_2CHCH_3, J = 6.4 Hz), 2.69-2.80 (m, 2H, CH_2CHCH_3), 3.16 (q, 1H, CH_2CHCH_3, J = 10.1 Hz), 4.66 (s, 2H, NCH_2CO), 5.94 (s, 2H, OCH_2O), 6.72 (d, 1H, Ar, J = 8.3 Hz), 6.80 (dd, 1H, Ar, J = 6.2 Hz, J = 2.1 Hz), 7.24 (s, 1H, Ar), 7.44 (t, 3H, Ar, J = 2.8 Hz), 7.77-7.79 (m, 2H, Ar), 8.01 (exch, br, s, 1H, NH).

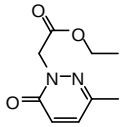
General procedure for 55a-c. A mixture of compound type **42** [**42c,d** and **(±)-42m**] (2.27 mmol), K_2CO_3 (4.54 mmol), and ethyl bromoacetate (3.41 mmol) in CH_3CN (3 mL) was refluxed under stirring for 2-3 h. The mixture was then concentrated in vacuo, diluted with cold water and extracted with CH_2Cl_2 (3 x 15 mL). The organic layer was evaporated in vacuo, and compounds **55a-c** were used for the following reactions without further purification.

5.2.160 Ethyl-2-[3-cyclohexyl-6-oxopyridazin-1(6*H*)-yl]acetate (55a)



Yield ~ 100 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.28-1.46 (m, 8H, (2 x CH_2 + CH-*H*) cyclohexyl + CH_2CH_3), 1.71-1.86 (m, 5H, 2 x CH_2 + CH-*H* cyclohexyl), 2.51-2.60 (m, 1H, CH, cyclohexyl), 4.23 (dt, 2H, CH_2CH_3, J = 4.3 Hz, J = 1.4 Hz), 4.83 (s, 2H, NCH_2CO), 6.90 (d, 1H, Ar, J = 9.0 Hz), 7.17 (d, 1H, Ar, J = 9.6 Hz).

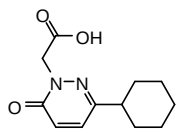
5.2.161 Ethyl-2-[3-methyl-6-oxopyridazin-1(6*H*)-yl]acetate (55b)



Yield ~ 100 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.30 (t, 3H, CH_2CH_3, J = 7.1 Hz), 2.37 (s, 3H, 3-CH_3), 4.26 (q, 2H, CH_2CH_3, J = 7.1 Hz), 4.87 (s, 2H, NCH_2CO), 7.02 (d, 1H, Ar, J = 9.4 Hz), 7.20 (d, 1H, Ar, J = 9.4 Hz).

General procedure for 56a-c. A suspension of derivative **55** (**55a-c**) (0.91 mmol) in 6 N NaOH (4 mL) was stirred at rt to 80 °C for 2 h. The mixture was then diluted with cold water and acidified with 6 N HCl. Products **56a,c** were filtered off by suction and recrystallized from ethanol.

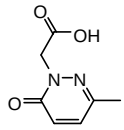
5.2.162 2-[3-Cyclohexyl-6-oxopyridazin-1(6*H*)-yl]acetic acid (56a)



Yield = 82 %; mp = 195-97 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.25-1.34 (m, 1H, CH-*H* cyclohexyl), 1.37-1.46 (m, 4H, 2 x CH_2 cyclohexyl), 1.76 (m, 1H, CH-*H* cyclohexyl), 1.79-1.93 (m, 4H, 2 x CH_2

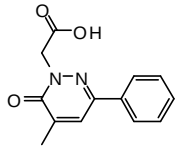
cyclohexyl), 2.50-2.60 (m, 1H, CH cyclohexyl), 4.94 (s, 2H, NCH_2CO), 6.99 (d, 1H, Ar, *J* = 9.5 Hz), 7.24 (d, 1H, Ar, *J* = 9.6 Hz).

5.2.163 2-[3-Methyl-6-oxopyridazin-1(6*H*)-yl]acetic acid (56b)



Yield = 72 %; mp = 247-49 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.37 (s, 3H, 3-CH_3), 4.94 (s, 2H, NCH_2CO), 6.97 (d, 1H, Ar, *J* = 9.5 Hz), 7.19 (d, 1H, Ar, *J* = 9.6 Hz).

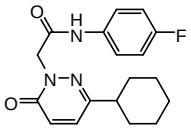
5.2.164 2-[5-Methyl-6-oxo-3-phenylpyridazin-1(6*H*)-yl]acetic acid (56c)



Yield = 90 %; mp = 92-94 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.34 (s, 3H, CH_3), 3.48 (exch br s, 1H, OH), 5.05 (s, 2H, CH_2COO), 7.45-7.50 (m, 3H, Ar), 7.63 (s, 1H, Ar), 7.78 (d, 2H, Ar, *J* = 4.5 Hz).

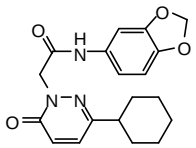
General procedure for 57a-f. To a cooled (-5 °C) and stirred solution of the appropriate compound **56** (**56a-c**) (0.59 mmol) in anhydrous tetrahydrofuran (3 mL), Et_3N (2.06 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C and ethyl chloroformate (0.65 mmol) was added. After 1 h, the appropriately substituted arylamine (1.18 mmol) was added. The reaction was carried out at room temperature for 12 h. The mixture was then concentrated in vacuo, diluted with cold water (10-15 mL), and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated to afford final compounds **57a-f**, which were purified by column chromatography using cyclohexane/ethyl acetate 1:1 (for compound **57a,d-f**), cyclohexane/ethyl acetate 1:2 (for compound **57b**) and CH_2Cl_2/CH_3OH 9.9:0.1 (for compound **57c**) as eluents.

5.2.165 *N*-(4-Fluorophenyl)-2-[3-cyclohexyl-6-oxopyridazin-1(6*H*)-yl]acetamide (57a)



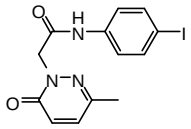
Yield = 98 %; mp = 149-51 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.21-1.30 (m, 1H, CH-*H* cyclohexyl), 1.39-1.48 (m, 4H, 2 x CH_2 cyclohexyl), 1.77 (d, 1H, CH-*H* cyclohexyl, *J* = 12.6 Hz), 1.85-1.93 (m, 4H, 2 x CH_2 cyclohexyl), 2.56-2.63 (m, 1H, CH cyclohexyl), 4.96 (s, 2H, NCH_2CO), 6.94-7.02 (m, 3H, Ar), 7.27 (d, 1H, Ar, *J* = 9.7 Hz), 7.46-7.49 (m, 2H, Ar), 9.10 (exch br s, 1H, NH).

5.2.166 *N*-(1,3-Benzodioxol-5-yl)-2-[3-cyclohexyl-6-oxopyridazin-1(6*H*)-yl]acetamide (57b)



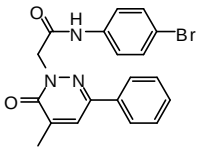
Yield ~ 100 %; mp = 185-87 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.20-1.28 (m, 1H, CH-*H* cyclohexyl), 1.30-1.48 (m, 4H, 2 x CH_2 cyclohexyl), 1.76 (d, 1H, CH-*H* cyclohexyl, *J* = 12.8 Hz), 1.85-1.93 (m, 4H, 2 x CH_2 cyclohexyl), 2.59-2.61 (m, 1H, CH cyclohexyl), 4.93 (s, 2H, NCH_2CO), 5.94 (s, 2H, OCH_2O), 6.72 (d, 1H, Ar, *J* = 8.4 Hz), 6.81 (dd, 1H, Ar, *J* = 6.3 Hz, *J* = 2.1 Hz), 7.00 (d, 1H, Ar, *J* = 9.5 Hz), 7.25-7.28 (m, 2H, Ar), 8.86 (exch br s, 1H, NH).

5.2.167 *N*-(4-Iodophenyl)-2-[3-methyl-6-oxopyridazin-1(6*H*)-yl]acetamide (57c)



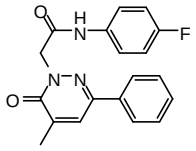
Yield ~ 100 %; mp = 160-61 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.39 (s, 3H, 3-CH_3), 4.96 (s, 2H, NCH_2CO), 6.97 (d, 1H, Ar, *J* = 9.5 Hz), 7.20-7.26 (m, 3H, Ar), 7.50 (dt, 2H, Ar, *J* = 5.2 Hz, *J* = 1.8 Hz), 9.27 (exch br s, 1H, NH).

5.2.168 *N*-(4-Bromophenyl)-2-[5-methyl-6-oxo-3-phenylpyridazin-1(6*H*)-yl]acetamide (57d)



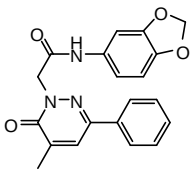
Yield = 38 %; mp = 149-51 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.37 (s, 3H, CH_3), 5.08 (s, 2H, CH_2CO), 7.40-7.49 (m, 7H, Ar), 7.68 (s, 1H, Ar), 7.82 (dd, 2H, Ar, *J* = 5.8 Hz, *J* = 1.4 Hz), 9.02 (exch br s, 1H, NH).

5.2.169 *N*-(4-Fluorophenyl)-2-[5-methyl-6-oxo-3-phenylpyridazin-1(6*H*)-yl]acetamide (57e)



Yield = 45 %; mp = 117-19 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.34 (s, 3H, CH_3), 5.11 (s, 2H, CH_2CO), 6.89-6.93 (td, 2H, Ar, *J* = 3.7 Hz, *J* = 2.1 Hz), 7.44-7.49 (m, 5H, Ar), 7.65 (s, 1H, Ar), 7.79-7.82 (dd, 2H, Ar, *J* = 3.1 Hz, *J* = 1.9 Hz), 9.21 (exch br s, 1H, NH).

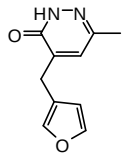
5.2.170 *N*-(1,3-Benzodioxol-5-yl)-2-[5-methyl-6-oxo-3-phenylpyridazin-1(6*H*)-yl]acetamide (57f)



Yield = 25 %; mp = 215-17 °C (EtOH). ^{1}H NMR (DMSO-d_6) δ 2.19 (s, 3H, CH_3), 4.95 (s, 2H, OCH_2O), 5.99 (s, 2H, CH_2CO), 6.87 (d, 1H, Ar, J = 8.3 Hz), 6.96 (d, 1H, Ar, J = 8.5 Hz), 7.29 (s, 1H, Ar), 7.49 (q, 3H, Ar, J = 7.5 Hz), 7.89 (d, 2H, Ar, J = 7.1 Hz), 8.04 (s, 1H, Ar), 10.26 (exch br s, 1H, NH).

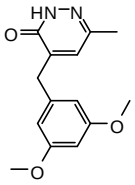
General Procedure for 58a-m. To 12 mL of KOH in absolute EtOH (5%, w/v), **42d** (4.46 mmol) and the appropriate substituted aromatic aldehyde (4.46 mmol) were added. The mixture was refluxed under stirring for 3-5 h. After cooling, it was concentrated in vacuo, diluted with ice-cold water (20-25 mL) and acidified with 2 N HCl. The suspension was extracted with CH_2Cl_2 (3 x 25 mL). Removal of the solvent afforded compounds **58a-m**, which were purified by crystallization in ethanol. For compound **58f** was necessary to perform an additional purification step by flash column chromatography using cyclohexane/ethyl acetate 2:1 as eluent.

5.2.171 4-(Furan-3-ylmethyl)-6-methylpyridazin-3(2*H*)-one (58a)



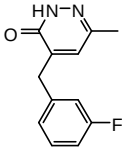
Yield = 24 %; mp = 137-39 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.29 (s, 3H, CH_3), 3.74 (s, 2H, CH_2), 6.33 (s, 1H, Ar), 6.86 (s, 1H, Ar), 7.39 (s, 1H, Ar), 7.46 (s, 1H, Ar).

5.2.172 4-(3,5-Dimethoxybenzyl)-6-methylpyridazin-3(2*H*)-one (58f)



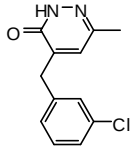
Yield = 56 %; mp = 167-69 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H, CH_3), 3.81 (s, 6H, 2 x OCH_3), 3.85 (s, 2H, CH_2), 6.41 (s, 3H, Ar), 6.77 (s, 1H, Ar).

5.2.173 4-(3-Fluorobenzyl)-6-methylpyridazin-3(2*H*)-one (58g)



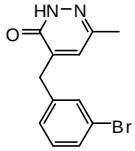
Yield = 95 %; mp = 114-16 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.28 (s, 3H, CH_3), 3.91 (s, 2H, CH_2), 6.75 (s, 1H, Ar), 6.98-7.07 (m, 3H, Ar), 7.30-7.36 (m, 1H, Ar), 11.31 (exch br s, 1H, NH).

5.2.174 4-(3-Chlorobenzyl)-6-methylpyridazin-3(2*H*)-one (58h)



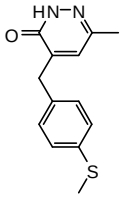
Yield = 81 %; mp = 124-26 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.28 (s, 3H, CH_3), 3.89 (s, 2H, CH_2), 6.75 (s, 1H, Ar), 7.16-7.18 (m, 1H, Ar), 7.26-7.31 (m, 3H, Ar), 11.09 (exch br s, 1H, NH).

5.2.175 4-(3-Bromobenzyl)-6-methylpyridazin-3(2*H*)-one (58j)



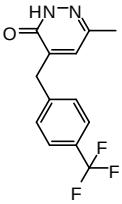
Yield = 28 %; mp = 147-49 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.28 (s, 3H, CH_3), 3.88 (s, 2H, CH_2), 6.75 (s, 1H, Ar), 7.24 (d, 2H, Ar, J = 5.2 Hz), 7.45 (d, 2H, Ar, J = 9.1 Hz), 10.82 (exch br s, 1H, NH).

5.2.176 6-Methyl-4-[4-(methylthio)benzyl]pyridazin-3(2*H*)-one (58k)



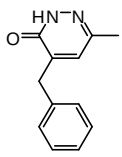
Yield = 46 %; mp = 151-53 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.,26 (s, 3H, CH_3), 2.51 (s, 3H, SCH_3), 3.87 (s, 2H, CH_2), 6.71 (s, 1H, Ar), 7.18 (d, 2H, Ar, J = 8.2 Hz), 7.27 (dd, 3H, Ar, J = 9.3 Hz, J = 1.8 Hz).

5.2.177 6-Methyl-4-[4-(trifluoromethyl)benzyl]pyridazin-3(2*H*)-one (58l)



Yield = 94 %; mp = 153-55 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.29 (s, 3H, CH_3), 3.97 (s, 2H, CH_2), 6.77 (s, 1H, Ar), 7.40 (d, 2H, Ar, J = 8.0 Hz), 7.62 (d, 2H, Ar, J = 8.1 Hz), 11.19 (exch br s, 1H, NH).

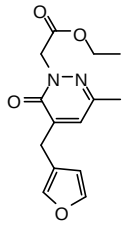
5.2.178 4-Benzyl-6-methylpyridazin-3(2*H*)-one (58m)



Yield ~ 100 %; mp = 113-15 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H, CH_3), 3.92 (s, 2H, CH_2), 6.73 (s, 1H, Ar), 7.26-7.33 (m, 3H, Ar), 7.28 (t, 2H, Ar, J = 6.9 Hz).

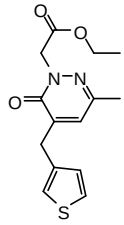
General Procedure for 59a-m. A mixture of the suitable intermediate type **58** (**58a-m**) (4.50 mmol), K_2CO_3 (9.00 mmol) and ethyl bromoacetate (6.75 mmol) in CH_3CN (10 mL) was refluxed under stirring for 2-4 h. The mixture was then concentrated in vacuo, diluted with cold water and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated in vacuo and compounds **59a-m** were purified by crystallization from ethanol (compounds **59a,g,h,j,k**) or by flash column chromatography using cyclohexane/ethyl acetate 2:1 (for **59b-e**) and cyclohexane/ethyl acetate 1:1 (for **59f,i,l,m**) as eluents.

5.2.179 Ethyl-2-[5-(furan-3-ylmethyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetate (59a)



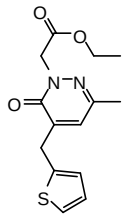
Yield = 69 %; mp = 61-67 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, $CH_2\mathit{CH_3}$, J = 7.1 Hz), 2.27 (s, 3H, 3-CH_3), 3.73 (s, 2H, CHC$\mathit{CH_2}$), 4.27 (q, 2H, O$\mathit{CH_2}CH_3$, J = 7.1 Hz), 4.87 (s, 2H, NCH_2CO), 6.32 (s, 1H, Ar), 6.82 (s, 1H, Ar), 7.37 (s, 1H, Ar), 7.45 (s, 1H, Ar).

5.2.180 Ethyl-2-[3-methyl-6-oxo-5-(thiophen-3-ylmethyl)pyridazin-1(6*H*)-yl]acetate (59b)



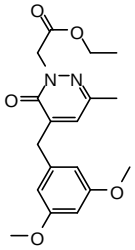
Yield = 91 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, $CH_2\mathit{CH_3}$, J = 7.1 Hz), 2.25 (s, 3H, 3-CH_3), 3.93 (s, 2H, CHC$\mathit{CH_2}$), 4.25 (q, 2H, $\mathit{CH_2}CH_3$, J = 5.8 Hz), 4.86 (s, 1H, NCH_2CO), 6.72 (s, 1H, Ar), 6.97 (dd, 1H, Ar, J = 3.64 Hz, J = 1.2 Hz), 7.11 (s, 1H, Ar), 7.33 (dd, 1H, Ar, J = 2.0 Hz, J = 4.6 Hz).

5.2.181 Ethyl-2-[3-methyl-6-oxo-5-(thiophen-2-ylmethyl)pyridazin-1(6*H*)-yl]acetate (59c)



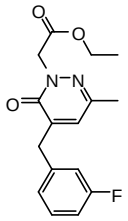
Yield ~ 100 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.30 (t, 3H, $CH_2\mathit{CH_3}$, J = 7.1 Hz), 2.26 (s, 3H, 3-CH_3), 4.11 (s, 2H, CHC$\mathit{CH_2}$), 4.25 (q, 2H, $\mathit{CH_2}CH_3$, J = 7.1 Hz), 4.86 (s, 2H, NCH_2CO), 6.82 (s, 1H, Ar), 6.92 (d, 1H, Ar, J = 3.3 Hz), 7.00 (t, 1H, Ar, J = 3.9 Hz), 7.22 (d, 1H, Ar, J = 5.1 Hz).

5.2.182 Ethyl-2-[5-(3,5-dimethoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetate (59f)



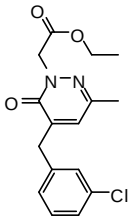
Yield = 95 %; brown oil. ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, CH_2CH_3), 2.24 (s, 3H, 3-CH_3), 3.79 (s, 6H, 2 x OCH_3), 3.84 (s, 2H, CHCCH_2), 4.24 (q, 2H, OCH_2CH_3, J = 7.1 Hz), 4.86 (s, 2H, NCH_2CO), 6.39 (s, 3H, Ar), 6.70 (s, 1H, Ar).

5.2.183 Ethyl-2-[5-(3-fluorobenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetate (59g)



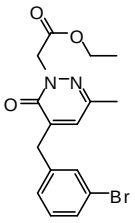
Yield = 89 %; mp = 74-76 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, CH_2CH_3, J = 7.2 Hz), 2.26 (s, 3H, 3-CH_3), 3.91 (s, 2H, CHCCH_2), 4.26 (q, 2H, OCH_2CH_3, J = 7.2 Hz), 4.87 (s, 2H, NCH_2CO), 6.71 (s, 1H, Ar), 6.95-7.05 (m, 3H, Ar), 7.30 (q, 1H, Ar, J = 6.5 Hz).

5.2.184 Ethyl-2-[5-(3-chlorobenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetate (59h)



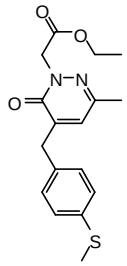
Yield ~ 100 %; mp = 89-91 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, CH_2CH_3, J = 7.2 Hz), 2.27 (s, 3H, 3-CH_3), 3.88 (s, 2H, CHCCH_2), 4.27 (q, 2H, OCH_2CH_3, J = 7.2 Hz), 4.86 (s, 2H, NCH_2CO), 6.71 (s, 1H, Ar), 7.15 (dd, 1H, Ar, J = 4.5 Hz, J = 1.8 Hz), 7.24-7.30 (m, 3H, Ar).

5.2.185 Ethyl-2-[5-(3-bromobenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetate (59j)



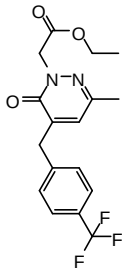
Yield = 82 %; mp = 98-100 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, CH_2CH_3, J = 7.0 Hz), 2.27 (s, 3H, 3-CH_3), 3.88 (s, 2H, CHCCH_2), 4.27 (q, 2H, OCH_2CH_3, J = 7.0 Hz), 4.86 (s, 2H, NCH_2CO), 6.71 (s, 1H, Ar), 7.21-7.25 (m, 2H, Ar), 7.42 (d, 2H, Ar, J = 11.9 Hz).

5.2.186 Ethyl-2-{3-methyl-5-[4-(methylthio)benzyl]-6-oxopyridazin-1(6*H*)-yl}acetate (59k)



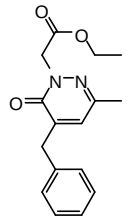
Yield ~ 100 %; mp = 129-31 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, CH_2CH_3, J = 6.8 Hz), 2.24 (s, 3H, 3-CH_3), 2.51 (s, 3H, SCH_3), 3.87 (s, 2H, CHCCH_2), 4.27 (q, 2H, OCH_2CH$_3$, J = 6.8 Hz), 4.86 (s, 2H, NCH_2CO), 6.68 (s, 1H, Ar), 7.17 (d, 2H, Ar, J = 7.3 Hz), 7.25-7.29 (m, 2H, Ar).

5.2.187 Ethyl-2-{3-methyl-6-oxo-5-[4-(trifluoromethyl)benzyl]pyridazin-1(6*H*)-yl}acetate (59l)



Yield ~ 100 %; brown oil. ^{1}H NMR ($CDCl_3$) δ 1.30 (t, 3H, CH_2CH_3, J = 7.0 Hz), 2.27 (s, 3H, 3-CH_3), 3.96 (s, 2H, CHCCH_2), 4.26 (q, 2H, OCH_2CH$_3$, J = 7.1 Hz), 4.86 (s, 2H, NCH_2CO), 6.73 (s, 1H, Ar), 7.38 (d, 2H, Ar, J = 8.0 Hz), 7.60 (d, 2H, Ar, J = 8.0 Hz).

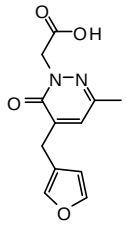
5.2.188 Ethyl-2-[5-benzyl-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetate (59m)



Yield = 98 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, CH_2CH_3, J = 7.1 Hz), 2.22 (s, 3H, 3-CH_3), 3.90 (s, 2H, CHCCH_2), 4.24 (q, 2H, CH_2CH$_3$, J = 7.1 Hz), 4.85 (s, 2H, NCH_2CO), 6.66 (s, 1H, Ar), 7.23-7.28 (m, 3H, Ar), 7.33-7.37 (m, 2H, Ar).

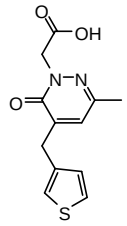
General Procedure for 60a-m. A suspension of the suitable intermediate type **59** (**59a-m**) (4.4 mmol) in 6 N NaOH (10 mL) was stirred at rt to 80 °C for 1-2 h. The mixture was firstly diluted with ice-cold water and then acidified with 6 N HCl. Products **60a-m** were filtered off by suction and recrystallized from ethanol.

5.2.189 2-[5-(Furan-3-ylmethyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetic acid (60a)



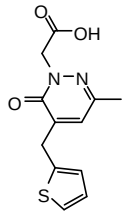
Yield = 69 %; mp = 62-64 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.29 (s, 3H, 3-CH_3), 3.46 (exch br s, 1H, OH), 3.75 (s, 2H, CHC*CH_2*), 4.93 (s, 2H, NCH_2CO), 6.31 (s, 1H, Ar), 6.86 (s, 1H, Ar), 7.37 (s, 1H, Ar), 7.45 (s, 1H, Ar).

5.2.190 2-[3-Methyl-6-oxo-5-(thiophen-3-ylmethyl)pyridazin-1(6*H*)-yl]acetic acid (60b)



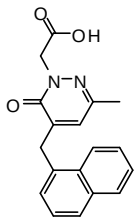
Yield ~ 100 %; mp = 123-25 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H, 3-CH_3), 3.95 (s, 2H, CHC*CH_2*), 4.93 (s, 2H, NCH_2CO), 5.21 (exch br s, 1H, OH), 6.76 (s, 1H, Ar), 6.97 (d, 1H, Ar, *J* = 4.8 Hz), 7.12 (s, 1H, Ar), 7.35 (t, 1H, Ar, *J* = 4.4 Hz).

5.2.191 2-[3-Methyl-6-oxo-5-(thiophen-2-ylmethyl)pyridazin-1(6*H*)-yl]acetic acid (60c)



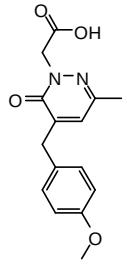
Yield = 88 %; mp = 123-25 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.28 (s, 3H, 3-CH_3), 4.13 (s, 2H, CHC*CH_2*), 4.93 (s, 2H, NCH_2CO), 6.85 (s, 1H, Ar), 6.94 (d, 1H, Ar, *J* = 3.4 Hz), 7.01 (dd, 1H, Ar, *J* = 1.6 Hz, *J* = 3.4 Hz), 7.24 (dd, 1H, Ar, *J* = 4.0 Hz, *J* = 1.0 Hz), 8.41 (exch br s, 1H, OH).

5.2.192 2-[3-Methyl-5-(naphthalen-1-ylmethyl)-6-oxopyridazin-1(6*H*)-yl]acetic acid (60d)



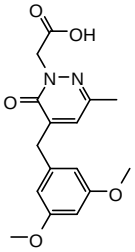
Yield = 98 %; mp = 198-200 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.11 (s, 3H, 3-CH_3), 4.39 (s, 2H, CHC*CH_2*), 5.01 (s, 2H, NCH_2CO), 5.83 (exch br s, 1H, OH), 6.42 (s, 1H, Ar), 7.41 (d, 1H, Ar, *J* = 6.8 Hz), 7.47-7.54 (m, 3H, Ar), 7.79 (d, 1H, Ar, *J* = 7.4 Hz), 7.86 (d, 1H, Ar, *J* = 8.2 Hz), 7.91 (d, 1H, Ar, *J* = 7.3 Hz).

5.2.193 2-[5-(4-Methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetic acid (60e)



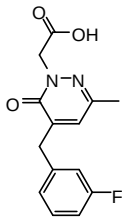
Yield = 96 %; mp = 144-45 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H, 3-CH_3), 3.84 (s, 3H, OCH_3), 3.87 (s, 2H, CHC*CH_2*), 4.93 (s, 2H, NCH_2CO), 6.69 (s, 1H, Ar), 6.91 (d, 2H, Ar, *J* = 8.5 Hz), 7.16 (d, 2H, Ar, *J* = 8.5 Hz).

5.2.194 2-[5-(3,5-Dimethoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetic acid (60f)



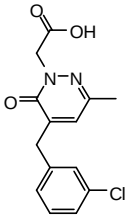
Yield = 76 %; mp = 112-14 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H, 3-CH_3), 3.37 (exch br s, 1H, OH), 3.80 (s, 6H, 2 x OCH_3), 3.85 (s, 2H, CHC*CH_2*), 4.93 (s, 2H, NCH_2CO), 6.40 (s, 3H, Ar), 6.75 (s, 1H, Ar).

5.2.195 2-[5-(3-Fluorobenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetic acid (60g)



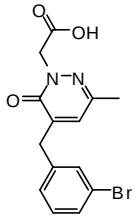
Yield = 82 %; mp = 124-26 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H, 3-CH_3), 3.92 (s, 2H, CHC*CH_2*), 4.92 (s, 2H, NCH_2CO), 6.35 (exch br s, 1H, OH), 6.72 (s, 1H, Ar), 6.95-7.04 (m, 3H, Ar), 7.30-7.36 (q, 1H, Ar, *J* = 6.1 Hz).

5.2.196 2-[5-(3-Chlorobenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetic acid (60h)



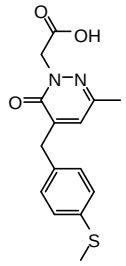
Yield = 75 %; mp = 162-64 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.28 (s, 3H, 3-CH_3), 3.90 (s, 2H, CHC*CH_2*), 4.93 (s, 2H, NCH_2CO), 6.72 (s, 1H, Ar), 7.14 (d, 1H, Ar, *J* = 5.6 Hz), 7.23-7.45 (m, 1H, Ar).

5.2.197 2-[5-(3-Bromobenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetic acid (60j)



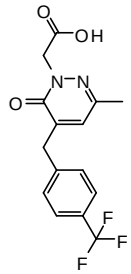
Yield = 89 %; mp = 173-75 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.29 (s, 3H, 3-CH_3), 3.90 (s, 2H, CHC*CH_2*), 4.92 (s, 2H, NCH_2CO), 6.74 (s, 1H, Ar), 7.19-7.26 (m, 2H, Ar), 7.43 (t, 2H, Ar, *J* = 7.4 Hz).

5.2.198 2-{3-Methyl-5-[4-(methylthio)benzyl]-6-oxopyridazin-1(6*H*)-yl}acetic acid (60k)



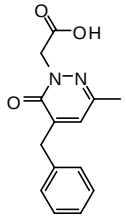
Yield ~ 100 %; mp = 87-89 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H, 3-CH_3), 2.51 (s, 3H, SCH_3), 3.87 (s, 2H, CHC*CH_2*), 4.91 (s, 2H, NCH_2CO), 6.69 (s, 1H, Ar), 7.16 (d, 2H, Ar, *J* = 7.6 Hz), 7.25 (d, 2H, Ar, *J* = 7.6 Hz).

5.2.199 2-{3-Methyl-6-oxo-5-[4-(trifluoromethyl)benzyl]pyridazin-1(6*H*)-yl}acetic acid (60l)



Yield = 82 %; mp = 127-29 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.28 (s, 3H, 3-CH_3), 3.98 (s, 2H, CHC*CH_2*), 4.93 (s, 2H, NCH_2CO), 6.74 (s, 1H, Ar), 7.37 (d, 2H, Ar, *J* = 8.0 Hz), 7.61 (d, 2H, Ar, *J* = 8.0 Hz).

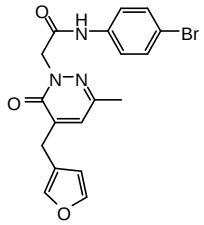
5.2.200 2-[5-Benzyl-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetic acid (60m)



Yield = 92 %; mp = 120-22 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H, 3-CH_3), 3.93 (s, 2H, CHC*CH_2*), 4.94 (s, 2H, NCH_2CO), 5.50 (exch br s, 1H, OH), 6.70 (s, 1H, Ar), 7.24-7.32 (m, 3H, Ar), 7.35-7.39 (m, 2H, Ar).

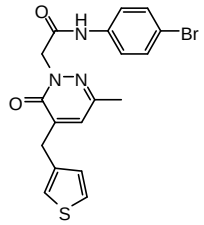
General Procedure for 61a-p. To a cooled (-5 °C) and stirred solution of the appropriate compound **60** (**60a-m**) (0.90 mmol), in anhydrous tetrahydrofuran (6 mL), Et_3N (3.15 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C and ethyl chloroformate (0.99 mmol) was added. After 1 h, the appropriately substituted arylamine (1.80 mmol) was added. The reaction was carried out at room temperature for 12 h. The mixture was then concentrated in vacuo, diluted with cold water (10-15 mL), and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated to afford final compounds **61a-p**, which were purified by column chromatography using cyclohexane/ethyl acetate 1:1 for compounds **61a,b,f,j,n,o**, CH_2Cl_2/CH_3OH 9.8:0.2 for compound **48c**, cyclohexane/ethyl acetate 2:1 for compounds **61d,g-i,l,m**, toluene/NH_4OH/EtOH/CH_2Cl_2/petroleum ether 7:0.05:0.30:2:0.65 for compound **61e** and cyclohexane/ethyl acetate 1:2 for compounds **61k,p**.

5.2.201 *N*-(4-Bromophenyl)-2-[5-(furan-3-ylmethyl)-3-methyl-6-oxo-pyridazin-1(6*H*)-yl]acetamide (61a)



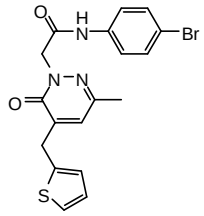
Yield = 50 %; mp = 67-69 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H, 3-CH_3), 3.76 (s, 2H, CHC*CH_2*), 4.96 (s, 2H, NCH_2CO), 6.31 (s, 1H, Ar), 6.91 (s, 1H, Ar), 7.38-7.40 (m, 5H, Ar), 7.46 (s, 1H, Ar), 9.05 (exch br s, 1H, NH).

5.2.202 *N*-(4-Bromophenyl)-2-[3-methyl-6-oxo-5-(thiophen-3-ylmethyl)- pyridazin-1(6*H*)-yl]acetamide (61b)



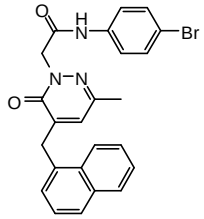
Yield = 24 %; mp = 93-95 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.31 (s, 3H, 3-CH_3), 3.96 (s, 2H, CHC*CH_2*), 4.95 (s, 2H, NCH_2CO), 6.84 (s, 1H, Ar), 6.97 (d, 1H, Ar, *J* = 1.9 Hz), 7.12 (s, 1H, Ar), 7.35 (q, 1H, Ar, *J* = 4.8 Hz), 7.39 (s, 4H, Ar), 9.04 (exch br s, 1H, NH).

5.2.203 *N*-(4-Bromophenyl)-2-[3-methyl-6-oxo-5-(thiophen-2-ylmethyl)-pyridazin-1(6*H*)-yl]acetamide (61c)



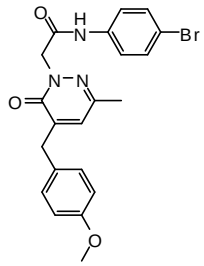
Yield = 80 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H, 3-CH_3), 4.14 (s, 2H, CHCCH_2), 4.97 (s, 2H, NCH_2CO), 6.93-6.95 (m, 2H, Ar), 6.99-7.01 (m, 1H, Ar), 7.24 (dd, 1H, Ar, *J* = 4.0 Hz, *J* = 1.0 Hz), 7.33 (q, 4H, Ar, *J* = 4.2 Hz), 9.25 (exch br s, 1H, NH).

5.2.204 *N*-(4-Bromophenyl)-2-[3-methyl-5-(naphthalen-1-ylmethyl)-6-oxo-pyridazin-1(6*H*)-yl]acetamide (61d)



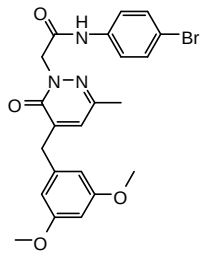
Yield = 77 %; mp = 208-09 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.16 (s, 3H, 3-CH_3), 4.39 (s, 2H, CHCCH_2), 5.04 (s, 2H, NCH_2CO), 6.54 (s, 1H, Ar), 7.33 (d, 2H, Ar, *J* = 8.8 Hz), 7.39 (d, 3H, Ar, *J* = 9.2 Hz), 7.45-7.54 (m, 3H, Ar), 7.80 (d, 1H, Ar, *J* = 8.2 Hz), 7.86 (d, 1H, Ar, *J* = 8.2 Hz), 7.92 (d, 1H, Ar, *J* = 7.8 Hz), 9.38 (exch br s, 1H, NH).

5.2.205 *N*-(4-Bromophenyl)-2-[5-(4-methoxybenzyl)-3-methyl-6-oxo-pyridazin-1(6*H*)-yl]acetamide (61e)



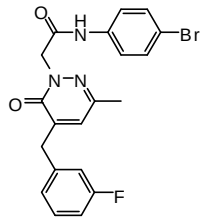
Yield = 73 %; mp = 139-141 °C (EtOH). IR (cm^{-1}) 3300 (NH), 1709 (CO), 1644 (CO). ^{1}H NMR ($CDCl_3$) δ 2.29 (s, 3H, 3-CH_3), 3.83 (s, 3H, OCH_3), 3.88 (s, 2H, CH_2-Ar), 4.94 (s, 2H, NCH_2CO), 6.78 (s, 1H, Ar), 6.90 (d, 2H, Ar, *J* = 8.6 Hz), 7.17 (d, 2H, Ar, *J* = 8.6 Hz), 7.40 (s, 4H, Ar), 9.01 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{21}H_{20}BrN_3O_3$, 442,31. Found: *m/z* 442.08 $[M]^+$.

5.2.206 *N*-(4-Bromophenyl)-2-[5-(3,5-dimethoxybenzyl)-3-methyl-6-oxo-pyridazin-1(6*H*)-yl]acetamide (61f)



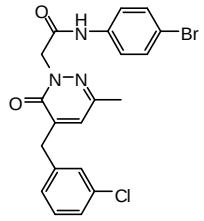
Yield = 62 %; mp = 87-89 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.29 (s, 3H, 3-CH_3), 3.77 (s, 6H, 2 x OCH_3), 3.83 (s, 2H, CHC*CH_2*), 4.94 (s, 2H, NCH_2CO), 6.37 (s, 3H, Ar), 6.85 (s, 1H, Ar), 7.27 (q, 4H, Ar, *J* = 11.4 Hz), 9.49 (exch br s, 1H, NH).

5.2.207 *N*-(4-Bromophenyl)-2-[5-(3-fluorobenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetamide (61g)



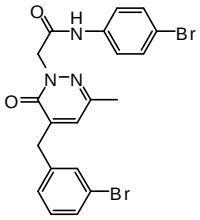
Yield = 47 %; mp = 88-89 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H, 3-CH_3), 3.93 (s, 2H, CHC*CH_2*), 4.95 (s, 2H, NCH_2CO), 6.85 (s, 1H, Ar), 6.97-7.05 (m, 3H, Ar), 7.28-7.34 (m, 5H, Ar), 9.14 (exch br s, 1H, NH).

5.2.208 *N*-(4-Bromophenyl)-2-[5-(3-chlorobenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetamide (61h)



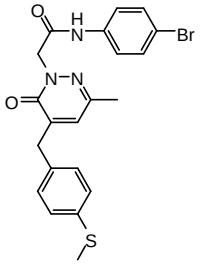
Yield = 26 %; mp = 185-187 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3H, 3-CH_3), 3.89 (s, 2H, CHC*CH_2*), 4.94 (s, 2H, NCH_2CO), 6.86 (s, 1H, Ar), 7.14 (s, 1H, Ar), 7.28 (dd, 7H, Ar, *J* = 1.3 Hz, *J* = 5.4 Hz), 9.26 (exch br s, 1H, NH).

5.2.209 *N*-(4-Bromophenyl)-2-[5-(3-bromobenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetamide (61i)



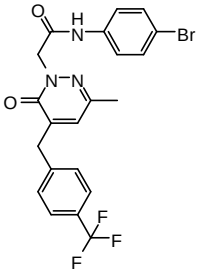
Yield = 47 %; mp = 99-101 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3H, 3-CH_3), 3.90 (s, 2H, CHC*CH_2*), 4.94 (s, 2H, NCH_2CO), 6.85 (s, 1H, Ar), 7.19-7.23 (m, 2H, Ar), 7.35-7.40 (m, 4H, Ar), 7.43 (t, 2H, Ar, *J* = 3.6 Hz), 9.00 (exch br s, 1H, NH).

5.2.210 *N*-(4-Bromophenyl)-2-{3-methyl-5-[4-(methylthio)benzyl]-6-oxo-pyridazin-1(6*H*)-yl}acetamide (61j)



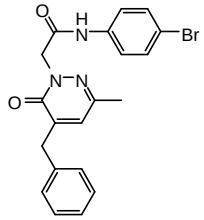
Yield = 10 %; mp = 97-99 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.30 (s, 3H, 3-CH_3), 2.50 (s, 3H, SCH_3), 3.89 (s, 2H, CHC*CH_2*), 4.94 (s, 2H, NCH_2CO), 6.80 (s, 1H, Ar), 7.17 (d, 2H, Ar, *J* = 8.2 Hz), 7.24 (d, 2H, Ar *J* = 8.2 Hz), 7.39 (dd, 4H, Ar, *J* = 2.6 Hz, *J* = 5.7 Hz), 9.01 (exch br s, 1H, NH).

5.2.211 *N*-(4-Bromophenyl)-2-{3-methyl-6-oxo-5-[4-(trifluoromethyl) benzyl]pyridazin-1(6*H*)-yl}acetamide (61k)



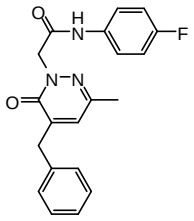
Yield = 44 %; mp = 81-83 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H, 3-CH_3), 3.99 (s, 2H, CHC*CH_2*), 4.94 (s, 2H, NCH_2CO), 6.84 (s, 1H, Ar), 7.32-7.40 (m, 6H, Ar), 7.61 (d, 2H, Ar, *J* = 8.1 Hz), 8.99 (exch br s, 1H, NH).

5.2.212 2-[5-Benzyl-3-methyl-6-oxopyridazin-1(6*H*)-yl]-*N*-(4-bromophenyl) acetamide (61 l)



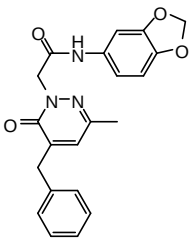
Yield = 47 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 2.30 (s, 3H, 3-CH_3), 3.93 (s, 2H, CHCCH_2), 4.94 (s, 2H, NCH_2CO), 6.82 (s, 1H, Ar), 7.24-7.28 (m, 2H, Ar), 7.30-7.38 (m, 7H, Ar), 9.18 (exch br s, 1H, NH).

5.2.213 *N*-(4-Fluorophenyl)-2-[5-benzyl-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetamide (61m)



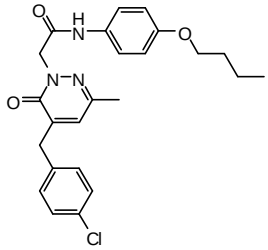
Yield = 53 %; mp = 175-76 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.29 (s, 3H, 3-CH_3), 3.94 (s, 2H, CHCCH_2), 4.95 (s, 2H, NCH_2CO), 6.80 (s, 1H, Ar), 6.97 (t, 2H, Ar, J = 8.7 Hz), 7.25-7.28 (m, 2H, Ar), 7.30-7.38 (m, 3H, Ar), 7.41-7.44 (m, 2H, Ar), 9.05 (exch br s, 1H, NH).

5.2.214 *N*-(1,3-Benzodioxol-5-yl)-2-[5-benzyl-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetamide (61n)



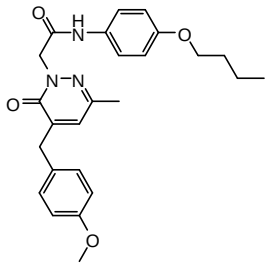
Yield = 26 %; mp = 63-65 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.22 (s, 3H, 3-CH_3), 3.80 (s, 2H, CHCCH_2), 4.79 (s, 2H, NCH_2CO), 5.99 (s, 2H, OCH_2O), 6.86 (d, 1H, Ar, J = 8.4 Hz), 6.93-6.95 (m, 1H, Ar), 7.08 (s, 1H, Ar), 7.22-7.34 (m, 7H, Ar), 10.19 (exch br s, 1H, NH).

5.2.215 *N*-(4-Butoxyphenyl)-2-[5-(4-chlorobenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetamide (61o)



Yield = 70 %. mp =146-147 °C (EtOH). IR (cm^{-1}) 3298 (NH), 1708 (CO), 1642 (CO). ^{1}H NMR ($CDCl_3$) δ 0.99 (t, 3H, $O(CH_2)_3CH_3$, J = 7.4 Hz), 1.49 (sext, 2H, $OCH_2CH_2CH_2CH_3$, J = 7.5 Hz), 1.76 (quint, 2H, $OCH_2CH_2CH_2CH_3$, J = 7.0 Hz), 2.29 (s, 3H, 3-CH_3), 3.88 (s, 2H, $CHCCH_2$), 3.93 (t, 2H, OCH_2, J = 6.5 Hz), 4.94 (s, 2H, NCH_2CO), 6.76 (s, 1H, Ar), 6.81 (d, 2H, Ar, J = 9.0 Hz), 7.18 (d, 2H, Ar, J = 8.4 Hz), 7.32 (d, 2H, Ar, J = 8.4 Hz), 7.38 (d, 2H, Ar, J = 9.0 Hz), 8.69 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{24}H_{26}ClN_3O_3$, 439,93. Found: *m/z* 440.17 $[M + H]^+$.

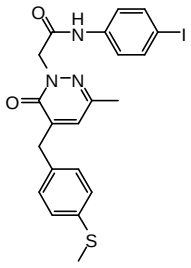
5.2.216 *N*-(4-Butoxyphenyl)-2-[5-(4-methoxybenzyl)-3-methyl-6-oxo-pyridazin-1(6*H*)-yl]acetamide (61p)



Yield = 60 %; mp = 160-161 °C (EtOH). IR (cm^{-1}) 3300 (NH), 1707 (CO), 1644 (CO). ^{1}H NMR ($CDCl_3$) δ 0.98 (t, 3H, $O(CH_2)_3CH_3$, J = 7.4 Hz), 1.45-1.54 (m, 2H, $OCH_2CH_2CH_2CH_3$), 1.77(quint, 2H, $OCH_2CH_2CH_2CH_3$, J = 7.1 Hz), 2.28 (s, 3H, 3-CH_3), 3.83 (s, 3H, OCH_3), 3.88 (s, 2H, $CHCCH_2$), 3.94 (t, 2H, OCH_2, J = 6.5 Hz), 4.93 (s, 2H, NCH_2CO), 6.74 (s, 1H, Ar), 6.82 (d, 2H, Ar, J = 9.0 Hz), 6.90 (d, 2H, Ar, J = 8.6 Hz), 7.17 (d, 2H, Ar, J = 8.5 Hz), 7.39 (d, 2H, Ar, J = 9.0 Hz), 8.67 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{25}H_{29}N_3O_4$, 435,52. Found: *m/z* 436.23 $[M + H]^+$.

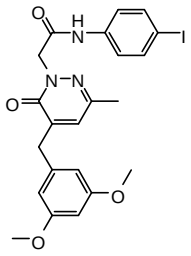
General Procedure for 62a-c. To 5 mL of KOH in absolute EtOH (5%, w/v), **53a** (0.13 mmol) and the appropriate substituted aromatic aldehyde (0.13 mmol) were added. The mixture was refluxed under stirring for 1-5 h. After cooling, the suspension was concentrated in vacuo, diluted with ice-cold water (5-10 mL), acidified with 2 N HCl and extracted with CH_2Cl_2 (3 x 25 mL). Removal of the solvent afforded compounds **62a-c**, which were purified by flash column chromatography using cyclohexane/ethyl acetate 2:1 (for **62a**) and cyclohexane/ethyl acetate 1:1 (for **62b,c**) as eluents.

5.2.217 *N*-(4-Iodophenyl)-2-{3-methyl-5-[4-(methylthio)benzyl]-6-oxo-pyridazin-1(6*H*)-yl}acetamide (62a)



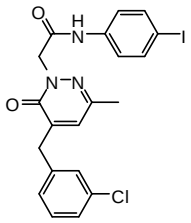
Yield = 46 %; mp = 68-70 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.30 (s, 3H, 3-CH_3), 2.51 (s, 3H, SCH_3), 3.89 (s, 2H, CHC*CH_2*), 4.93 (s, 2H, NCH_2CO), 6.80 (s, 1H, Ar), 7.18 (d, 2H, Ar, *J* = 8.4 Hz), 7.24-7.28 (m, 4H, Ar), 7.61 (d, 2H, Ar, *J* = 8.8 Hz), 8.92 (exch br s, 1H, NH).

5.2.218 *N*-(4-Iodophenyl)-2-[5-(3,5-dimethoxybenzyl)-3-methyl-6-oxo pyridazin-1(6*H*)-yl]- acetamide (62b)



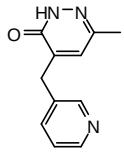
Yield = 30 %; mp = 63-65 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.30 (s, 3H, 3-CH_3), 3.79 (s, 6H, 2 x OCH_3), 3.87 (s, 2H, CHC*CH_2*), 4.94 (s, 2H, NCH_2CO), 6.40 (s, 3H, Ar), 6.82 (s, 1H, Ar), 7.28 (d, 2H, Ar, *J* = 5.7 Hz), 7.58 (d, 2H, Ar, *J* = 8.6 Hz), 9.01 (exch br s, 1H, NH).

5.2.219 *N*-(4-Iodophenyl)-2-[5-(3-chlorobenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetamide (62c)



Yield = 25 %; mp = 58-60 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3H, 3-CH_3), 3.90 (s, 2H, CHC*CH_2*), 4.94 (s, 2H, NCH_2CO), 6.86 (s, 1H, Ar), 7.13-7.16 (m, 1H, Ar), 7.20 (d, 2H, Ar, *J* = 7.0 Hz), 7.28 (d, 3H, Ar, *J* = 4.2 Hz), 7.52 (d, 2H, Ar, *J* = 8.7 Hz), 9.14 (exch br s, 1H, NH).

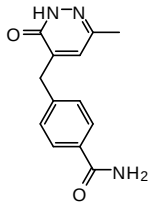
5.2.220 6-Methyl-4-(pyridin-3-ylmethyl)pyridazin-3(2*H*)-one (63)



To 6 mL of KOH in absolute EtOH (5%, w/v), **42d** (1.78 mmol) and commercially available pyridine-3-carbaldehyde (1.78 mmol) were added. The mixture was refluxed under stirring for 5 h. After cooling, the suspension was concentrated in vacuo, diluted with ice-cold water (10 mL), acidified with 2 N HCl and extracted with CH_2Cl_2 (3 x 25 mL). Removal of the solvent afforded compound **63**, which was purified by flash column chromatography using NH_4OH/EtOH/CH_2Cl_2/petroleum ether 4:25:150:50 as eluents. Yield = 53 %; mp = 181-183 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.28 (s, 3H, 6-CH_3), 3.93 (s, 2H, CHC*CH_2*),

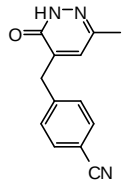
6.80 (s, 1H, Ar), 7.33 (dd, 1H, Ar, *J* = 2.7 Hz, *J* = 5.0 Hz), 7.70 (dd, 1H, Ar, *J* = 6.1 Hz, *J* = 1.8 Hz), 8.57 (s, 2H, Ar), 11.53 (exch br s, 1H, NH).

5.2.221 4-[(6-Methyl-3-oxo-2,3-dihydropyridazin-4-yl)methyl]benzamide (64)



To 6 mL of KOH in absolute EtOH (5%, w/v), **42d** (1.78 mmol) and commercially available 4-***cyanobenzaldehyde*** (3.56 mmol) were added. The mixture was refluxed under stirring for 4 h. After cooling, the suspension was concentrated in vacuo, diluted with ice-cold water (10 mL) and acidified with 2 N HCl. After 1 h stirring in ice-bath, the precipitate was filtered off and purified by crystallization in ethanol. Yield = 70 %; mp = 164-166 °C (EtOH). IR (cm^{-1}) 3300 (NH), 3200 (NH_2), 1649 (CO), 1608 (CO). ^{1}H NMR ($CDCl_3$) δ 2.18 (s, 3H, 6-CH_3), 3.80 (s, 2H, CHC*CH_2*), 7.06 (s, 1H, Ar), 7.33 (d, 2H, Ar, *J* = 8.2 Hz), 7.80 (d, 2H, Ar, *J* = 8.1 Hz), 12.72 (exch br s, 1H, NH_2).

5.2.222 4-[(6-Methyl-3-oxo-2,3-dihydropyridazin-4-yl)methyl]benzonitrile (65)

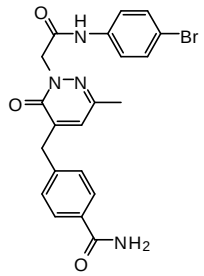


A suspension of **64** (0.82 mmol) in 5 mL of $POCl_3$ was stirred at 60 °C for 3 h. After cooling, the mixture was concentrated in vacuo, diluted with ice-cold water (10 mL) and extracted with CH_2Cl_2 (3 x 15 mL). The organic layer was dried (Na_2SO_4) and concentrated in vacuo, to afford a yellow solid which was purified by flash column chromatography using CH_2Cl_2/CH_3OH 7:3 as eluent. Yield = 60 %; mp = 213-215 °C (EtOH). IR (cm^{-1}) 2225 (CN), 1649 (CO), 1609 (CO). ^{1}H NMR ($CDCl_3$) δ 2.29 (s, 3H, 6-CH_3), 3.96 (s, 2H, CHC*CH_2*), 6.79 (s, 1H, Ar), 7.41 (d, 2H, Ar, *J* = 7.0 Hz), 7.65 (d, 2H, Ar, *J* = 6.9 Hz), 10.61 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{25}H_{29}N_3O_4$, 435,52. Found: *m/z* 436.23 $[M + H]^+$.

General Procedure for 66a-c. A mixture of compound **63**, **64** or **65** (0.79 mmol), K_2CO_3 (1.58 mmol) and *N*-(4-bromophenyl)-2-chloroacetamide **22** (1.19 mmol) in CH_3CN (2 mL), was refluxed under stirring for 2-3 h. The mixture was then concentrated in vacuo and diluted with cold water. After 1 h stirring in ice-bath, the precipitate was filtered off by suction and purified by flash column

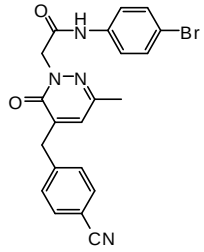
chromatography using alternatively CH_2Cl_2/CH_3OH 9.5:05 (for **66a**), CH_2Cl_2/CH_3OH 9.9:0.1 (for **66b**) or $CH_2Cl_2/CH_3OH/NH_4OH$ 9.5:0.5:0.05 (for **66c**) as eluents.

5.2.223 4-{2-[(4-Bromophenycarbamoyl)methyl]-6-methyl-3-oxo-2,3-dihydropyridazin-4-ylmethyl}benzamide (66a)



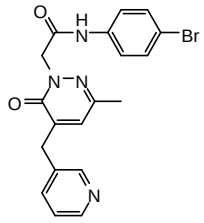
Yield = 59 %; mp = 168-171 °C (EtOH). IR (cm^{-1}) 3200 (NH_2), 1654 (CO), 1609 (CO). ^{1}H NMR ($CDCl_3$) δ 2.31 (s, 3H, 6-CH_3), 3.99 (s, 2H, CHC*CH_2*), 4.93 (s, 2H, NCH_2CO), 6.81 (s, 1H, Ar), 7.34-7.41 (m, 6H, Ar), 7.80 (d, 2H, Ar, *J* = 8.1 Hz), 8.89 (exch br s, 1H, NH).

5.2.224 *N*-(4-Bromophenyl)-2-[5-(4-cyanobenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl])acetamide (66b)



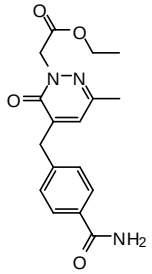
Yield = 19 %; mp = 210-211 °C (EtOH). IR (cm^{-1}) 2225 (CN), 1650 (CO), 1598 (CO). ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3H, 3-CH_3), 3.99 (s, 2H, CHC*CH_2*), 4.93 (s, 2H, NCH_2CO), 6.85 (s, 1H, Ar), 7.39 (q, 6H, Ar, *J* = 8.3 Hz), 7.65 (d, 2H, Ar, *J* = 8.2 Hz), 8.77 (exch br s, 1H, NH).

5.2.225 *N*-(4-Bromophenyl)-2-[3-methyl-6-oxo-5-(pyridin-3-ylmethyl)-pyridazin-1(6H)-yl]acetamide (66c)



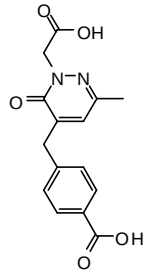
Yield = 61 %; mp = 216-218 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.30 (s, 3H, 3-CH_3), 3.92 (s, 2H, CHC*CH_2*), 4.93 (s, 2H, NCH_2CO), 6.86 (s, 1H, Ar), 7.27-7.33 (m, 5H, Ar), 7.61 (d, 1H, Ar, *J* = 7.8 Hz), 8.54 (d, 2H, Ar, *J* = 6.2 Hz), 9.29 (exch br s, 1H, NH).

5.2.226 Ethyl-2-[5-(4-carbamoylbenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetate (67)



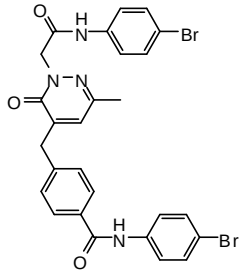
A mixture of compound **64** (1.56 mmol), K_2CO_3 (3.12 mmol) and ethyl bromoacetate (2.34 mmol) in CH_3CN (6 mL), was refluxed under stirring for 2 h. The mixture was then concentrated in vacuo and diluted with cold water. After 1h stirring in ice-bath, the yellow precipitate was filtered off by suction and purified by recrystallization in ethanol. Yield = 78 %; mp = 174-76 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, CH_2CH_3), 2.25 (s, 3H, 3-CH_3), 3.96 (s, 2H, CHCCH_2), 4.26 (q, 2H, CH_2CH_3, J = 7.2 Hz), 4.86 (s, 2H, NCH_2CO), 6.70 (s, 1H, Ar), 7.34 (d, 2H, Ar, J = 7.7 Hz), 7.80 (d, 2H, Ar, J = 7.7 Hz).

5.2.227 4-{[2-(Carboxymethyl)-6-methyl-3-oxo-2,3-dihydropyridazin-4-yl]methyl} benzoic acid (68)



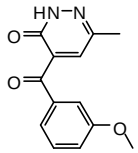
A suspension of the intermediate **67** (1.22 mmol) in 6 N NaOH (5 mL) was stirred at 60 °C for 2 h. The mixture was diluted with ice-cold water (3 mL), acidified with 6 N HCl and the final product **68** was then filtered off by suction and recrystallized from ethanol. Yield = 76 %; mp = 225-27 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.22 (s, 3H, 6-CH_3), 3.87 (s, 2H, CHCCH_2), 4.69 (s, 2H, NCH_2CO), 7.15 (s, 1H, Ar), 7.39 (d, 2H, Ar, J = 8.0 Hz), 7.88 (d, 2H, Ar, J = 8.0 Hz), 13.01 (exch br s, 1H, OH).

5.2.228 *N*-(4-Bromophenyl)-4-{2-[(4-bromophenylcarbamoyl)methyl]-6-methyl-3-oxo-2,3-dihydropyridazin-4-ylmethyl}benzamide (69)



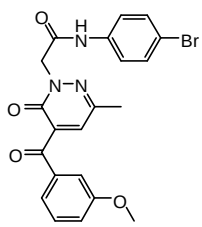
To a cooled (-5 °C) and stirred solution of compound **68** (0.93 mmol) in anhydrous tetrahydrofuran (7 mL), Et_3N (3.26 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C and ethyl chloroformate (1.02 mmol) was added. After 1 h, 4-bromo aniline (1.86 mmol) was added. The reaction was carried out at room temperature for 12 h. The mixture was then concentrated in vacuo, diluted with cold water (15 mL) and extracted with CH_2Cl_2 (3 x 15 mL). After removal of the solvent, the residue was purified by column chromatography using $CH_2Cl_2/CH_3OH/NH_4OH$ 9.5:0.5:0.05 as eluent. The analytical sample of compound **69** was obtained from a further purification through a silica gel preparative TLC (eluent: $CH_2Cl_2/CH_3OH/NH_4OH$ 9.5:0.5:0.05). Yield = 10 %; mp = 226-228 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.31 (s, 3H, 6-CH_3), 4.00 (s, 2H, CHC*CH_2*), 4.93 (s, 2H, NCH_2CO), 6.83 (s, 1H, Ar), 7.40 (t, 6H, Ar, *J* = 8.4 Hz), 7.51 (d, 2H, Ar, *J* = 8.7 Hz), 7.57 (d, 2H, Ar, *J* = 8.9 Hz), 7.84 (d, 2H, Ar, *J* = 7.7 Hz), 8.67 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{27}H_{22}Br_2N_4O_3$, 610.30. Found: *m/z* 609 $[M - H]^-$, 611.2 $[M + H]^+$.

5.2.229 4-(3-Methoxybenzoyl)-6-methylpyridazin-3(2*H*)-one (70)



To a stirred and heated (60 °C) suspension of compound **43** (2.39 mmol) in 15 mL of 50 % (v/v) acetic acid, $Ce(NH_4)_2(NO_3)_6$ (7.17 mmol) was slowly added over 0.5 h and the reaction is carried out at 60 °C for additional 1 h. The mixture was then diluted with ice-cold water (10 mL) and extracted with CH_2Cl_2 (15 mL). After washing with H_2O (3 x 10 mL), the organic layer was evaporated under vacuo and the residue was purified by CombyFlash® (eluent: cyclohexane/ethyl acetate, gradient 1:1 to 1:3). Yield = 17 %; oil. ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H, 6-CH_3), 3.92 (s, 3H, OCH_3), 6.74-6.95 (m, 4H, Ar), 7.31 (t, 1H, Ar, *J* = 7.8 Hz), 11.32 (exch br s, 1H, NH).

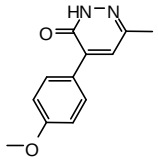
5.2.230 *N*-(4-Bromophenyl)-2-[5-(3-methoxybenzoyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetamide (71)



A mixture of intermediate **70** (0.41 mmol), K_2CO_3 (0.82 mmol) and *N*-(4-bromophenyl)-2-chloroacetamide **22** (0.61 mmol) in CH_3CN (5 mL), was refluxed under stirring for 6 h. The mixture was

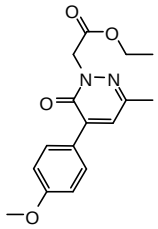
then concentrated in vacuo and extracted with CH_2Cl_2 (3 x 15 mL). After removal of the solvent under vacuo the residue was purified by flash column chromatography using NH_4OH/EtOH/CH_2Cl_2/petroleum ether 4:25:150:269 as eluent. Yield = 10 %; mp = 176-177 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H, 3-CH_3), 3.82 (s, 3H, OCH_3), 4.92 (s, 2H, NCH_2CO), 6.62 (s, 1H, Ar), 6.69 (t, 1H, Ar, J = 2.1 Hz), 6.74 (d, 1H, Ar, J = 7.5 Hz), 6.85 (dd, 1H, Ar, J = 6.3 Hz, J = 2.0 Hz), 7.29 (t, 1H, Ar, J = 8.2 Hz), 7.38-7.46 (m, 4H, Ar), 9.10 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{21}H_{18}BrN_3O_4$, 456,29. Found: *m/z* 457.20 [M + H]$^+$.

5.2.231 4-(4-Methoxyphenyl)-6-methylpyridazin-3(2*H*)-one (74)



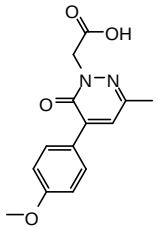
To an ice-cold solution of (*E*)-methyl 2-(4-methoxyphenyl)-4-oxopent-2-enoate **73** (0.34 mmol) in dry toluene (3 mL), hydrazine hydrate (0.68 mmol) was added drop-wise. The solution was stirred at reflux temperature for 2 h. The solvent was evaporated and ice-cold water (5 mL) was added to the residue. The aqueous layer was extracted with CH_2Cl_2 (3 x 15 mL) and the combined organic layers were dried over Na_2SO_4 and evaporated in vacuo. The crude product was purified by flash column chromatography (eluent: cyclohexane/ethyl acetate 1:3) to yield **74** as an amorphous white solid. Yield = 55 %; mp = 155-158 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.39 (s, 3H, 6-CH_3), 3.88 (s, 3H, OCH_3), 6.99 (d, 2H, Ar, J = 8.9 Hz), 7.24 (s, 1H, Ar), 7.86 (d, 2H, Ar, J = 8.9 Hz), 10.51 (exch br s, 1H, NH).

5.2.233 Ethyl-2-[5-(4-methoxyphenyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetate (75)



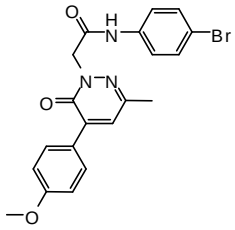
A mixture of the intermediate **74** (0.18 mmol), K_2CO_3 (0.36 mmol) and ethyl bromoacetate (0.28 mmol) in CH_3CN (2 mL), was refluxed under stirring for 1.5 h. The mixture was then concentrated in vacuo and extracted with CH_2Cl_2 (3 x 10 mL). After removal of the solvent under vacuo the residue was purified by flash column chromatography using cyclohexane/ethyl acetate 1:1 as eluent. Yield ~ 100 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.30 (t, 3H, CH_2CH_3, J = 7.0 Hz), 2.37 (s, 3H, 3-CH_3), 3.92 (s, 3H, OCH_3), 4.23 (q, 2H, CH_2CH_3, J = 7.0 Hz), 4.89 (s, 2H, NCH_2CO), 6.94 (d, 2H, Ar, J = 8.8 Hz), 7.21 (s, 1H, Ar), 7.81 (d, 2H, Ar, J = 8.8 Hz).

5.2.234 2-[5-(4-Methoxyphenyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetic acid (76)



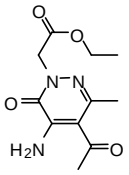
A suspension of the intermediate **75** (0.20 mmol) in 6 N NaOH (1.5 mL) was stirred at 60 °C for 0.5 h. The mixture was diluted with ice-cold water (1 mL), acidified with 6 N HCl and the final product **68** was then filtered off by suction and recrystallized from ethanol. Yield ~ 100 %; mp = 202-203 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.41 (s, 3H, 3-CH_3), 3.87 (s, 3H, OCH_3), 4.99 (s, 2H, NCH_2CO), 6.98 (d, 2H, Ar, J = 8.8 Hz), 7.26 (s, 1H, Ar), 7.82 (d, 2H, Ar, J = 8.8 Hz).

5.2.235 *N*-(4-Bromophenyl)-2-[5-(4-methoxyphenyl)-3-methyl-6-oxo-pyridazin-1(6*H*)-yl]acetamide (77)



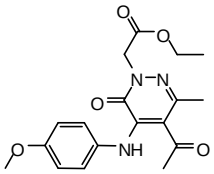
To a cooled (-5 °C) and stirred solutions of compound **76** (0.20 mmol) in anhydrous tetrahydrofuran (2 mL), Et_3N (0.70 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C and ethyl chloroformate (0.22 mmol) was added. After 1 h, 4-bromoaniline (0.40 mmol) was added and the reaction was carried out at room temperature for 12 h. The mixture was then concentrated in vacuo, diluted with cold water (5 mL) and extracted with CH_2Cl_2 (3 x 10 mL). After removal of the solvent, the residue was purified by column chromatography using cyclohexane/ethyl acetate 2:1 as eluent. Yield = 35 %; mp = 251-253 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 3H, 6-CH_3), 3.88 (s, 3H, OCH_3), 5.00 (s, 2H, NCH_2CO), 7.00 (d, 2H, Ar, J = 8.7 Hz), 7.27 (s, 1H, Ar), 7.40 (q, 4H, Ar, J = 9.2 Hz), 7.80 (d, 2H, Ar, J = 8.7 Hz), 9.07 (exch br s, 1H, NH).

5.2.236 Ethyl-2-[4-acetyl-5-amino-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetate (80)



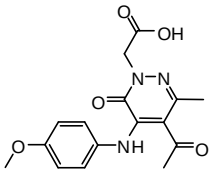
A mixture of the intermediate **79** (0.99 mmol), K_2CO_3 (1.98 mmol), and ethyl bromoacetate (1.34 mmol) in CH_3CN (3 mL) was refluxed under stirring for 3 h. The mixture was then concentrated in vacuo, diluted with cold water, and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated in vacuo, and compound **80** was purified by recrystallization from ethanol. Yield = 44 %; mp = 145-46 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.32 (t, 3H, CH_2CH_3, J = 7.2 Hz), 2.53 (s, 3H, 3-CH_3), 2.60 (s, 3H, $COCH_3$), 4.27 (q, 2H, OCH_2CH_3, J = 7.1 Hz), 4.83 (s, 2H, NCH_2CO), 7.75 (exch br s, 1H, NH).

5.2.237 Ethyl-2-[4-acetyl-5-(4-methoxyphenylamino)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetate (81)



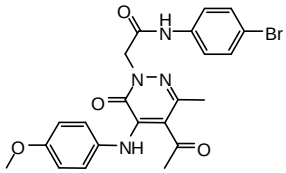
To the suspension of **80** (0.91 mmol), copper acetate (1.36 mmol) and 4-methoxyphenylboronic acid (1.82 mmol) in CH_2Cl_2 (4 mL), Et_3N (1.82 mmol) was added and the mixture was stirred at room temperature for 12 h. The suspension was extracted with 15% aqueous ammonia (3 x 10 mL), then the organic layer was washed with water (10 mL) and dried over Na_2SO_4. After removal of the solvent in vacuo, the residue was purified by flash column chromatography using cyclohexane/ethyl acetate 1:3 as eluent. Yield = 61 %; mp = 115-17 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.34 (t, 3H, CH_2CH_3, J = 7.2 Hz), 1.88 (s, 3H, $COCH_3$), 2.14 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 4.29 (q, 2H, OCH_2CH_3, J = 7.2 Hz), 4.88 (s, 2H, $COCH_2N$), 6.85 (d, 2H, Ar, J = 6.7 Hz), 7.04 (d, 2H, Ar, J = 8.9 Hz), 7.62 (exch br s, 1H, NH).

5.2.238 2-[4-Acetyl-5-(4-methoxyphenylamino)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetic acid (82)



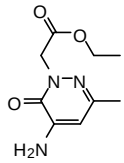
A suspension of the intermediate **81** (0.39 mmol) in 6 N NaOH (10 mL) was stirred at rt to 80 °C for 1.5 h. The mixture was diluted with cold water and acidified with 6 N HCl. After 1 h stirring in ice-bath, the product **82** was filtered off by suction and recrystallized from ethanol. Yield = 76 %; mp = 104-06 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.88 (s, 3H, $COCH_3$), 2.10 (exch br s, 1H, OH), 2.16 (s, 3H, 3-CH_3), 3.82 (s, 3H, OCH_3), 4.95 (s, 2H, $COCH_2N$), 6.86 (d, 2H, Ar, J = 8.6 Hz), 7.05 (d, 2H, Ar, J = 8.6 Hz), 7.62 (exch br s, 1H, NH).

5.2.239 *N*-(4-Bromophenyl)-2-[4-acetyl-5-(4-methoxyphenylamino)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetamide (83)



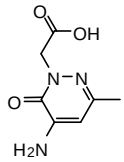
To a cooled (-5 °C) and stirred solution of **82** (0.30 mmol) in anhydrous tetrahydrofuran (3 mL), Et_3N (1.06 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C and ethyl chloroformate (0.33 mmol) was added. After 1 h, 4-bromo aniline (0.60 mmol) was added and the reaction was carried out at room temperature for 12 h. The mixture was then concentrated in vacuo, diluted with cold water (10 mL), and extracted with CH_2Cl_2 (3 x 15 mL). The organic layer was dried over Na_2SO_4 and the solvent was evaporated to afford final compound **83**, which was purified by flash column chromatography using cyclohexane/ethyl acetate 1:1 as eluent. Yield = 62 %; mp = 210-11 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.88 (s, 3H, $COCH_3$), 2.19 (s, 3H, 3-CH_3), 3.83 (s, 3H, OCH_3), 4.97 (s, 2H, $COCH_2N$), 6.87 (d, 2H, Ar, J = 8.9 Hz), 7.05 (d, 2H, Ar, J = 8.9 Hz), 7.44 (td, 4H, Ar, J = 2.8 Hz, J = 6.5 Hz), 7.65 (exch br s, 1H, NH), 8.64 (exch br s, 1H, NH).

5.2.240 Ethyl-2-[5-amino-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetate (85)



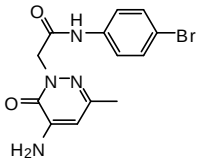
A mixture of **84** (0.80 mmol), K_2CO_3 (1.60 mmol) and ethyl bromoacetate (1.20 mmol) in CH_3CN (5 mL) was refluxed under stirring for 3 h. The solvent was removed under reduced pressure, then the crude mixture was diluted with cold water (10 mL) and extracted with CH_2Cl_2 (3 x 15 mL). The organic layer was dried over Na_2SO_4 and evaporated in vacuo; finally compound **85** was purified by column chromatography using CH_2Cl_2/CH_3OH 95:5 as eluent. Yield = 71 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.30 (t, 3H, CH_2CH_3, J = 7.1 Hz), 2.22 (s, 3H, 3-CH_3), 4.25 (qd, 2H, OCH_2CH_3, J = 4.1 Hz, J = 3.0 Hz), 4.84 (s, 2H, NCH_2CO), 6.17 (s, 1H, Ar).

5.2.241 2-[5-Amino-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetic acid (86)



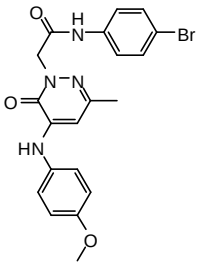
A suspension of compound **85** (0.28 mmol) in 6 NaOH (2 mL) was stirred at rt to 80 °C for 2 h. The mixture was diluted with ice-cold water (1 mL), acidified with 6 N HCl and the aqueous phase was evaporated in vacuo. The crude residue was dissolved in ethanol and the precipitate was filered off. Finally, evaporation of the solvent afforded compound **86**, which was purified by crystallization from cyclohexane. Yield ~ 100 %; mp = 234-36 °C (cyclohexane). ^{1}H NMR ($CDCl_3$) δ 2.09 (s, 3H, 3-CH_3), 4.62 (s, 2H, NCH_2CO), 6.13 (s, 1H, Ar), 6.37 (exch br s, 2H, NH_2), 6.55 (exch br s, 1H, OH).

5.2.242 *N*-(4-Bromophenyl)-2-[5-amino-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetamide (87)



To a cooled (-5 °C) and stirred solution of compound **86** (0.33 mmol), in anhydrous tetrahydrofuran (3 mL), Et_3N (1.15 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C and ethyl chloroformate (0.36 mmol) was added. After 1 h 4-bromo aniline (0.66 mmol) was added and the reaction was carried out at room temperature for 12 h. The mixture was then concentrated in vacuo, diluted with ice-cold water (10 mL) and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated to afford final compound **87**, which was purified by flash column chromatography using cyclohexane/ethyl acetate 1:1 as eluent. Yield = 50 %; mp = 244-45 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H, 3-CH_3), 4.93 (s, 2H, $COCH_2N$), 6.24 (s, 1H, Ar), 7.42 (s, 4H, Ar), 8.83 (exch br s, 1H, NH), 8.64 (exch br s, 1H, NH).

5.2.243 *N*-(4-Bromophenyl)-2-[5-(4-methoxyphenylamino)-3-methyl-6-oxopyridazin-1(6*H*)-yl]acetamide (88)

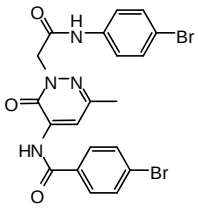


To a suspension of **87** (0.36 mmol), copper acetate (0.53 mmol) and 4-methoxyphenylboronic acid (0.36 mmol) in CH_2Cl_2 (3 mL), Et_3N (0.72 mmol) was added and the mixture was stirred at room temperature for 12 h. The suspension was extracted with 15% aqueous ammonia (3 x 10 mL), and the organic layer was washed with 10 mL of water and dried over Na_2SO_4. After removal of the solvent under reduced pressure, the residue was purified by flash column chromatography using CH_2Cl_2/CH_3OH 9.5:0.5 as eluent. The analytical sample of compound **88** was obtained from a further purification through a silica

gel preparative TLC (eluent: CH_2Cl_2/CH_3OH 9.5:0.5). Yield = 10 %; mp = 249-51 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H, 3-CH_3), 3.86 (s, 3H, OCH_3), 4.97 (s, 2H, $COCH_2N$), 6.38 (s, 1H, Ar), 6.97 (d, 2H, Ar, *J* = 8.9 Hz), 7.18 (d, 2H, Ar, *J* = 8.9 Hz), 7.35 (exch br s, 1H, NH), 7.44 (dd, 4H, Ar, *J* = 5.5 Hz, *J* = 9.1 Hz), 8.77 (exch br s, 1H, NH).

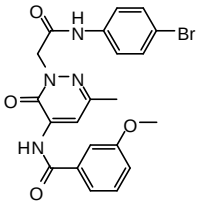
General Procedure for 89a,b. A solution of compound **87** (0.21 mmol), Et_3N (5 drops) and the appropriate substituted benzoyl chloride (0.25 mmol), in dry CH_2Cl_2 (5 mL), was stirred at 0 °C for 1 h. Extra benzoyl chloride (0.25 mmol) was added. The reaction was carried out always at 0 °C for additional 3 h and then, 12 h at room temperature. The mixture was extracted with 6 N NaOH (3 x 10 mL), then the organic layer was washed with water (10 mL) and dried over Na_2SO_4. After removal of the solvent in vacuo, compound **89a** was purified by two consecutive silica gel preparative TLC using in both cases cyclohexane/ethyl acetate 1:2 as eluent. Differently, in the case of compound **89b** the residue was purified by flash column chromatography using cyclohexane/ethyl acetate 1:3 as eluent.

5.2.244 4-Bromo-*N*-{2-[2-(4-bromophenylcarbamoyl)methyl]-6-methyl-3-oxo-2,3-dihydropyridazin-4-yl}benzamide (89a)



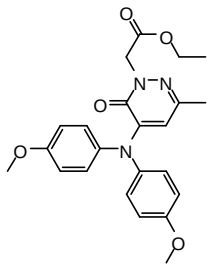
Yield = 10 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 3H, 6-CH_3), 4.98 (s, 2H, $COCH_2N$), 7.44 (s, 4H, Ar), 7.69 (d, 2H, Ar, *J* = 8.6 Hz), 7.82 (d, 2H, Ar, *J* = 8.6 Hz), 8.24 (s, 1H, Ar).

5.2.245 3-Metoxy-*N*-{2-[(4-bromophenylcarbamoyl)methyl]-6-methyl-3-oxo-2,3-dihydropyridazin-4-yl}benzamide (89b)



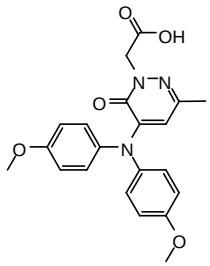
Yield = 13 %; mp = 226-28 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H, 6-CH_3), 3.89 (s, 3H, OCH_3), 4.93 (s, 2H, $COCH_2N$), 4.98 (exch br s, 1H, NH), 6.24 (s, 1H, Ar), 7.16-7.19 (m, 1H, Ar), 7.39-7.45 (m, 5H, Ar), 7.63 (t, 1H, Ar, *J* = 2.5 Hz), 7.73 (dd, 1H, Ar, *J* = 5.3 Hz, *J* = 1.0 Hz), 8.83 (exch br s, 1H, Ar).

5.2.246 Ethyl-2-{5-[bis(4-methoxyphenyl)amino]-3-methyl-6-oxopyridazin-1(6*H*)-yl}acetate (90)



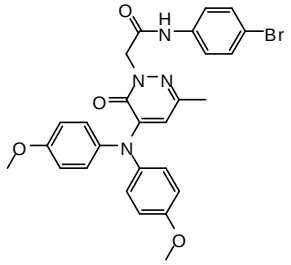
To a suspension of compound **85** (0.57 mmol), copper acetate (0.85 mmol) and 4-methoxyphenylboronic acid (1.14 mmol) in CH_2Cl_2 (3 mL), Et_3N (0.64 mmol) was added and the mixture was stirred at room temperature for 14 h. The mixture was extracted with 15% aqueous ammonia (3 x 10 mL) and the organic layer was washed with 10 mL of water and dried over Na_2SO_4. After removal of the solvent in vacuo, the residue was purified by flash column chromatography using cyclohexane/ethyl acetate 1:3 as eluent. Yield = 21 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.28 (t, 3H, *CH*$_2$*CH*$_3$, *J* = 7.2 Hz), 2.18 (s, 3H, 3-CH_3), 3.81 (s, 6H, 2 x OCH_3), 4.21 (q, 2H, *CH*$_2$$CH_3$, *J* = 7.2 Hz), 4.83 (s, 2H, NCH_2CO), 6.32 (s, 1H, Ar), 6.86 (dd, 4H, Ar, *J* = 3.4 Hz, *J* = 2.3 Hz), 6.99 (dd, 4H, Ar, *J* = 4.5 Hz, *J* = 2.3 Hz).

5.2.247 2-{5-[Bis-(4-methoxyphenyl)amino]-3-methyl-6-oxopyridazin-1(6*H*)-yl}acetic acid (91)



A suspension of the intermediate **90** (0.12 mmol), 6 NaOH (10 mL) and EtOH (3 mL) was stirred at rt 12 h. After removal of the solvent under vacuo, the mixture was diluted with ice-cold water and acidified with 6 N HCl. After 1 h stirring in ice-bath, the product **91** was filtered off by suction and recrystallized from ethanol. Yield = 84 %; mp = 192-93 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.20 (s, 3H, 3-CH_3), 3.82 (s, 6H, 2 x OCH_3), 4.88 (s, 2H, NCH_2CO), 6.34 (s, 1H, Ar), 6.86 (d, 4H, Ar, *J* = 8.8 Hz), 6.99 (d, 4H, Ar, *J* = 8.8 Hz).

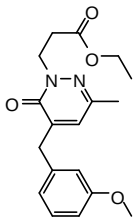
5.2.248 *N*-(4-Bromophenyl)-2-{5-[bis(4-methoxyphenyl)amino]-3-methyl-6-oxopyridazin-1(6*H*)-yl}acetamide (92)



To a cooled (-5 °C) and stirred solution of compound **91** (0.10 mmol) in anhydrous tetrahydrofuran (4 mL), Et_3N (0.35 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C and ethyl chloroformate (0.11 mmol) was added. After 1 h 4-bromo aniline (0.20 mmol) was added and the reaction was carried out at room temperature for 12 h. The mixture was then concentrated in vacuo, diluted with cold water (10 mL) and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated to afford final compound **92**, which was purified by flash column chromatography using cyclohexane/ethyl acetate 1:1 as eluent. Yield = 55 %; mp = 244-245 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.23 (s, 3H, 3-CH_3), 3.79 (s, 6H, 2 x OCH_3), 4.81 (s, 2H, NCH_2CO), 6.39 (s, 1H, Ar), 6.84 (dd, 4H, Ar, J = 4.6 Hz, J = 2.2 Hz), 6.99 (dd, 4H, Ar, J = 2.2 Hz, J = 3.4 Hz), 7.25-7.38 (m, 4H, Ar), 9.00 (exch br s, 1H, NH).

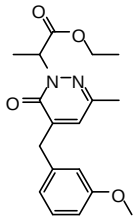
General Procedure for 93a,b. A mixture of **43** (1.13 mmol), K_2CO_3 (2.26 mmol), and the appropriate alkyl halide (1.70 mmol) in CH_3CN (3 mL) was refluxed under stirring for 6 h. The mixture was then concentrated in vacuo, diluted with cold water and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated in vacuo and compounds **93a,b** were purified by column chromatography using cyclohexane/ethyl acetate 1:1 as eluent.

5.2.249 Ethyl-3-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]propanoate (93a)



Yield = 86 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.26 (t, 3H, OCH_2CH_3, J = 7.1 Hz), 2.22 (s, 3H, 3-CH_3), 2.84 (t, 2H, NCH_2CH_2CO, J = 7.2 Hz), 3.83 (s, 3H, OCH_3), 3.87 (s, 2H, CH_2-Ar), 4.17 (q, 2H, OCH_2CH_3, J = 7.1 Hz), 4.45 (t, 2H, NCH_2CH_2COO, J = 7.3 Hz), 6.64 (s, 1H, Ar), 6.80 (s, 1H, Ar), 6.83 (d, 2H, Ar, J = 7.9 Hz), 7.26-7.30 (m, 1H, Ar).

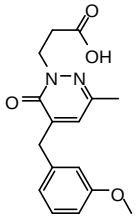
5.2.250 Ethyl-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6H)-yl]propanoate (93b)



Yield = 85 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.22 (t, 3H, OCH_2CH_3, J = 7.1 Hz), 1.65 (d, 3H,CH_3CHN, J = 7.2 Hz), 2.21 (s, 3H, 3-CH_3), 3.78 (s, 3H, OCH_3), 3.84 (s, 2H, CH_2-Ar), 4.19 (q, 2H, OCH_2CH_3, J = 6.7 Hz), 5.51 (q, 1H, CH_3CHN, J = 7.2 Hz), 6.64 (s, 1H, Ar), 6.77-6.81 (m, 3H, Ar), 7.22-7.26 (m, 1H, Ar).

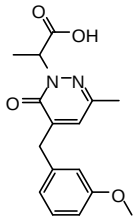
General Procedure for 94a,b. A suspension of the appropriate intermediate type **93** (**93a,b**) (0.5 mmol), and 6 N NaOH (6 mL) in ethanol (2 mL) was stirred at rt to 80 °C for 1 h. The mixture was then concentrated in vacuo, diluted with cold water and acidified with 6 N HCl. Compound **94a** was filtered off by suction and recrystallized from ethanol. For compound **94b** differently, after acidification with 6 N HCl, the mixture was extracted with CH_2Cl_2 (3 x 15 mL) and the solvent was evaporated in vacuo to give the pure compound as an oil.

5.2.251 3-[5-(3-Methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]propanoic acid (94a)



Yield = 96 %; mp = 86-88 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.22 (s, 3H, 3-CH_3), 2.89 (t, 2H, NCH_2CH_2CO, J = 7.2 Hz), 3.81 (s, 3H, OCH_3), 3.87 (s, 2H, CH_2-Ar), 4.46 (t, 2H, NCH_2CH_2CO, J = 7.2 Hz), 6.66 (s, 1H, Ar), 6.78 (s, 1H, Ar), 6.81-6.85 (m, 2H, Ar), 7.25-7.29 (m, 1H, Ar), 9.99 (exch br s, 1H, OH).

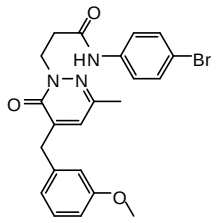
5.2.252 2-[5-(3-Methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]propanoic acid (94b)



Yield = 85 %; oil. ^{1}H NMR ($CDCl_3$) δ 1.71 (d, 3H, CH_3CHN, J = 7.2 Hz), 2.25 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.88 (s, 2H, CH_2-Ar), 5.54 (q, 1H, CH_3CHN, J = 7.2 Hz), 6.67 (s, 1H, Ar), 6.79-6.85 (m, 3H, Ar), 7.26-7.29 (m, 1H, Ar).

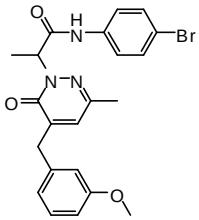
General Procedure for 95a,b. To a cooled (-5 °C) and stirred solution of compound **94a** or **94b** (0.35 mmol), in anhydrous tetrahydrofuran (3-5 mL), Et_3N (1.22 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C and ethyl chloroformate (0.38 mmol) was added. After 1 h, 4-bromoaniline (0.7 mmol) was added and the reaction was carried out at room temperature for 12 h. The mixture was then concentrated in vacuo, diluted with cold water (20-30 mL) and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated to afford the final compounds **95a,b**, which were purified by column chromatography using cyclohexane/ethyl acetate 1:2 as eluent for compound **95a**, cyclohexane/ethyl acetate 2:1 for compound **95b**.

5.2.253 *N*-(4-Bromophenyl)-3-[5-(3-methoxybenzyl)-3-methyl-6-oxo-pyridazin-1(6*H*)-yl]propanamide (95a)



Yield = 82 %; mp = 123-125 °C (EtOH). IR (cm^{-1}) 3297 (NH), 1710 (CO), 1644 (CO). ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H, 3-CH_3), 3.00 (t, 2H, NCH_2CH_2CO, J = 6.3 Hz), 3.80 (s, 3H, OCH_3), 3.88 (s, 2H, CH_2-Ar), 4.54 (t, 2H, NCH_2CH_2COO, J = 6.3 Hz), 6.76-6.84 (m, 4H, Ar), 7.25 (t, 1H, Ar, J = 7.9 Hz), 7.40 (d, 2H, Ar, J = 8.8 Hz), 7.50 (d, 2H, Ar, J = 8.8 Hz), 9.29 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{22}H_{22}BrN_3O_3$, 456.33. Found: *m/z* 456.09 $[M]^+$.

5.2.254 (±)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-pyridazin-1(6*H*)-yl]propanamide (95b)

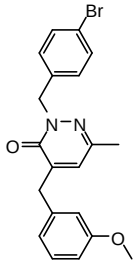


Yield = 53 %; oil; IR (cm^{-1}) 3300 (NH), 1709 (CO), 1643 (CO). ^{1}H NMR ($CDCl_3$) δ 1.71 (d, 3H, CH_3CHN, J = 7.1 Hz), 2.31 (s, 3H, 3-CH_3), 3.80 (s, 3H, OCH_3), 3.90 (s, 2H, CH_2-Ar), 5.71 (q, 1H, CH_3CHN, J = 7.0 Hz), 6.79-6.85 (m, 4H, Ar), 7.25-7.29 (m, 1H, Ar), 7.35-7.36 (m, 4H, Ar), 9.18 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{22}H_{22}BrN_3O_3$, 456.33. Found: *m/z* 456.09 $[M]^+$.

General Procedure for 96a,b. A mixture of **43** (1.13 mmol), K_2CO_3 (2.26 mmol), and appropriate alkyl halide (1.70 mmol) in CH_3CN (3 mL) was refluxed under stirring for 6 h. The mixture was then concentrated in vacuo, diluted with cold water and extracted with CH_2Cl_2 (3 x 15 mL). The

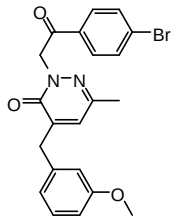
solvent was evaporated in vacuo and compounds **96a,b** were purified by column chromatography using cyclohexane/ethyl acetate 2:1 for **96a** and cyclohexane/ethyl acetate 1:1 for **96b** as eluents.

5.2.255 2-(4-Bromobenzyl)-4-(3-methoxybenzyl)-6-methyl-pyridazin-3(2*H*)-one (96a)



Yield = 68 %; mp = 112-114 °C (EtOH). IR (cm^{-1}) 1638 (CO). ^{1}H NMR ($CDCl_3$) δ 2.23 (s, 3H, 6-CH_3), 3.81 (s, 3H, OCH_3), 3.86 (s, 2H, *CH_2*-Ar), 5.25 (s, 2H, NCH_2Ar), 6.64 (s, 1H, Ar), 6.78-6.85 (m, 3H, Ar), 7.26-7.30 (m, 1H, Ar), 7.35 (d, 2H, Ar, *J* = 8.4 Hz), 7.45-7.47 (m, 2H, Ar). MS (ESI) calcd. For $C_{20}H_{19}BrN_2O_2$, 399.28. Found: *m/z* 399.07 $[M]^+$.

5.2.256 2-[2-(4-Bromophenyl)-2-oxo-ethyl]-4-(3-methoxybenzyl)-6-methyl-pyridazin-3(2*H*)-one (96b)

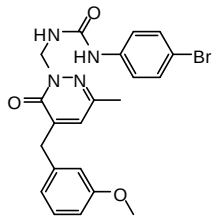


Yield = 95 %; oil. IR (cm^{-1}) 1715 (CO), 1644 (CO). ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H, 6-CH_3), 3.83 (s, 3H, OCH_3), 3.89 (s, 2H, CH_2-Ar), 5.52 (s, 2H, NCH_2CO), 6.72 (s, 1H, Ar), 6.80 (s, 1H, Ar), 6.84 (d, 2H, Ar, *J* = 7.9 Hz), 7.27-7.31 (m, 1H, Ar), 7.66 (d, 2H, Ar, *J* = 8.6 Hz), 7.88 (d, 2H, Ar, *J* = 8.5 Hz). MS (ESI) calcd. For $C_{21}H_{19}BrN_2O_3$, 427.29. Found: *m/z* 427.07 $[M]^+$.

General Procedure for 97a,b. A mixture of **43a** (0.32 mmol), 40% formaldehyde (3 mL) and 33% NH_3 (1.5 mL) in dioxane (1.5-2 mL) was heated at 50 °C for 1 h. The solvent was then evaporated in vacuo, and the residue was extracted with CH_2Cl_2 (3 x 15 mL). The organic layer was dried with Na_2SO_4 and evaporated to afford an oil. For compound **97a**, the residual oil was dissolved in 2 mL of anhydrous CH_2Cl_2 and 4-bromophenyl isocyanate (0.35 mmol) was added. The mixture was stirred at room temperature for 12 h, then the solid residue was filtered off and the solution was evaporated in vacuo to afford compound **97a**, which was purified by flash chromatography using cyclohexane/ethyl acetate 3:1 as eluent. For compound **97b**, the residual oil was dissolved in 3 mL of anhydrous CH_2Cl_2 and, after cooling (0 °C), 4-bromobenzoyl chloride (0.44 mmol) was added and the mixture was stirred at 0 °C for 6 h. Finally, the residue was washed with cold 0.5 N NaOH (3 x 10 mL) and with cold water (2 x 10 mL).

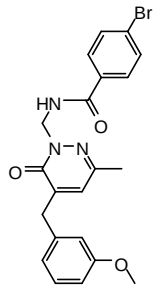
Evaporation of the organic layer afforded compound **97b**, which was purified by flash chromatography using cyclohexane/ethyl acetate 3:1 as eluent.

5.2.257 1-(4-Bromophenyl)-3-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-ylmethyl]-urea (97a)



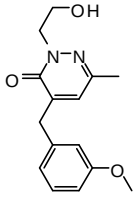
Yield = 27 %; mp = 112- 114 °C (EtOH). IR (cm^{-1}) 3270 (NH), 3265 (NH), 1708 (CO), 1630 (CO). ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H, 3-CH_3), 3.82 (s, 3H, OCH_3), 3.88 (s, 2H, *CH_2*-Ar), 6.13 (s, 2H,NCH_2N), 6.68 (s, 1H, Ar), 6.78-6.81 (m, 1H, Ar), 6.83-6.86 (m, 2H, Ar), 6.99 (exch br s, 1H, NH), 7.27-7.31 (m, 3H, Ar), 7.41 (m, 2H, Ar, *J* = 8.8 Hz). MS (ESI) calcd. For $C_{21}H_{21}BrN_4O_3$, 457.32. Found: *m/z* 458.07 $[M + H]^+$.

5.2.258 4-Bromo-*N*-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-ylmethyl]-benzamide (97b)



Yield = 30 %; oil. IR (cm^{-1}) 3290 (NH), 1708 (CO), 1640 (CO). ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H, 3-CH_3), 3.83 (s, 3H, OCH_3), 3.90 (s, 2H, *CH_2*-Ar), 6.29 (s, 2H, NCH_2N), 6.69 (s, 1H, Ar), 6.81-6.87 (m, 3H, Ar), 7.28-7.32 (m, 1H, Ar), 7.58 (d, 2H, Ar, J = 8.6 Hz), 7.94 (d, 2H, Ar, *J* = 8.6 Hz). MS (ESI) calcd. For $C_{21}H_{20}BrN_3O_3$, 442.31. Found: *m/z* 443.06 $[M + H]^+$.

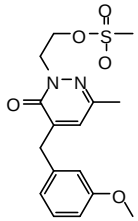
5.2.259 2-(2-Hydroxyethyl)-4-(3-methoxybenzyl)-6-methyl-pyridazin-3(2*H*)-one (98)



To a refluxed mixture of compound **44** (0.47 mmol) and $NaBH_4$ (2.64 mmol) in anhydrous THF (6 mL), CH_3OH (1.45 mL) was slowly added. After stirring for 1 h at 60 °C, the mixture was concentrated in vacuo, diluted with cold water (10-15 mL), and extracted with CH_2Cl_2 (3 x 15 mL). Evaporation of the

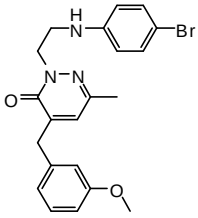
solvent afforded compound **98**. Yield = 95 %; oil. ^{1}H NMR ($CDCl_3$) δ 2.23 (s, 3H, 6-CH_3), 3.80 (s, 3H,OCH_3), 3.86 (s, 2H, *CH_2*-Ar), 4.00 (t, 2H, NCH_2*CH_2*OH, *J* = 5.1 Hz), 4.35 (t, 2H, N*CH_2*CH_2OH, *J* = 5.0 Hz), 4.66 (exch br s, 1H, OH), 6.68 (s, 1H, Ar), 6.78-6.84 (m, 3H, Ar), 7.25-7.29 (m, 1H, Ar).

5.2.260 Methanesulfonic acid 2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]ethyl ester (99)



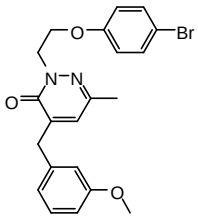
To a cooled (0 °C) and stirred solution of **98** (0.47 mmol) and pyridine (0.5 mmol) in anhydrous CH_2Cl_2 (2 mL), methanesulfonyl chloride (0.61 mmol) was added dropwise, and the mixture was stirred at room temperature for 4 h. Then ice cold water was added and the mixture was extracted with CH_2Cl_2 (3 x 15 mL): evaporation of the solvent afforded the desired compound. Yield = 85 %; oil. ^{1}H NMR ($CDCl_3$) δ 2.22 (s, 3H, 3-CH_3), 2.98 (s, 3H, CH_3SO_3), 3.80 (s, 3H, OCH_3), 3.86 (s, 2H, *CH_2*-Ar), 3.87-3.90 (m, 2H, NCH_2*CH_2*O), 4.45 (t, 2H, N*CH_2*CH_2O, *J* = 6.6 Hz), 6.66 (s, 1H, Ar), 6.78 (s, 1H, Ar), 6.82 (d, 2H, Ar, *J* = 8.1 Hz), 7.24-7.28 (m, 1H, Ar).

5.2.261 2-[2-(4-Bromophenylamino)-ethyl]-4-(3-methoxybenzyl)-6-methyl-pyridazin-3(2*H*)-one (100)



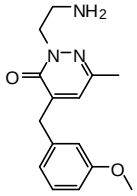
A solution of **99** (0.4 mmol) and 4-bromoaniline (0.8 mmol) in 2-propanol (2 mL) was heated under stirring for 6 h at 60 °C. Then, the mixture was concentrated in vacuo, cold water (30 mL) was added and the suspension was extracted with CH_2Cl_2 (3 x 15 mL). Evaporation of the solvent afforded the final compound **100**, which was purified by column chromatography using toluene/ethyl acetate 8:2 as eluent. Yield = 58 %; mp = 86-88 °C (EtOH). IR (cm^{-1}) 3350 (NH), 1643 (CO). ^{1}H NMR ($CDCl_3$) δ 2.24 (s, 3H, 6-CH_3), 3.58 (t, 2H, NCH_2*CH_2*NHAr, *J* = 5.7 Hz), 3.82 (s, 3H, OCH_3), 3.86 (s, 2H, *CH_2*-Ar), 4.46 (t, 2H, N*CH_2*CH_2NHAr, *J* = 5.8 Hz), 6.65-6.68 (m, 3H, Ar), 6.79-6.86 (m, 3H, Ar), 7.27-7.31 (m, 3H, Ar). MS (ESI) calcd. For $C_{21}H_{22}BrN_3O_2$, 428.32. Found: *m/z* 428.10 $[M]^+$.

5.2.262 2-[2-(4-Bromophenoxy)-ethyl]-4-(3-methoxybenzyl)-6-methyl-pyridazin-3(2*H*)-one (101)



To a suspension of **98** (0.55 mmol), copper acetate (0.82 mmol) and 4-bromophenylboronic acid (1.08 mmol) in CH_2Cl_2 (2 mL), Et_3N (1.08 mmol) was added and the mixture was stirred at room temperature for 12 h. The suspension was extracted with 15% aqueous ammonia (10 mL), then the organic layer was washed with water (10 mL) and dried over Na_2SO_4. After removal of the solvent, the residue was purified by flash column chromatography using toluene/ethyl acetate 8:2 as eluent. Yield = 26 %; mp = 82-83 °C (EtOH). IR (cm^{-1}) 1643 (CO). ^{1}H NMR ($CDCl_3$) δ 2.24 (s, 3H, 6-CH_3), 3.82 (s, 3H, OCH_3), 3.87 (s, 2H, CH_2-Ar), 4.36 (t, 2H, NCH_2CH_2O, J = 5.9 Hz), 4.54 (t, 2H, NCH_2CH_2O, J = 5.8 Hz), 6.66 (s, 1H, Ar), 6.79-6.86 (m, 5H, Ar), 7.27-7.35 (m, 1H, Ar), 7.35-7.37 (m, 2H, Ar). MS (ESI) calcd. For $C_{21}H_{21}BrN_2O_3$, 429.31. Found: *m/z* 430.08 $[M + H]^+$.

5.2.263 2-(2-Aminoethyl)-4-(3-methoxybenzyl)-6-methyl-pyridazin-3(2*H*)-one (102)

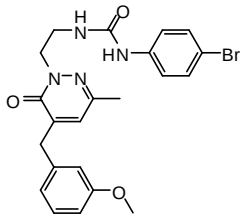


A mixture of **99** (0.43 mmol) and 33% NH_3 (3 mL) in isopropanol (2 mL) was stirred at 60 °C for 3 h. After concentration of the solvent and dilution with cold water (20 mL), the mixture was extracted with CH_2Cl_2 (3 x 15 mL). Evaporation of the solvent afforded desired compound **102**. Yield = 68 %; oil. ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H, 6-CH_3), 3.82 (s, 3H, OCH_3), 3.88 (s, 2H, CH_2-Ar), 4.02-4.04 (m, 2H, $NCH_2CH_2NH_2$), 4.37 (t, 2H, $NCH_2CH_2NH_2$, J = 4.9 Hz), 5.38 (exch br s, 2H, NH_2), 6.69 (s, 1H, Ar), 6.79-6.85 (m, 3H, Ar), 7.26-7.30 (m, 1H, Ar).

General Procedure for 103a,b. Compounds **103a,b** were obtained starting from the intermediate **102**. For compound **103a**, to a cooled (0 °C) and stirred solution of compound **102** (0.35 mmol) in anhydrous CH_2Cl_2 (2 mL), 4-bromophenyl isocyanate (0.40 mmol) was added. The mixture was stirred at 0 °C to rt 6 h. Removal of the solvent gave a residue that was purified by column chromatography using cyclohexane/ethyl acetate 1:1 as eluent. For compound **103b**, Et_3N (1.8 mmol) and 4-bromobenzoyl chloride (1.43 mmol) were added to a cooled (0 °C) and stirred solution of **102** (0.73 mmol) in anhydrous

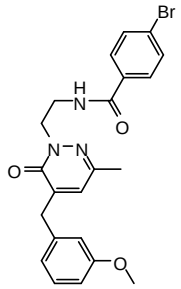
CH_2Cl_2 (2 mL) and the mixture was stirred at 0 °C for 6 h. The solid residue was filtered off and, in turn, the organic layer was washed with 6 N NaOH (3 x 10 mL) and with cold water (2 x 10 mL). The organic layer was dried with Na_2SO_4 and evaporated in vacuo to afford compound **103b**, which was purified by flash chromatography using CH_2Cl_2/CH_3OH 99:1 as eluent.

5.2.264 1-(4-Bromophenyl)-3-{2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-ethyl}-urea (103a)



Yield = 15 %; mp = 114-115 °C (EtOH). IR (cm^{-1}) 3270 (NH), 3265 (NH), 1705 (CO), 1630 (CO). ^{1}H NMR ($CDCl_3$) δ 2.23 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.87 (s, 2H, *CH*$_2$-Ar), 4.47 (t, 2H, NCH$_2$*CH*$_2$NH, *J* = 5.2 Hz), 4.57 (t, 2H, N*CH*$_2$CH$_2$NH, *J* = 5.1 Hz), 6.69 (s, 1H, Ar), 6.78-6.84 (m, 3H, Ar), 7.04 (exch br s, 1H, NH), 7.25-7.28 (m, 3H, Ar), 7.41 (d, 2H, Ar, *J* = 8.8 Hz). MS (ESI) calcd. For $C_{22}H_{23}BrN_4O_3$, 471.35. Found: *m/z* 472.08 $[M + H]^+$.

5.2.265 4-Bromo-*N*-{2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]-ethyl}-benzamide (103b)

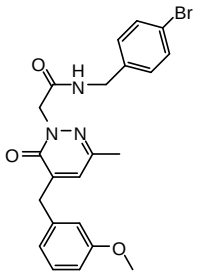


Yield = 13 %; oil. IR (cm^{-1}) 3300 (NH), 1707 (CO), 1643 (CO). ^{1}H NMR ($CDCl_3$) δ 2.18 (s, 3H, 3-CH_3), 3.82 (s, 3H, OCH_3), 3.87 (s, 2H, *CH*$_2$-Ar), 4.55 (t, 2H, NCH$_2$*CH*$_2$NH, *J* = 5.4 Hz), 4.70 (t, 2H, N*CH*$_2$CH$_2$NH, *J* = 5.4 Hz), 6.67 (s, 1H, Ar), 6.68-6.85 (m, 3H, Ar), 7.26 (d, 1H, Ar, *J* = 7.8 Hz), 7.56 (d, 2H, Ar, *J* = 8.5 Hz), 7.86 (d, 2H, Ar, *J* = 8.5 Hz). MS (ESI) calcd. For $C_{22}H_{22}BrN_3O_3$, 456.33. Found: *m/z* 457.08 $[M + H]^+$.

General Procedure for 104a-c. To a cooled (-5 °C) and stirred solution of compound **45** (0.35 mmol) in anhydrous tetrahydrofuran (3-5 mL), Et_3N (1.22 mmol) was added. After 30 min, the mixture was allowed to warm up to 0 °C, and ethyl chloroformate (0.38 mmol) was added. After 1 h, the 4-Bromobenzylamine, 4-Bromophenol or 4-Bromo-*N*-methylaniline (0.7 mmol) were respectively added. The reaction was carried out at room temperature for 12 h. The mixture was then concentrated in vacuo,

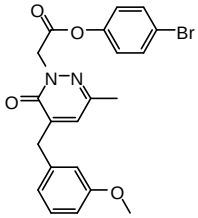
diluted with cold water (20-30 mL) and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated to afford final compounds **104a-c**, which were purified by column chromatography using cyclohexane/ethyl acetate 1:2 for compounds **104a** and cyclohexane/ethyl acetate 2:1 for **104b,c** as eluents.

5.2.266 *N*-(4-Bromobenzyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]acetamide (104a)



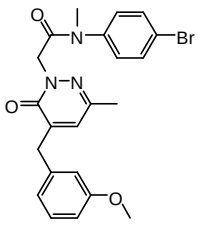
Yield = 97 %; mp = 184-185 °C (EtOH). IR (cm^{-1}) 3300 (NH), 1708 (CO), 1644 (CO). ^{1}H NMR (DMSO-d_6) δ 2.21 (s, 3H, 3-CH_3), 3.73 (s, 3H,OCH_3), 3.76 (s, 2H, *CH_2*NHCO), 4.27 (d, 2H, OCH_3-C_6H_4-*CH_2*), 4.68 (s, 2H, N*CH_2*CO), 6.80-6.86 (m, 3H, Ar), 7.05 (s, 1H, Ar), 7.23-7.26 (m, 3H, Ar), 7.51 (d, 2H, Ar, *J* = 8.3 Hz), 8.63 (exch br t, 1H, NH, *J* = 5.8 Hz). MS (ESI) calcd. For $C_{22}H_{22}BrN_3O_3$, 456.33. Found: *m/z* 456.09 $[M]^+$.

5.2.267 [5-(3-Methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]acetic acid-(4-bromo-phenyl)ester (104b)



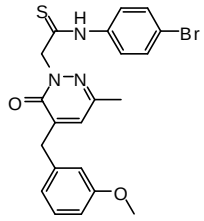
Yield = 97 %; mp = 111-112 °C (EtOH). IR (cm^{-1}) 3300 (NH), 1745 (CO), 1644 (CO). ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.90 (s, 2H, *CH_2*-Ar), 5.10 (s, 2H, NCH_2COO), 6.73 (s, 1H, Ar), 6.80 (s, 1H, Ar), 6.83-6.86 (m, 2H, Ar), 7.05 (d, 2H, Ar, *J* = 8.7 Hz), 7.26-7.30 (m, 1H, Ar), 7.50 (d, 2H, Ar, *J* = 8.7 Hz). MS (ESI) calcd. For $C_{21}H_{19}BrN_2O_4$, 443.29. Found: *m/z* 443.06 $[M]^+$.

5.2.268 *N*-(4-Methoxyphenyl)-*N*-methyl-2-[5-(3-Methoxybenzyl)-3-methyl-6-oxo-6*H*-pyridazin-1-yl]acetamide (104c)



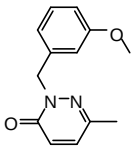
Yield = 45 %; mp = 125-127 °C (EtOH). IR (cm^{-1}) 1709 (CO), 1644 (CO). ^{1}H NMR ($CDCl_3$) δ 2.21 (s, 3H, 3-CH_3), 3.32 (s, 3H, CH_3N), 3.82 (s, 3H, OCH_3), 3.86 (s, 2H, *CH_2*-Ar), 4.64 (s, 2H, NCH_2CO), 6.62 (s, 1H, Ar), 6.77 (s, 1H, Ar), 6.80-6.85 (m, 2H, Ar), 7.26-7.30 (m, 3H, Ar), 7.60 (d, 2H, Ar, *J* = 8.4 Hz). MS (ESI) calcd. For $C_{22}H_{22}BrN_3O_3$, 456.33. Found: *m/z* 456.09 $[M]^+$.

5.2.269 *N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo-pyridazin-1(6*H*)-yl]ethanethioamide (105)



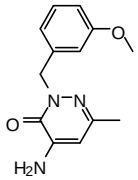
Lawesson's reagent (0.28 mmol) was slowly added to a stirred solution of compound **46a** (0.14 mmol) in toluene (3 mL) and the reaction was carried out at reflux for 3 h. The solvent was removed in vacuo and the mixture was diluted with ice-cold water and extracted with CH_2Cl_2 (3 x 10 mL). The crude product was finally purified by flash column chromatography using cyclohexane/ethyl acetate 1:1 as eluent, to yield **105** as an amorphous solid. Yield = 30 %; mp = 68-70 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.92 (s, 2H, *CH_2*-Ar), 5.37 (s, 2H, NCH_2CS), 6.81 (s, 1H, Ar), 6.83-6.87 (m, 3H, Ar), 7.28 (t, 1H, Ar, *J* = 5.0 Hz), 7.48 (d, 2H, Ar, *J* = 8.8 Hz), 7.74 (d, 2H, Ar, *J* = 8.8 Hz), 11.46 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{22}H_{22}BrN_3O_3$, 458.37. Found: *m/z* 458.17 $[M + H]^+$, 482.36 $[M + Na]^+$, 378.45 $[M - Br]^+$, 231.17 $[M - C_8H_7BrNS]^+$.

5.2.270 2-(3-Methoxybenzyl)-6-methylpyridazin-3(2*H*)-one (106)



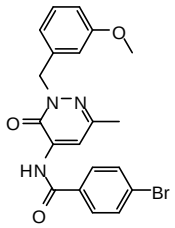
A mixture of **54b** (1.13 mmol), K_2CO_3 (2.26 mmol) and 3-methoxybenzyl chloride (1.70 mmol) in CH_3CN (3 mL) was refluxed under stirring for 6 h. The mixture was then concentrated in vacuo, diluted with cold water and extracted with CH_2Cl_2 (3 x 15 mL). The solvent was evaporated in vacuo and compound **106** was purified by flash chromatography using CH_2Cl_2/CH_3OH 9.9:0.1 as eluent. Yield = 86 %; mp = 53-55 °C (cyclohexane). ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H, 6-CH_3), 3.80 (s, 3H, OCH_3), 5.26 (s, 2H, NCH_2), 6.81-6.84 (m, 1H, Ar), 6.87 (d, 1H, Ar, *J* = 9.4 Hz), 6.97-7.05 (m, 2H, Ar), 7.07 (d, 1H, Ar, *J* = 9.4 Hz), 7.22-7.26 (m, 1H, Ar).

5.2.271 4-Amino-2-(3-methoxybenzyl)-6-methylpyridazin-3(2*H*)-one (107)



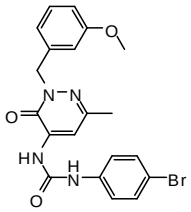
A suspension of **106** (0.78 mmol) and hydrazine hydrate (3.12 mmol) was stirred in a sealed tube at 180 °C for 12 h. After cooling, ice-cold water was added. The suspension was kept at 0 °C in ice-bath for 2 h and the precipitate was then filtered off to give a first batch of **107**. The solution was saturated with NH_4Cl and extracted with CH_2Cl_2 (3 x 25 mL). Removal of the solvent afforded a second batch of product. Yield = 89 %; mp = 96-98 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.23 (s, 3H,CH_3), 3.81 (s, 3H,OCH_3), 4.85 (exch br s, 2H, NH_2), 5.27 (s, 2H,CH_2), 6.16 (s, 1H, Ar), 6.81-6.85 (m, 1H, Ar), 6.97-7.02 (m, 2H, Ar), 7.23-7.28 (m, 1H, Ar).

5.2.272 4-Bromo-*N*-[2-(3-methoxybenzyl)-6-methyl-3-oxo-2,3-dihydro-pyridazin-4-yl]benzamide (108)



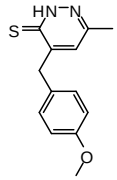
Et_3N (1.8 mmol) and 4-bromobenzoyl chloride (1.43 mmol) were added to a cooled (0 °C) and stirred solution of **107** (0.73 mmol) in anhydrous CH_2Cl_2 (2 mL), and the mixture was monitored under stirring at 0 °C for 10 h. The solid residue was removed by filtration. The organic layer was washed with 6 N NaOH (3 x 10 mL) and with cold water (2 x 10 mL). Drying with Na_2SO_4 and evaporation of the solvent in vacuo afforded compound **108**, which was purified by flash chromatography using cyclohexane/ethyl acetate 3:1 as eluent. Yield = 28 %; mp = 162-164 °C (EtOH). IR (cm^{-1}) 3300 (NH), 1709 (CO), 1644 (CO). ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3H, CH_3), 3.82 (s, 3H, OCH_3), 5.32 (s, 2H,CH_2), 6.84-6.87 (m, 1H, Ar), 6.98-7.02 (m, 2H, Ar), 7.26-7.30 (m, 1H, Ar), 7.66 (d, 1H, Ar, J = 8.7 Hz), 7.80 (d, 2H, Ar, J = 8.6 Hz), 8.14 (s, 1H, Ar), 9.37 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{20}H_{18}BrN_3O_3$, 428.28. Found: *m/z* 428.06 $[M]^+$.

5.2.273 1-(4-Bromophenyl)-3-[2-(3-methoxybenzyl)-6-methyl-3-oxo-2,3-dihydro pyridazin-4-yl]urea (109)



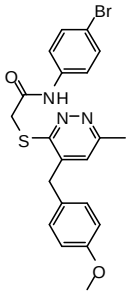
To a stirred solution of compound **107** (0.35 mmol) in anhydrous toluene (2 mL), 4-bromophenyl isocyanate (0.40 mmol) was added. The mixture was refluxed for 7 h and after cooling, the solvent was removed under reduced pressure. The mixture was diluted with ice-cold water and extracted with CH_2Cl_2 (3 x 15 mL). Evaporation of the solvent gave a crude product that was purified by column chromatography using firstly CH_2Cl_2, to remove the 4-bromophenyl urea, and then cyclohexane/ethyl acetate 2:1 as eluent. Yield = 56 %; mp = 207-209 °C (EtOH). IR (cm^{-1}) 3270 (NH), 3265 (NH), 1705 (CO), 1630 (CO). ^{1}H NMR ($CDCl_3$) δ 2.12 (s, 3H,CH_3), 3.57 (s, 3H, OCH_3), 5.08 (s, 2H, CH_2), 6.51-6.58 (m, 3H, Ar), 6.97- 7.04 (m, 3H, Ar), 7.23 (d, 2H, Ar, *J* = 8.7 Hz), 7.86 (s, 1H, Ar), 8.70 (exch br s, 1H, NH), 8,94 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{20}H_{19}BrN_4O_3$, 443.29. Found: *m/z* 443.07 $[M]^+$.

5.2.274 4-(4-methoxybenzyl)-6-methylpyridazine-3(2*H*)-thione (110)



Lawesson's reagent (0.87 mmol) was slowly added to a stirred solution of compound **58e** (0.87 mmol) in toluene (3 mL) and the reaction was carried out at reflux for 2 h. The mixture was cooled and after 1 h stirring in ice-bath the precipitate was filtered off and purified by recrystallization from ethanol. Yield = 47 %; mp = 191-93 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.30 (s, 3H, 3-CH_3), 3.85 (s, 3H, OCH_3), 4.11 (s, 2H, *CH_2*-Ar), 6.57 (s, 1H, Ar), 6.93 (d, 2H, Ar, *J* = 8.5 Hz), 7.17 (d, 2H, Ar, *J* = 8.5 Hz), 12.12 (exch br s, 1H, SH).

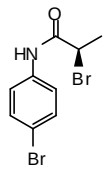
5.2.275 *N*-(4-Bromophenyl)-2-[4-(4-methoxybenzyl)-6-methylpyridazin-3-ylthio] acetamide (111)



A mixture of **110** (0.41 mmol), K_2CO_3 (0.82 mmol), and *N*-(4-bromophenyl)-2-chloro acetamide **22** (0.61 mmol) in CH_3CN (4 mL) was refluxed under stirring for 1.5 h. After cooling, the solvent was evaporated and the mixture was diluted with cold water. The precipitate was filtered off and purified by flash chromatography using cyclohexane/ethyl acetate 1:2 as eluent. Yield = 96 %; mp = 116-118 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.62 (s, 3H, 6-CH_3), 3.84 (s, 3H, OCH_3), 3.87 (s, 2H, CH_2-Ar), 4.08 (s, 2H, SCH_2CO), 6.84 (s, 1H, Ar), 6.92 (d, 2H, Ar, *J* = 8.5 Hz), 7.11 (d, 2H, Ar, *J* = 8.5 Hz), 7.38 (d, 2H, Ar, *J* = 8.8 Hz), 7.48 (d, 2H, Ar, *J* = 8.8 Hz), 10.26 (exch br s, 1H, NH).

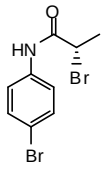
General Procedure for R-(+)-112 and **S-(-)-112.** To a stirred solution of the suitable bromopropionic acid [R-(+)- or S-(-)-] (1.31 mmol), in anhydrous CH_2Cl_2 (5 mL), $SOCl_2$ (7.86 mmol) was added dropwise and the reaction was carried out at 40 °C for 4 h. The mixture was allowed to cool down and then the solvent and the excess of $SOCl_2$ were removed in vacuo. The residue was dissolved in anhydrous THF (5 mL) and 4-bromoaniline (2.62 mmol) was added. The reaction was stirred for additional 4 h at room temperature. After removal of the solvent under reduced pressure, the mixture was dissolved in CH_2Cl_2 and washed, in turn, with 2 N HCl (3 x 15 mL), 2 N NaOH (3 x 15 mL) and with H_2O (15 mL). The organic layer was dried over Na_2SO_4 and evaporated under vacuo to give the crude products which were purified by flash column chromatography using toluene/ethyl acetate 6:1 as eluents.

5.2.276 R-(+)-2-Bromo-*N*-(4-bromophenyl)propanamide [R-(+)-112]



Yield = 12 %; mp = 150-51 °C (EtOH). $[\alpha]^{20}_D$ = + 25° (*c* = 1, $CHCl_3$). e.e. > 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 1.98 (d, 3H, CH*CH*$_3$, *J* = 7.0 Hz), 4.56 (q, 1H, *CH*CH_3, *J* = 7.0 Hz), 7.45-7.50 (dd, 4H, Ar, *J* = 6.3 Hz, *J* = 2.9 Hz), 8.09 (exch br s, 1H, NH).

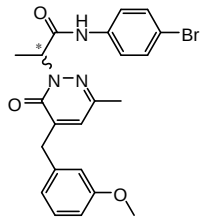
5.2.277 S-(-)-2-Bromo-*N*-(4-bromophenyl)propanamide [S-(-)-112]



Yield = 15 %; mp = 150-51 °C (EtOH). $[\alpha]^{20}_D$ = - 25° (*c* = 1, $CHCl_3$). e.e. > 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 1.99 (d, 3H, CH*CH*$_3$, *J* = 7.0 Hz), 4.56 (q, 1H, *CH*CH_3, *J* = 7.0 Hz), 7.46-7.50 (dd, 4H, Ar, *J* = 3.7 Hz, *J* = 2.6 Hz), 8.07 (exch br s, 1H, NH).

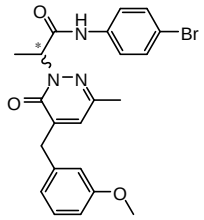
General procedure for (+)-113 [rac 70:30, from S-(-)-112] and (-)-113 [rac 60:40, from R-(+)-112]. A mixture of intermediate **43** (0.17 mmol), K_2CO_3 (0.34 mmol) and **R-(+)-112** or **S-(-)-112** (0.19 mmol) respectively, in CH_3CN (3 mL) were refluxed under stirring for 2 h. After cooling, the solvent was evaporated and the mixture was diluted with ice-cold water and extracted using CH_2Cl_2 (3 x 10 mL). The organic layer was collected, dried over Na_2SO_4 and evaporated in vacuo. The crude residues were purified by flash column chromatography using cyclohexane/ethyl acetate 2:1 as eluent.

5.2.278 (+)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]propanamide [(+)-113, rac 70:30]



Yield = 90 %; mp = 63-64 °C (*n*-hexane). $[\alpha]^{20}_D$ = + 35° (*c* = 1, $CHCl_3$). e.e. = 70 % (of the (+)-enantiomer, determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 1.71 (d, 3H, CH*CH*$_3$, *J* = 7.1 Hz), 2.30 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.91 (s, 2H, *CH*$_2$-Ar), 5.71 (q, 1H, *CH*CH_3, *J* = 7.1 Hz), 6.79 (d, 2H, Ar, *J* = 7.6 Hz), 6.84 (d, 2H, Ar, *J* = 8.3 Hz), 7.28 (t, 1H, Ar, *J* = 9.1 Hz), 7.38 (d, 4H, Ar, *J* = 6.0 Hz), 9.09 (exch br s, 1H, NH).

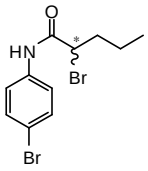
5.2.279 (-)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]propanamide [(-)-113, rac 60:40]



Yield = 93 %; mp = 63-64 °C (*n*-hexane). $[\alpha]^{20}_D$ = - 30° (*c* = 1, $CHCl_3$). e.e. = 60 % (of the n (-)-enantiomer, determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 1.71 (d, 3H, CH*CH*$_3$, *J* = 7.1 Hz), 2.31 (s, 3H, 3-CH_3), 3.80 (s, 3H, OCH_3), 3.90 (s, 2H, *CH*$_2$-Ar), 5.71 (q, 1H, *CH*CH_3, *J* = 7.1 Hz), 6.79 (q, 2H, Ar, *J* = 1.0 Hz), 6.84 (dd, 2H, Ar, *J* = 6.0 Hz, *J* = 1.0 Hz), 7.27 (t, 1H, Ar, *J* = 8.2 Hz), 7.36 (d, 4H, Ar, *J* = 6.0 Hz), 9.16 (exch br s, 1H, NH).

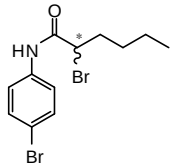
General Procedure for racemates (±)-115a-f and 116. Compounds **(±)-115a-f** and **116** were synthesized using the suitable carboxylic acid and following the same procedure exploited for **R-(+)-** and **S-(-)-112**.

5.2.280 (±)-2-Bromo-N-(4-bromophenyl)pentanamide [(±)-115b]



Yield = 10 %; mp = 94-96 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 1.00 (t, 3H, CH_2CH_3, J = 7.4 Hz), 1.50-1.65 (m, 2H, $CH_2CH_2CH_2CH3$), 2.05-2.16 (m, 1H, COCHCH-HCH$_2$), 2.20-2.25 (m, 1H, COCHCH-HCH$_2$), 4.47 (dd, 1H, COCHCH$_2$, J = 3.1 Hz, J = 5.2 Hz), 7.43-7.50 (m, 4H, Ar), 8.10 (exch br s, 1H, NH).

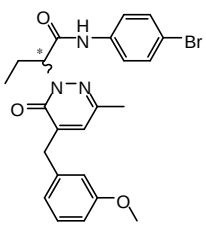
5.2.281 (±)-2-Bromo-N-(4-bromophenyl)hexanamide [(±)-115d]



Yield = 10 %; mp = 114-15 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 0.95 (t, 3H, CH_2CH_3, J = 7.2 Hz), 1.37-1.44 (m, 2H, $CH_2CH_2CH_2CH3$), 1.48-1.57 (m, 2H, $CH_2CH_2CH_2CH3$), 2.06-2.14 (m, 1H, COCHCH-HCH$_2$), 2.20-2.29 (m, 1H, COCHCH-HCH$_2$), 4.45 (dd, 1H, COCHCH$_2$, J = 7.9 Hz, J = 5.2 Hz), 7.44-7.50 (m, 4H, Ar), 8.07 (exch br s, 1H, NH).

General Procedure for racemates (±)-117a-f and 118. A mixture of compound **43** (0.13 mmol), K_2CO_3 (0.26 mmol) and the suitable intermediate type **115** [**(±)-115a-f**] or **116** (0.14 mmol), in CH_3CN (3 mL) was refluxed under stirring for 4-5 h. After cooling, the solvent was evaporated and ice-cold water was added to the mixture. For racemate **(±)-117a**, after 1 h stirring in ice-bath the precipitate was filtered off and purified by crystallization in cyclohexane. For racemates **(±)-117b-f** and compound **118**, differently, the mixture was extracted using CH_2Cl_2 (3 x 10 mL). The organic layer was collected, dried over Na_2SO_4 and evaporated in vacuo. The crude residue was purified by flash column chromatography using as eluent cyclohexane/ethyl acetate 2:1 for racemates **(±)-117b-d**, cyclohexane/ ethyl acetate 1:3 for racemate **(±)-117e** and cyclohexane/ethyl acetate 1:1 for compounds **(±)-117f** and **118**.

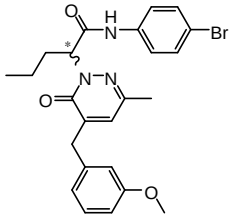
5.2.282 (±)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]butanamide [(±)-117a]



Yield = 98 %; mp = 79-81 °C (cyclohexane). ^{1}H NMR ($CDCl_3$) δ 0.95 (t, 3H, CHCH_3, J = 7.4 Hz), 2.19-2.38 (m, 5H, 3-CH_3 + CH_2CH_3), 3.81 (s, 3H, OCH_3), 3.91 (s, 2H, CH_2-Ar), 5.46 (dd, 1H, NCHCH$_2$, J =

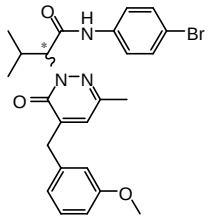
1.4 Hz, *J* = 6.9 Hz), 6.77-6.85 (m, 4H, Ar), 7.27 (t, 1H, Ar, *J* = 7.9 Hz), 7.30-7.44 (m, 4H, Ar), 9.13 (exch br s, 1H, NH).

5.2.283 (±)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]pentanamide [(±)-117b]



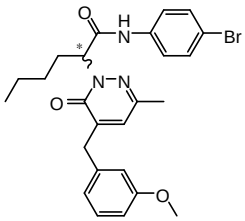
Yield = 94 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.98 (t, 3H, CHCH_3, *J* = 7.6 Hz), 1.26-1.40 (m, 2H, $CH_2CH_2CH_3$), 2.17-2.28 (m, 2H, $CHCH_2CH_2$), 2.30 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.90 (s, 2H, CH_2-Ar), 5.56 (t, 1H, N$CH$$CH_2$, *J* = 8.2 Hz), 6.75 (s, 1H, Ar), 6.79 (s, 1H, Ar), 6.82-6.86 (m, 2H, Ar), 7.27 (t, 1H, Ar, *J* = 7.8 Hz), 7.38 (s, 4H, Ar), 9.03 (exch br s, 1H, NH).

5.2.284 (±)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]-3-methylbutanamide [(±)-117c]



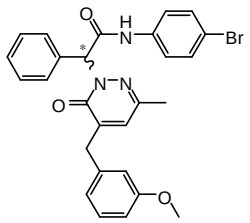
Yield = 80 %; mp = 161-62 °C (*n*-hexane). ^{1}H NMR ($CDCl_3$) δ 0.83 (d, 3H, CHCH_3, *J* = 6.6 Hz), 1.16 (d, 3H, CHCH_3, *J* = 6.6 Hz), 2.30 (s, 3H, 3-CH_3), 2.85-2.98 (m, 1H, $CH$$CH_3$), 3.81 (s, 3H, OCH_3), 3.90 (s, 2H, CH_2-Ar), 5.14 (d, 1H, NCHCHCH, *J* = 11.0 Hz), 6.72 (s, 1H, Ar), 6.78-6.85 (m, 3H, Ar), 7.28 (t, 1H, Ar, *J* = 7.8 Hz), 7.39-7.44 (m, 4H, Ar), 9.06 (exch br s, 1H, NH).

5.2.285 (±)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]hexanamide [(±)-117d]



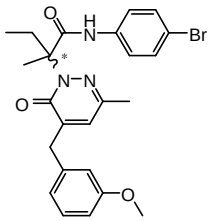
Yield = 93 %; colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.92 (t, 3H, CHCH_3, *J* = 7.3 Hz), 1.24-1.43 (m, 4H, $CH_2CH_2CH_2CH_3$), 2.22-2.30 (m, 5H, 3-CH_3 + $CHCH_2CH_2CH_2CH_3$), 3.81 (s, 3H, OCH_3), 3.91 (s, 2H, CH_2-Ar), 5.53 (dd, 1H, N$CH$$CH_2$, *J* = 1.3 Hz, *J* = 6.9 Hz), 6.74 (s, 1H, Ar), 6.80-6.86 (m, 3H, Ar), 7.28 (t, 1H, Ar, *J* = 8.5 Hz), 7.35-7.45 (m, 4H, Ar), 8.98 (exch br s, 1H, NH).

5.2.286 (±)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]-2-phenylacetamide [(±)-117e]



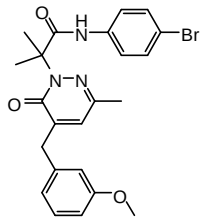
Yield = 42 %; mp = 117-19 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.89 (s, 2H, *CH_2*-Ar), 6.67 (s, 1H, Ar), 6.87-6.87 (m, 4H, NCHCO + 3 x Ar), 7.28 (t, 1H, Ar, *J* = 7.6 Hz), 7.36-7.45 (m, 7H, Ar), 7.56-7.59 (m, 2H, Ar), 8.33 (exch br s, 1H, NH).

5.2.287 (±)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]-2-methylbutanamide [(±)-117f]



Yield = 60 %; mp = 141-42 °C (*n*-hexane). ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 3H, CH_2CH_3, *J* = 7.3 Hz), 1.83 (s, 3H, $COCCH_3$), 2.25 (sext, 1H, NCCH-*H*CH_3, *J* = 6.9 Hz), 2.38 (sext, 1H, NCCH-*H*CH_3, *J* = 7.4 Hz), 2.59 (s, 3H, 3-CH_3), 3.78 (s, 3H, OCH_3), 3.97 (qd, 2H, *CH_2*-Ar, *J* = 3.3 Hz, *J* = 16.2 Hz), 6.73 (s, 1H, Ar), 6.77 (d, 1H, Ar, *J* = 7.6 Hz), 6.85 (dd, 1H, Ar, *J* = 6.0 Hz, *J* = 1.9 Hz), 7.09 (s, 1H, Ar), 7.23 (d, 2H, Ar, *J* = 8.9 Hz), 7.29 (t, 1H, Ar, *J* = 8.0 Hz), 7.37 (d, 2H, Ar, *J* = 8.8 Hz), 8.15 (exch br s, 1H, NH).

5.2.288 *N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]-2-methylpropanamide [(±)-118]

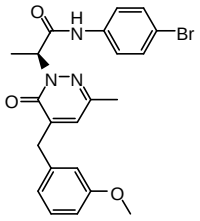


Yield = 65 %; mp = 58-60 °C (*n*-hexane). ^{1}H NMR ($CDCl_3$) δ 1.83 (s, 6H, 2 x $NCCH_3$), 2.61 (s, 3H, 3-CH_3), 3.80 (s, 3H, OCH_3), 3.97 (s, 2H, *CH_2*-Ar, *J* = 3.3 Hz), 6.76-6.88 (m, 3H, Ar), 7.11 (s, 1H, Ar), 7.27-7.32 (m, 3H, Ar), 7.37 (dd, 2H, Ar, *J* = 4.7 Hz, *J* = 2.0 Hz), 8.26 (exch br s, 1H, NH).

HPLC resolution of (±)-95b and (±)-117a by Chiral Phase HPLC. Both racemates were separated by chiral-phase HPLC with a Chiralcel OD® (250mm x 4.6mm I.D., 10 μm particle size) column. The eluent mixture *n*-hexane/IPA 95:5 was used in isocratic mode with the flow 1.2 mL/min at

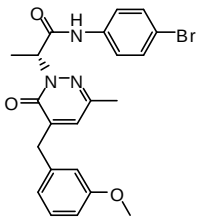
25 °C and the UV signal was followed at λ = 250 nm. Racemates were dissolved in ethanol (1 mg/mL solution) and 50 µL were injected each time. In order to separate about 20 mg of racemic mixtures 600 injections were needed and it took about 200 h to obtain the resolved enantiomeric pairs **S-(+)95b/R-(-)-95b** (t_R = 19.2, t_R = 23.2) and **S-(+)-117a/R-(-)-117a** (t_R = 15.9, t_R = 17.9) (see **section 3.3.6.4** for the assignment of the absolute configuration).

5.2.289 S-(+)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]propanamide [S-(+)-95b]



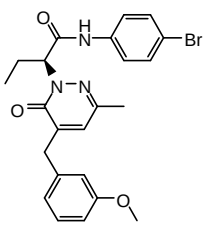
Light-yellow oil. $[\alpha]^{20}_D$ = + 80° (*c* = 1, $CHCl_3$). e.e. > 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 1.71 (d, 3H, CH*CH*$_3$, *J* = 7.0 Hz), 2.29 (s, 3H, 3-CH_3), 3.82 (s, 3H, OCH_3), 3.91 (s, 2H, *CH*$_2$-Ar), 5.71 (q, 1H, *CH*CH_3, *J* = 7.0 Hz), 6.78 (s, 1H, Ar), 6.81(s, 1H, Ar), 6.84 (d, 2H, Ar, *J* = 7.4 Hz), 7.28 (t, 1H, Ar, *J* = 9.0 Hz), 7.40 (d, 4H, Ar, *J* = 4.9 Hz), 8.99 (exch br s, 1H, NH).

5.2.290 R-(-)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]propanamide [R-(-)-95b]



Light-yellow oil. $[\alpha]^{20}_D$ = - 78° (*c* = 1, $CHCl_3$). e.e. = 97.4 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 1.71 (d, 3H, CH*CH*$_3$, *J* = 7.0 Hz), 2.30 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.91 (s, 2H, *CH*$_2$-Ar), 5.71 (q, 1H, *CH*CH_3, *J* = 7.0 Hz), 6.78 (s, 1H, Ar), 6.81(s, 1H, Ar), 6.84 (d, 2H, Ar, *J* = 8.3 Hz), 7.28 (t, 1H, Ar, *J* = 8.8 Hz), 7.40 (d, 4H, Ar, *J* = 3.0 Hz), 9.01 (exch br s, 1H, NH).

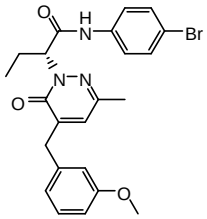
5.2.291 S-(+)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]butanamide [S-(+)-117a]



Light-yellow oil. $[\alpha]^{20}_D$ + 129° (*c* = 1, $CHCl_3$). e.e. > 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 3H, CH*CH*$_3$, *J* = 7.4 Hz), 2.18-2.39 (m, 5H, 3-CH_3 + *CH*$_2$$CH_3$), 3.81 (s, 3H,

OCH_3), 3.90 (s, 2H, CH_2-Ar), 5.47 (dd, 1H, N*CH*CH_2, *J* = 1.5 Hz, *J* = 6.9 Hz), 6.75 (s, 1H, Ar), 6.79 (d, 1H, Ar, *J* = 1.9 Hz), 6.82-6.86 (m, 2H, Ar), 7.28 (t, 1H, Ar, *J* = 7.9 Hz), 7.35-7.41 (m, 4H, Ar), 9.08 (exch br s, 1H, NH).

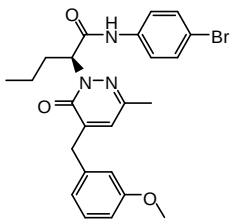
5.2.292 R-(-)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]butanamide [R-(-)-117a]



Light-yellow oil. $[\alpha]^{20}_D$ - 129° (*c* = 1, $CHCl_3$). e.e. = 98.0 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 3H, CH*CH*$_3$, *J* = 7.4 Hz), 2.18-2.39 (m, 5H, 3-CH_3 + *CH*$_2$$CH_3$), 3.81 (s, 3H, OCH_3), 3.91 (s, 2H, *CH*$_2$-Ar), 5.47 (dd, 1H, N*CH*CH_2, *J* = 1.2 Hz, *J* = 7.0 Hz), 6.75 (s, 1H, Ar), 6.79 (s, 1H, Ar), 6.82-6.86 (m, 2H, Ar), 7.28 (t, 1H, Ar, *J* = 8.1 Hz), 7.36-7.41 (m, 4H, Ar), 9.08 (exch br s, 1H, NH).

HPLC resolution of (±)-117b-f by Chiral Phase HPLC. Racemates were separated by chiral-phase HPLC with a Lux Amylose-2® (250 mm x 4.6 mm I.D., 5 μm particle size) column. The eluent mixture *n*-hexane/IPA 60:40 was used in isocratic mode with the flow 1.5 mL/min at 40 °C and the UV signal was followed at λ = 250 nm. Racemates were dissolved in ethanol (2 mg/mL solution), then 100 μL of **(±)-117b-e** and 80 μL of **(±)-117f** were injected each time. In order to separate about 20 mg of racemic mixtures 50 injections were needed and it took about 10 h to obtain the resolved enantiomeric pairs **S-(+)117b/R-(-)-117b** (t_R = 5.1, t_R = 9.4), **S-(+)-117c/R-(-)-117c** (t_R = 3.9, t_R = 10.1), **S-(+)-117d/R-(-)-117d** (t_R = 5.4, t_R = 9.7), **R-(-)-117e/S-(+)-117e** (t_R = 7.6, t_R = 12.3) and **R-(-)-117f/S-(+)-117f** (t_R = 6.7, t_R = 8.6) (see **section 3.3.6.4** for the assignment of the absolute configuration).

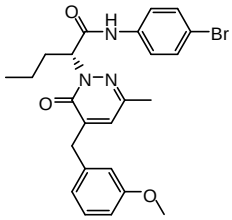
5.2.293 S-(+)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]pentanamide [S-(+)-117b]



Light-yellow oil. $[\alpha]^{20}_D$ + 139° (*c* = 1, $CHCl_3$). e.e. > 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 0.98 (t, 3H, CH*CH*$_3$, *J* = 7.3 Hz), 1.27-1.42 (m, 2H, CH_2*CH*$_2$$CH_3$), 2.15-2.28 (m, 2H, CH*CH*$_2$$CH_2$), 2.30 (s, 3H, 3-$CH_3$), 3.81 (s, 3H, OCH_3), 3.90 (s, 2H, *CH*$_2$-Ar), 5.56 (t, 1H, N*CH*CH_2, *J* =

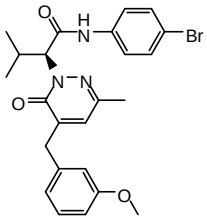
8.2 Hz), 6.75 (s, 1H, Ar), 6.79 (d, 1H, Ar, *J* = 1.9 Hz), 6.82-6.86 (m, 2H, Ar), 7.28 (t, 1H, Ar, *J* = 9.0 Hz), 7.39 (s, 4H, Ar), 9.02 (exch br s, 1H, NH).

5.2.294 R-(-)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]pentanamide [R-(-)-117b]



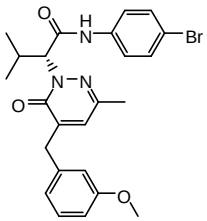
Light-yellow oil. $[\alpha]^{20}_{D}$ - 137° (*c* = 1, $CHCl_3$). e.e. = 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 0.98 (t, 3H, CH*CH*$_3$, *J* = 7.4 Hz), 1.28-1.40 (m, 2H, CH_2*CH*$_2$$CH_3$), 2.17-2.28 (m, 2H, CH*CH*$_2$$CH_2$), 2.30 (s, 3H, 6-$CH_3$), 3.81 (s, 3H, OCH_3), 3.90 (s, 2H, *CH*$_2$-Ar), 5.56 (t, 1H, N*CH*CH_2, *J* = 7.2 Hz), 6.75 (s, 1H, Ar), 6.79 (d, 1H, Ar, *J* = 1.5 Hz), 6.82-6.86 (m, 2H, Ar), 7.28 (t, 1H, Ar, *J* = 9.0 Hz), 7.39 (s, 4H, Ar), 9.14 (exch br s, 1H, NH).

5.2.295 S-(+)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]-3-methylbutanamide [S-(+)-117c]



mp 63-65 °C. $[\alpha]^{20}_{D}$ + 99° (*c* = 1, $CHCl_3$). e.e. > 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 0.83 (d, 3H, CH*CH*$_3$, *J* = 6.6 Hz), 1.16 (d, 3H, CH*CH*$_3$, *J* = 6.6 Hz), 2.31 (s, 3H, 3-CH_3), 2.86-2.96 (m, 1H, *CH*CH_3), 3.81 (s, 3H, OCH_3), 3.90 (s, 2H, *CH*$_2$-Ar), 5.14 (d, 1H, N*CH*CHCH, *J* = 11.0 Hz), 6.71 (s, 1H, Ar), 6.78 (s, 1H, Ar), 6.81-6.86 (m, 2H, Ar), 7.28 (t, 1H, Ar, *J* = 7.5 Hz), 7.38-7.44 (m, 4H, Ar), 9.06 (exch br s, 1H, NH).

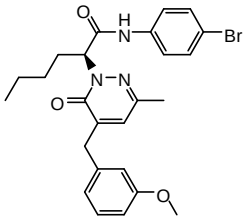
5.2.296 R-(-)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]-3-methylbutanamide [R-(-)-117c]



mp 63-65 °C. $[\alpha]^{20}_{D}$ - 97° (*c* = 1, $CHCl_3$). e.e. = 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 0.83 (d, 3H, CH*CH*$_3$, *J* = 6.6 Hz), 1.16 (d, 3H, CH*CH*$_3$, *J* = 6.6 Hz), 2.30 (s, 3H, 3-CH_3), 2.86-2.96 (m, 1H, *CH*CH_3), 3.81 (s, 3H, OCH_3), 3.90 (s, 2H, *CH*$_2$-Ar), 5.14 (d, 1H, N*CH*CHCH, *J* = 11.0 Hz),

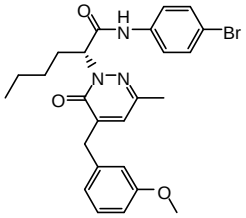
6.71 (s, 1H, Ar), 6.78 (s, 1H, Ar), 6.81-6.86 (m, 3H, Ar), 7.28 (t, 1H, Ar, *J* = 7.8 Hz), 7.38-7.44 (m, 4H, Ar), 9.06 (exch br s, 1H, NH).

5.2.297 S-(+)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]hexanamide [S-(+)-117d]



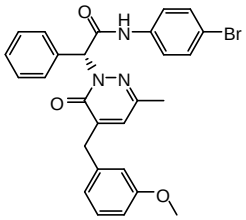
Light-yellow oil. $[\alpha]^{20}_{D}$ + 111° (*c* = 1, $CHCl_3$). e.e. > 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 0.92 (t, 3H, CH*C*H_3, *J* = 7.3 Hz), 1.17-1.46 (m, 4H, CH_2*C*H_2*C*H_2CH_3), 2.14-2.34 (m, 5H, 6-CH_3 + CH*C*$H_2CH_2CH_2CH_3$), 3.81 (s, 3H, OCH_3), 3.91 (s, 2H, *C*H_2-Ar), 5.54 (dd, 1H, N*C*HCH_2, *J* = 1.3 Hz, *J* = 6.9 Hz), 6.75 (d, 1H, Ar, *J* = 1.1 Hz), 6.79 (t, 1H, Ar, *J* = 1.7 Hz), 6.82-6.86 (m, 2H, Ar), 7.28 (t, 1H, Ar, *J* = 9.4 Hz), 7.38 (s, 4H, Ar), 9.04 (exch br s, 1H, NH).

5.2.298 R-(-)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxopyridazin-1(6*H*)-yl]hexanamide [R-(-)-117d]



Light-yellow oil. $[\alpha]^{20}_{D}$ - 110° (*c* = 1, $CHCl_3$). e.e. = 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 0.92 (t, 3H, CH*C*H_3, *J* = 7.3 Hz), 1.17-1.46 (m, 4H, CH_2*C*H_2*C*H_2CH_3), 2.16-2.34 (m, 5H, 6-CH_3 + CH*C*$H_2CH_2CH_2CH_3$), 3.81 (s, 3H, OCH_3), 3.90 (s, 2H, *C*H_2-Ar), 5.54 (dd, 1H, N*C*HCH_2, *J* = 1.2 Hz, *J* = 6.9 Hz), 6.75 (s, 1H, Ar), 6.79 (d, 1H, Ar, *J* = 2.0 Hz), 6.82-6.86 (m, 2H, Ar), 7.28 (t, 1H, Ar, *J* = 9.3 Hz), 7.36-7.41 (s, 4H, Ar), 9.04 (exch br s, 1H, NH).

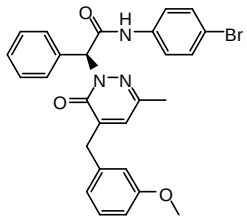
5.2.299 R-(-)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo pyridazin-1(6*H*)-yl]-2-phenylacetamide [R-(-)-117e]



Light-yellow oil. $[\alpha]^{20}_{D}$ - 17° (*c* = 1, $CHCl_3$). e.e. = 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H, 3-CH_3), 3.81 (s, 3H, OCH_3), 3.89 (s, 2H, *C*H_2-Ar), 6.68 (s, 1H, Ar), 6.77 (s, 1H, Ar), 6.80-6.85 (m, 2H, Ar), 6.88 (s, 1H, NCHCO), 7.28 (t, 1H, Ar, *J* = 7.6 Hz), 7.36-7.45 (m, 7H,

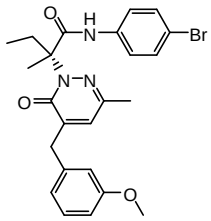
Ar), 7.56-7.59 (m, 2H, Ar), 8.33 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{27}H_{24}BrN_3O_3$, 518.40. Found: *m/z* 519.10 $[M + H]^+$, 541.30 $[M + Na]^+$.

5.2.300 S-(-)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo pyridazin-1(6*H*)-yl]-2-phenylacetamide [S-(+)-117e]



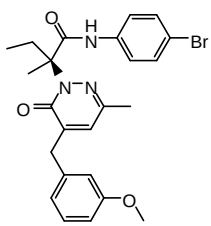
Light-yellow oil. $[\alpha]^{20}_D$ + 16° (*c* = 1, $CHCl_3$). e.e. > 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H, 3-CH_3), 3.80 (s, 3H, OCH_3), 3.89 (s, 2H, *CH_2*-Ar), 6.68 (s, 1H, Ar), 6.77 (s, 1H, Ar), 6.80-6.85 (m, 2H, Ar), 6.88 (s, 1H, NCHCO), 7.28 (t, 1H, Ar, *J* = 7.6 Hz), 7.36-7.45 (m, 7H, Ar), 7.56-7.59 (m, 2H, Ar), 8.33 (exch br s, 1H, NH). MS (ESI) calcd. For $C_{27}H_{24}BrN_3O_3$, 518.40. Found: *m/z* 519.10 $[M + H]^+$, 541.30 $[M + Na]^+$.

5.2.301 R-(-)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo pyridazin-1(6*H*)-yl]-2-methylbutanamide [R-(-)-117f]



mp 108-09 °C. $[\alpha]^{20}_D$ - 18° (*c* = 1, $CHCl_3$). e.e. > 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 3H, CH_2CH_3, *J* = 7.4 Hz), 1.83 (s, 3H, $NCCH_3$), 2.26 (sext, 1H, NCCH-*H*CH_3, *J* = 7.4 Hz), 2.39 (sext, 1H, NCCH-*H*CH_3, *J* = 7.4 Hz), 2.60 (s, 3H, 6-CH_3), 3.79 (s, 3H, OCH_3), 3.97 (d, 2H, *CH_2*-Ar, *J* = 3.0 Hz, *J* = 16.2 Hz), 6.73 (s, 1H, Ar), 6.77 (d, 1H, Ar, *J* = 7.6 Hz), 6.85 (d, 1H, Ar, *J* = 8.1 Hz), 7.10 (s, 1H, Ar), 7.23 (d, 2H, Ar, *J* = 8.8 Hz), 7.30 (t, 1H, Ar, *J* = 8.1 Hz), 7.38 (d, 2H, Ar, *J* = 8.7 Hz), 8.13 (exch br s, 1H, NH).

5.2.302 S-(+)-*N*-(4-Bromophenyl)-2-[5-(3-methoxybenzyl)-3-methyl-6-oxo pyridazin-1(6*H*)-yl]-2-methylbutanamide [S-(+)-117f]

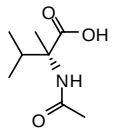


mp 108-09 °C. $[\alpha]^{20}_D$ + 17 (*c* = 1, $CHCl_3$). e.e. = 99.3 % (determined by analytical chiral HPLC). ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 3H, CH_2CH_3, *J* = 7.4 Hz), 1.83 (s, 3H, $NCCH_3$), 2.26 (sext, 1H, NCCH-*H*CH_3, *J*

= 7.4 Hz), 2.39 (sext, 1H, NCCH-*H*CH_3, *J* = 7.1 Hz), 2.60 (s, 3H, 6-CH_3), 3.79 (s, 3H, OCH_3), 3.97 (qd, 2H, *CH_2*-Ar, *J* = 3.0 Hz, *J* = 16.2 Hz), 6.73 (s, 1H, Ar), 6.77 (d, 1H, Ar, *J* = 7.5 Hz), 6.85 (dd, 1H, Ar, *J* = 6.3 Hz, *J* = 1.9 Hz), 7.10 (s, 1H, Ar), 7.23 (d, 2H, Ar, *J* = 8.7 Hz), 7.29 (t, 1H, Ar, *J* = 7.9 Hz), 7.38 (d, 2H, Ar, *J* = 8.7 Hz), 8.13 (exch br s, 1H, NH).

General Procedure for reference compounds R-(-)-119 and S-(-)-120. To an aqueous (75 mL) solution of R-(-)-2-phenylglycine or S-(-)-α-Methylvaline (99.33 mmol) respectively, acetic anhydride (0.75 mol) was added. The mixture was stirred for 0.5 h at 70 °C until all the amino acid was dissolved. When the reaction mixture was cooled to 5 °C, the crystallized amide was separated by filtration and the final products were purified by recrystallization from ethanol.

5.2.303 S-(-)-2-Acetamido-2,3-dimethylbutanoic acid [S-(-)-120]



Yield = 36 %; mp = 213-14 °C (EtOH). $[\alpha]^{20}_D$ = - 1.4° (*c* = 1, EtOH). e.e. > 99.9 % (determined by analytical chiral HPLC). ^{1}H NMR (DMSO-d_6) δ 0.84 (d, 3H, CH*CH_3*, *J* = 6.8 Hz), 0.91 (d, 3H, CH*CH_3*, *J* = 6.8 Hz), 1.25 (s, 3H, CHC*CH_3*), 1.81 (s, 3H, $COCH_3$), 1.91-1.99 (m, 1H, C*CH*$(CH_3)_2$), 7.07 (exch br s, 1H, NH), 12.08 (exch br s, 1H, OH).

6. BIOLOGICAL METHODS

6.1 Cell Culture

Human promyelocytic leukemia HL-60 cells stably transfected with FPR1 (HL-60-FPR1), FPR2 (HL-60-FPR2), or FPR3 (HL-60-FPR3) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES, 100 μg/ml streptomycin, 100 U/ml penicillin, and G418 (1 mg/mL), as previously described.[315] Wild-type HL-60 cells were cultured under the same conditions, but without G418.

6.2 Isolation of Human Neutrophils

Blood was collected from healthy donors in accordance with a protocol approved by the Institutional Review Board at Montana State University. Neutrophils were purified from the blood using dextran sedimentation, followed by Histopaque 1077 gradient separation and hypotonic lysis of red blood cells, as previously described.[204] Isolated neutrophils were washed twice and resuspended in HBSS without Ca^{2+} and Mg^{2+} ($HBSS^-$). Neutrophil preparations were routinely > 95 % pure, as determined by light microscopy, and > 98 % viable, as determined by trypan blue exclusion.

6.3 Ca^{2+} Mobilization Assay

Changes in intracellular Ca^{2+} were measured with a FlexStation II scanning fluorometer using a FLIPR 3 calcium assay kit (Molecular Devices, Sunnyvale, CA) for human neutrophils and HL-60 cells. All active compounds were evaluated in parent (wild-type) HL-60 cells for supporting that the agonists are inactive in non-transfected cells. Human neutrophils or HL-60 cells, suspended in $HBSS^-$ containing 10 mM HEPES, were loaded with Fluo-4 AM dye (Invitrogen) (1.25 μg/mL final concentration) and incubated for 30 min in the dark at 37 °C. After dye loading, the cells were washed with $HBSS^-$ containing 10 mM HEPES, resuspended in HBSS containing 10 mM HEPES and Ca^{2+} and Mg^{2+} ($HBSS^+$), and aliquotted into the wells of a flat-bottomed, half-area-well black microtiter plates (2×10^5 cells/well). The compound source plate contained dilutions of test compounds in $HBSS^+$. Changes in fluorescence were monitored (λ_{ex} = 485 nm, λ_{em} = 538 nm) every 5 s for 240 s at room temperature after automated addition of compounds. Maximum change in fluorescence, expressed in arbitrary units over baseline, was used to determine agonist response. Responses were normalized to the response induced by 5 nM fMLF (Sigma Chemical Co., St. Louis, MO) for HL-60-FPR1 and neutrophils, or 5 nM WKYMVm (Calbiochem, San Diego, CA) for HL-60-FPR2 and HL-60-FPR3 cells, which were assigned a value of 100%. Curve fitting (5-6 points) and calculation of median effective concentration values (EC_{50}) were performed by nonlinear regression analysis of the dose-response curves generated using Prism 5 (GraphPad Software, Inc., San Diego, CA).

6.4 Chemotaxis Assay

Neutrophils were suspended in $HBSS^+$ containing 2% (v/v) fetal bovine serum (FBS) (2 x 106 cells/mL), and chemotaxis was analyzed in 96-well ChemoTx chemotaxis chambers (Neuroprobe, Gaithersburg, MD), as previously described.[204] In brief, lower wells were loaded with 30 μL of $HBSS^+$ containing 2% (v/v) FBS and the indicated concentrations of test compound, DMSO (negative control), and 1 nM fMLF as a positive control. The number of migrated cells was determined by measuring ATP in lysates of transmigrated cells using a luminescence-based assay (CellTiter-Glo; Promega, Madison, WI), and luminescence measurements were converted to absolute cell numbers by comparison of the values with standard curves obtained with known numbers of neutrophils. The results are expressed as percentage of negative control and were calculated as follows: (number of cells migrating in response to test compounds/spontaneous cell migration in response to control medium) x 100. EC_{50} values were determined by nonlinear regression analysis of the dose-response curves generated using Prism 5 software.

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UNIVERSITY OF CAMBRIDGE

8. SUPPLEMENT :

Solid-Phase Synthesis of Transition State Mimetics in the Quorum Sensing System of Staphylococcus Aureus to Develop Catalytic Antibodies

7-months training period at Chemistry Department of the University of Cambridge

Supervisor: Dr. David R. Spring

Tutor: Dr. Albert Isidro-Llobet

8.1 INTRODUCTION

8.1.1 Antibiotics today: an overview

The development of antibacterial agents is arguably one of the greatest successes of the 20th century medicine.[1] Antibiotic drugs have played an essential role in the global increase in life expectancy and quality that has occurred over the last century.[2] However, since the "glory days" of antibiotic discovery in the 1960s and 1970s, there has been a sharp decline in investment for new antibacterial interventions by the large pharmaceutical companies. Concomitantly, the incidence of resistance among clinical isolates against conventional antibiotics is on the increase.[3] Indeed, bacteria have quickly become resistant to the most commonly prescribed antibiotics.[4] As a result, we are left with a legacy of relatively few efficacious drugs and,[1] unfortunately, pathogens continue to adapt faster than new antimicrobial agents can be developed to control them.[5] Thus, bacterial infection, particularly from multi-drug resistant strains, remains a serious threat to human lives.[1,4] Indeed, the scarcity of new antibiotic products has prompted some commentators to refer to an impending "pharmageddon" and to an impending return to the "pre-antibiotic" era, at least for the treatment of some bacterial organisms. Consequently, the development of novel therapies for the treatment of human bacterial infections is of paramount importance.[6]

Existing antibiotics generally inhibit bacterial cellular processes that are essential for microbial survival.[7,8] An inherent problem with this approach is that it creates a selection pressure for drug-resistant mutations.[9,10] Antivirulence therapies seek to address this issue.[7,8] These methods aim to target bacterial systems associated with virulence rather than essential cellular process and the hope is that such strategies will reduce selective survival pressures and slow the development of resistance.[11]

Over the last decade, the work of many groups has shown that systems associated with virulence in bacteria are valid targets for the development of new antibacterial agents. Although virulence is a multi-factorial phenotype, in many clinically-relevant bacteria the trait is controlled by a relatively small number of signalling pathways.[12,13] Crucially for the current proposal, small molecules are often found at the apex of these signalling pathways, so that theoretically by eliminating these compounds from the growth medium, the virulence of the organism in question should be reduced. Indeed, disruption of these signalling pathways, either through mutation or through chemical intervention, is a proven way of diminishing the pathogenicity of a number of organisms and facilitating their immune clearance from the infected host. In addition, to reduce the selection pressure for drug-resistant mutations, non-lethal alternatives to antibiotic interventions are an attractive therapeutic strategy as well because, by specifically targeting virulence, it is possible to decrease the potential negative impact of treatment on the patient.[12] Recent years have witnessed a growing realisation that antivirulence therapies represent a

potentially valuable alternative to traditional antibiotic methods for the treatment of bacterial infections. In this context, bacterial quorum sensing (QS) systems offer an attractive target.[14,15]

8.1.2 Quorum sensing (QS)

QS is a method of intercellular communication employed by many species of bacteria. This signalling process is used by bacterial colonies to coordinate gene expression in a cell density-dependent manner.[16-18] Several clinically relevant pathogens use quorum-sensing systems to regulate processes associated with virulence.[19] However, quorum sensing is not directly involved in biological processes that are essential for bacterial survival.[20,21] Thus, selective disruption of QS (so-called *"quorum quenching"*) using non-native small molecule entities represents a strategy to attenuate bacterial pathogenicity without imposing an intense selective pressure for the development of resistant mutants,[22,23] compared to existing antibiotic treatments.[24]

Quorum sensing is mediated by small diffusible molecules termed *autoinducers*. These small molecule entities are synthesized intracellularly (throughout the growth of the bacteria) and released into the surrounding growth medium. The extracellular concentration of the small molecules led to an increase of the cell density. In fact, when this concentration exceeds a certain value, the accumulated signal molecules are sensed by specific receptors on the bacterial.[25] The binding of the autoinducers to the receptors occurs and starting from this, a signal transduction cascade leads to a change in gene expression.[15,26,27] In this way, the bacterial population reaches a critical "threshold" cell density which is of primary importance for its virulence.

In the case of pathogens, QS-controlled genes often encode virulence factors.[28] Indeed, virulence factor production in several clinically relevant pathogenic bacteria including *Staphylococcus aureus*, *Clostridium difficile* and *Pseudomonas aeruginosa* is known to be regulated by QS systems. In this scenario, at low cell densities, the cells do not produce virulence factors and therefore appear "innocent" to the host. However, once the population becomes quorate, the QS system simultaneously stimulates virulence factor production, ensuring that the population as a whole produces a welter of tissue-damaging proteins in a highly coordinated and cooperative manner.[29-32] Nature is known to have evolved quorum-quenching enzyme that are capable of hydrolyzing quorum sensing QS molecules and,[23,33-35] recently, the concept of quorum quenching using the catalytic antibody technology directed towards the development of functionally equivalent "unnatural" enzymes, has been introduced.[36-38] This approach uses small molecules as haptens to elicit antibodies capable of catalyzing QS molecules hydrolysis and thus inhibit quorum sensing. The hypothesis underpinning this aim is that by selectively depleting these small molecules from the site of bacterial infection, the invading organism will be made less aggressive

allowing the host immune system a better chance of clearing the infection before the bacteria cause too much tissue damage.

8.1.3 Catalytic antibodies through Transition State (TS) mimetics

Catalytic antibodies or "abzymes" are essentially tailor-made biological catalysts capable of accelerating the rate of a specific chemical reaction by preferentially stabilizing the transition state of the intended "natural" substrate to product conversion,[37] following the same way of enzymes. Although several abzymes, that catalyze a variety of chemical transformations, have been generated, the most active antibodies have usually been found to provide rate enhancements for reactions with intrinsically low activation energy barriers.[36] That is, abzymes generally catalyze reactions that happen at a slow, but measurable rate under ambient conditions. The procurement of catalytic antibodies for a specific reaction typically requires the use of a molecule which has been designed to be a stable mimic of the transition state structure associated with the reaction.[39] The aim is to obtain antibodies that bind to this transition state analogue with high affinity; such antibodies should therefore be able to bind tightly to, and preferentially stabilise, the transition state of the reaction of interest thus decreasing the activation energy barrier to the process (**figure 8.1**).[40-43] Since abzymes are far more structurally-constrained than their enzyme counterparts, their catalytic activity is often relatively poor in comparison with the latter. However, they still provide rate enhancements of 10^4 or more over the uncatalyzed reaction and they have proven therapeutic potential in the treatment of drug addiction.[44]

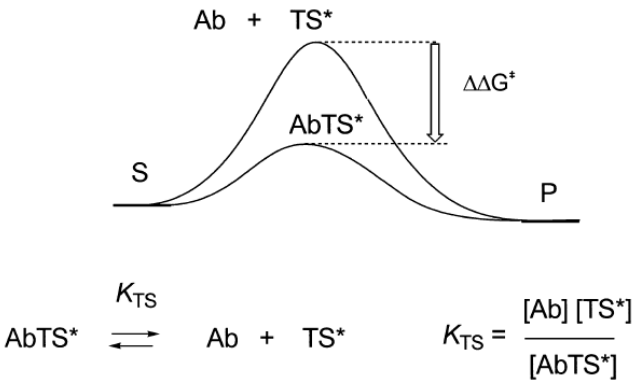


Figure 8.1. Energy diagram for an antibody-catalyzed and uncatalyzed transformation of a substrate S to a product P proceeding via a transition state (TS*) or an antibody-bound transition state (Ab.TS*). The catalytic effect of the antibody is given by ΔΔG*, which corresponds to the transition state dissociation constant K_{TS}. Adapted from Reymond (2002). [44]

The improved pharmacological potential of many abzymes (compared to non host-derived enzymes) comes from the fact that, as in much of the proposed work, they can be based around a human antibody framework and are therefore not targets for immune clearance. A more recent work has shown that the

kinetic parameters of abzymes can be improved through targeted design strategies, and it is now clear that in some cases, residues in the substrate-binding site can also make additional catalytic contributions through, for example, acid-base catalysis.[45]

8.1.4 Transition state mimetics and *quorum quenching*

In principle, if stable small molecules that mimic the transition states associated with possible decomposition pathways of autoinducers can be synthesized, it should be possible to use these molecules to generate catalytic antibodies capable of degrading said autoinducers (*"quorum quenching"*), thereby interfering with the quorum sensing process. Since different species of bacteria generally use different autoinducer molecules, the nature of the transition state mimics required to elicit catalytic antibodies is species-dependent. In this work we focused upon quorum sensing in the Gram positive bacterium *Staphylococcus aureus* where stable structural moieties that mimic the transition states associated with the degradation reaction of the autoinducers need to be identified.[5]

8.1.5 Quorum sensing in Staphylococcus aureus

Staphylococcus aureus is a prevalent and highly adaptable Gram-positive bacterium responsible for numerous clinical infections.[46] It is one of the most dreaded pathogens in hospitals and causes more nosocomial infections than any other Gram-positive bacterium. Methicillin-resistant strains of *S. aureus* (MRSA) represent an important problem, since infections can advance so rapidly that they become life-threatening before clinical intervention can be realised. The treatment issue is compounded by the fact that MRSA strains are resistant to most clinically-used β-lactam antibiotics, so vancomycin, *"the antibiotic of last resort"*, is often the necessary choice of treatment. Worryingly, vancomycin-resistant *S. aureus* strains are now also appearing in hospitals, reinforcing the need for new anti-staphylococcal interventions. The emergence of antibiotic resistance in this and other species has become a serious concern in the medical community.[6]

Various aspects of virulence in *S. aureus* are known to be regulated by a quorum sensing system which employs small autoinducing (oligo)peptides (AIPs), comprised of 7-10 amino acid residues, as the signalling molecules. AIPs function as extracellular signalling molecules that allow individual cells to sense the surrounding population density. Once a *"quorum"* of cells has been achieved, the bacteria modulate their gene expression to facilitate cooperative behaviours that confer survivability to the developing colony.[47] The discovery of this global regulatory system for virulence in *S. aureus* has provided an avenue for interrupting these defenses.[48,49] AIP mimics that perturb this system would be useful chemical probes and could potentially be developed for therapeutic applications.[5]

8.1.6 Autoinducing (oligo)peptides (AIPs)

The quorum sensing circuit outlined in **figure 8.2** represent the basic paradigm for AIP-mediated signalling in *S. aureus*. QS in *S. aureus* is encoded by the accessory gene regulator (*agr*) locus.[50] Each bacterium secretes AIPs that accumulate in the extracellular environment. Once these ligands reach a threshold concentration, they will bind productively to a cognate receptor protein located on the cell exterior named AgrC. AIP binding activates a two-component intracellular signalling system that up-regulates the production of virulence determinant-encoding genes (including genes that encode toxins, host cell-adhesion factors and extracellular hemolytic/proteolytic enzymes) and also up-regulates production of the four *agr* proteins (AgrA-D).

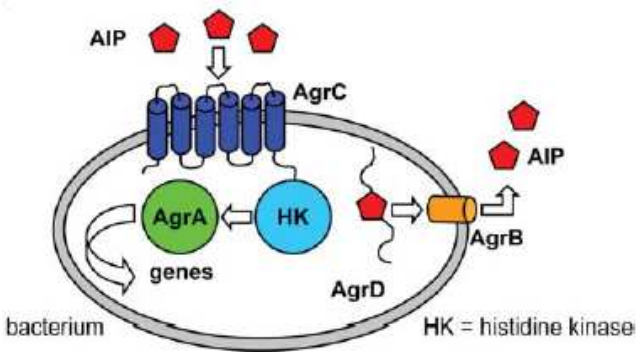


Figure 8.2. Proposed mechanism of the two-component *agr* autoinduction system in *S. aureus.* AgrB processes the propeptide AgrD to generate an AIP and secretes it into the extracellular environment. The AIPs bind to the AgrC receptor, a histidine-kinase that phosphorylates the intracellular response regulator AgrA. This second signalling component then promotes gene transcription that induces virulence and produces the *agr* proteins, completing the autoinduction circuit. Adapted from Gorske and Blackwell (2006).[5]

The *agr*-mediated QS systems in *S. aureus* have evolutionarily diverged into four different sub-groups (AIP-I/IV) (**figure 8.3**).

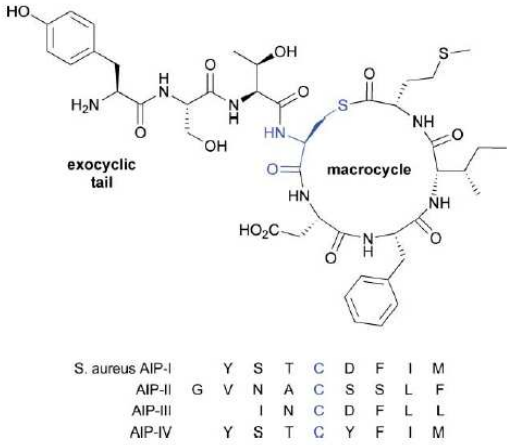


Figure 8.3. Chemical structure of AIP-I and peptide sequences of AIPs I through IV. The cysteine residue that forms the thiolactone is highlighted in blue. Adapted from Gorske and Blackwell (2006).[5]

Each sub-group is associated with a distinct AIP. However, in all cases, the AIPs have a similar core structure; they all comprise a macrocylic thiolactone and a conjoined linear tail moiety. The macrocyle

appears to be the main determinant required for molecular recognition by the membrane receptor AgrC, while the exocyclic peptide moiety is required for receptor activation. The conserved thiolactone is labile and it is known that cleavage of this ring abolishes agonistic activity in the AIPs. Thus the thiolactone ring moiety is an excellent target for the development of catalytic antibodies. If stable small molecules that mimic possible transition states associated with the rate determining steps of thiolactone ring hydrolysis can be identified, in theory, these could be used to elicit antibodies that are capable of catalysing this hydrolysis step, thereby inhibiting QS in S. aureus and thus attenuating its virulence.[5,51]

8.2 BACKGROUND AND AIMS OF THE PROJECT

The main goal of the proposed study is the synthesis of transition state (TS) mimetics of the degradation reaction of the AIPs, that could potentially be used to elicit catalytic antibodies capable to attenuate the expression of virulence determinants in the Gram positive bacterium *S. aureus*. To achieve this aim, our primary experimental objective was the synthesis of macrocyclic phosphopeptides as new structural peptidomimetic analogues that mimic the *transition state* associated with the degradation of AIPs involved in the population cell density-control of *S. aureus*. This approach will hopefully reduce or abolish the expression of virulence determinants associated with the AIPs and allow the establishment of structure-activity relationships (SARs) in these systems, to better understand quorum sensing signalling pathway and be able to develop new peptidomimetic inhibitors.

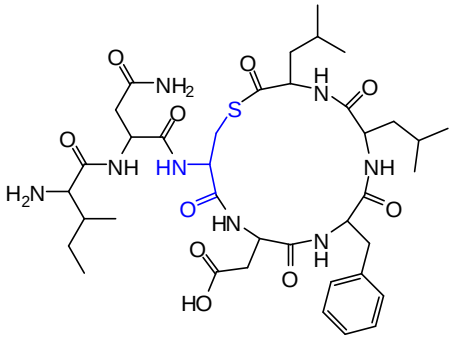


Figure 8.4. Structure of the natural autoinducing (oligo)peptide **AIP-III**. The cysteine residue that forms the thiolactone is highlighted in blue.

AIP-III (**figure 8.4**) was chosen as the reference peptide, because it is the substrate of EMRSA-16, an important Methicillin-resistant S. aureus strain available at the Biochemistry Department of the University of Cambridge and because all the AIP peptides have a similar core structure being the macrocycle the main determinant required for the activity. Therefore, one of the aims of the project was the total synthesis of the natural substrate (**AIP-III**),[52] to use it as reference in the biological tests, following a Fmoc/*t*-Bu solid-phase synthesis.[51] The thioester group (**figure 8.4**), as previously mentioned, is an excellent target to develop catalytic antibodies since cleavage of the thiolactone ring

abolishes agonistic activity of the AIPs. As phosphate derivatives are structurally similar to thioester bonds, the most important aim of this project is the synthesis, using a Fmoc/*t*-Bu solid-phase strategy, of two phosphorus **AIP-III** analogues as potential TS mimetics (**figure 8.5**).[53]

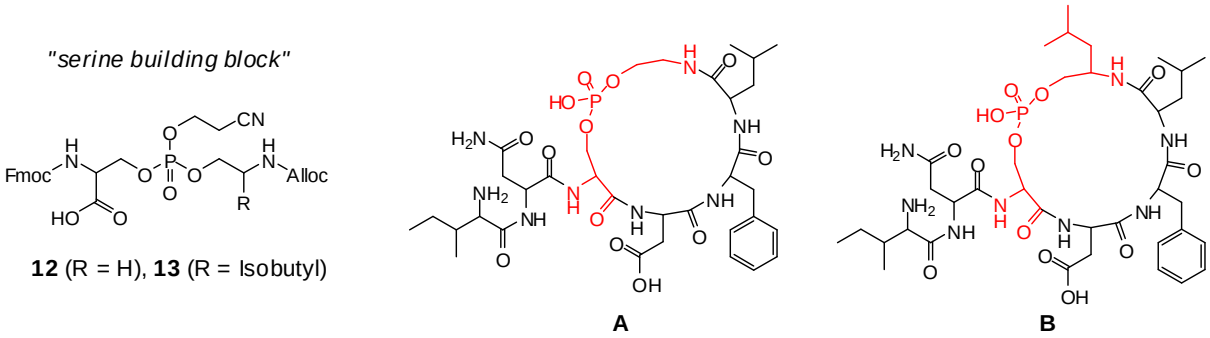


Figure 8.5. Structures of the *"serine building blocks"* and the two **AIP-III** analogues designed (**A** and **B**). The phosphodipeptide inserted in the macrocycle is highlighted in red. The structure **A** is reported as **23** in the **section 8.3.2.2** (**scheme S.5**).

In these new molecules the thioester group is replaced by a phosphate group, previously built in a *"serine building block"* (**figure 8.5**). It should be noted that the two new macrocycles, compared to the natural peptide, have two members more, one O and one CH_2 but the size of the cycle does not seem to be crucial for the activity.[52]

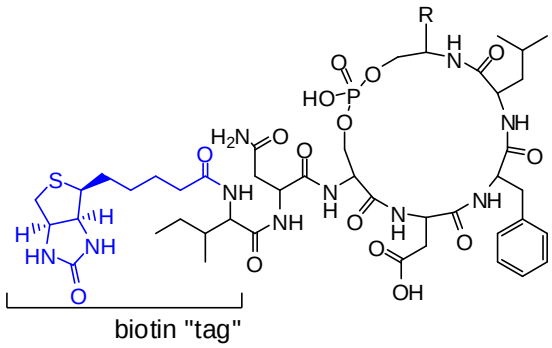


Figure 8.6. The **biotinylated variants** of the **AIP-III** analogue designed.

The transition state mimetics will be tested to evaluate their activity as quorum quenching agents and later on will be attached to a biotin-tag (**figure 8.6**) for phage library screening.[23]

8.3 CHEMISTRY

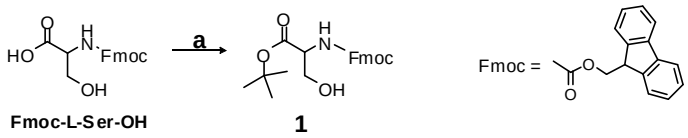
The chemistry of this project is divided in 3 parts: synthesis of the *"serine building blocks"* (**section 8.3.1**), synthesis of the phosphorus cyclic peptides (**section 8.3.2**), synthesis of the natural substrate (**AIP-III**) (**section 8.3.3**).

8.3.1 Synthesis of the *"serine building blocks"*

The two *"serine building blocks"* (**figure 8.5)** which will be incorporated in the phosphorus cyclic peptides were prepared following the synthetic pathways depicted in **schemes S.1-S.3**.

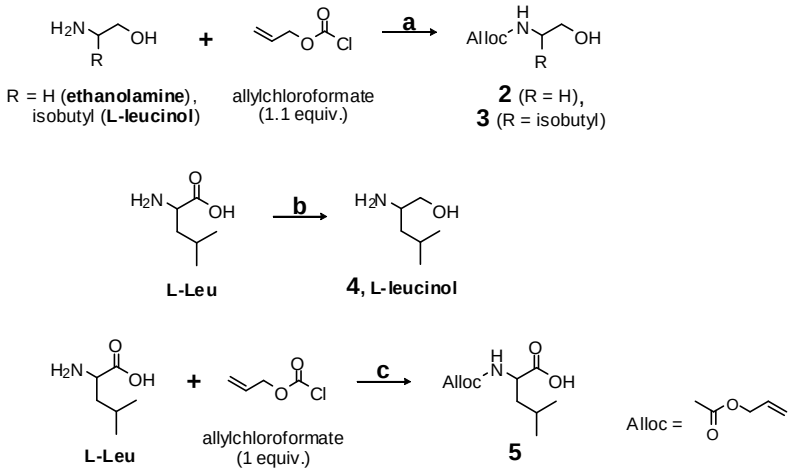
8.3.1.1 Protection of the starting amino acids and amino alcohols

N-α-Fmoc-serine-*t*-butyl ester **1** (**scheme S.1**) was readily obtained in quantitative yield following the literature.[54]



Scheme S.1. Reagents and conditions: a) *t*-Butyl-trichloroacetimidate (4 equiv), CHX/EtOAc, rt, o/n.

On the other hand, reaction of the amino alcohols, ethanolamine and L-leucinol (obtained in quantitative yield by reduction of L-leucine,[55] **scheme S.2**), with allyl chloroformate yielded the corresponding Alloc protected ethanolamine (**2**) and L-leucinol (**3**) with 97% and 86% yield respectively after purification by flash chromatography (**scheme S.2**).[56] The Alloc group was chosen because its Pd catalyzed removal is compatible with Fmoc/*t*-Bu solid-phase strategy that will be used in the phosphopeptides synthesis.[57]



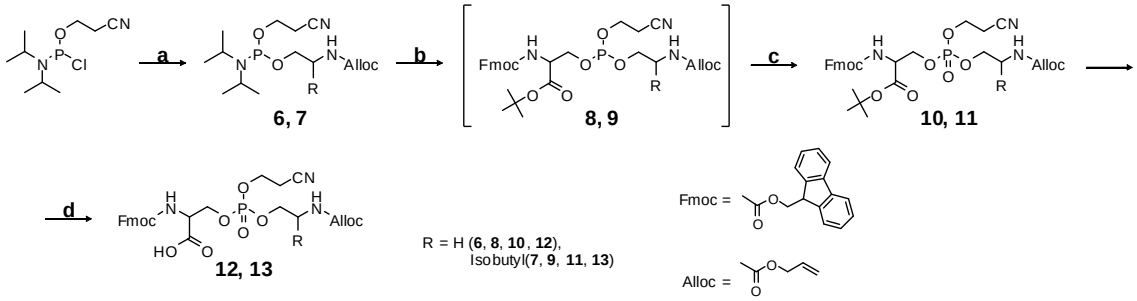
Scheme S.2. Reagents and conditions: a) $NaHCO_3$ (1.1 equiv), dioxane/water (1:1), 0 °C → rt, 3 h, **b)** LiBH4 (2 equiv), trimethylsilyl chloride (4 equiv), THF anhydrous, 0 °C → rt , 16 h, **c)** NaN_3 (1.5 equiv), dioxane/water, Na_2CO_3 1% in water, rt, 1 d (10% Na_2CO_3 in water is added to keep the pH between 8-10).

Another necessary building block for the phosphopeptide synthesis was Alloc-L-Leu-OH, which was obtained in 77% yield by reaction of in situ generated allyl carbonazidate with L-leucine (**scheme S.2**).[58]

8.3.1.2 Phosphorus couplings

The desired *"serine building blocks"* were obtained in 4 synthetic steps (**scheme S.3**) using the commercially available 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite as the phosphorus source.[59-61] The first reaction is a coupling of the Alloc-ethanolamine (**2**) or Alloc-L-leucinol (**3**) on the 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite (**scheme S.3**).[62,63] The purified products must be stored under N_2 at -20 °C, since they decompose to complex mixtures after few days at room temperature.

The following reaction of of *N*-α-Fmoc-serine-*t*-butyl ester **1** with compounds **6** and **7** (**scheme S.3**) was performed in the presence of tetrazole.[64,65]



Scheme S.3. Reagents and conditions: a) Alloc-ethanolamine (**2**) or Alloc-L-leucinol (**3**) (0.7 equiv), DIPEA (4.7 equiv), CH_2Cl_2 anhydrous, N_2, rt, 2-3 h; **b)** Fmoc-L-Ser-*t*-Bu ester (**1**) (1 equiv), tetrazole (1.2 equiv), THF anhydrous, N_2, rt, o/n; **c)** *t*-BuOOH, THF anhydrous, N_2, rt, 3-4 h; **d)** TFA (20 equiv), CH_2Cl_2, rt, 2-3h.

When explosive tetrazole was replaced by 4,5-dicyanoimidazole, the crude product obtained was much less pure both by ^{1}H NMR and TLC.[66] The obtained phosphite intermediates **8** and **9** are unstable trivalent phosphorus species which could be isolated by flash chromatography on silica gel, but it is more convenient to oxidyze them straight away to afford the more stable pentavalent species **10** and **11** (**scheme S.3**).[61,67] *m*-CPBA was initially used as an oxidizing agent but the yields were very low. In contrast, when *tert*-butyl hydroperoxide was used, fully protected phospho-amino acids **10** (R = H) and **11** (R = Isobutyl) were obtained in 57 and 20% yield respectively over two steps (**scheme S.3**). Subsequent treatment of **10** and **11** with 50% trifluoroacetic acid afforded the free acids **12** (R = H) and **13** (R = Isobutyl). It is important to note that compounds **10**, **11** and the corresponding free carboxylic acids (**12** and **13**) were obtained as diastereomeric mixtures because the chiral centre on the phosphorus was present in its 2 configurations. These diastereomeric mixtures were inseparable by flash chromatography or HPLC and made the ^{1}H NMR analysis more complex. However, once the cyanoethyl group was removed during the treatment with piperidine to remove the Fmoc group, performed in the phosphopeptide synthesis (**scheme S.4**), the phosphorus center is no longer chiral, leading to only one stereoisomer of the desired phosphopeptide.

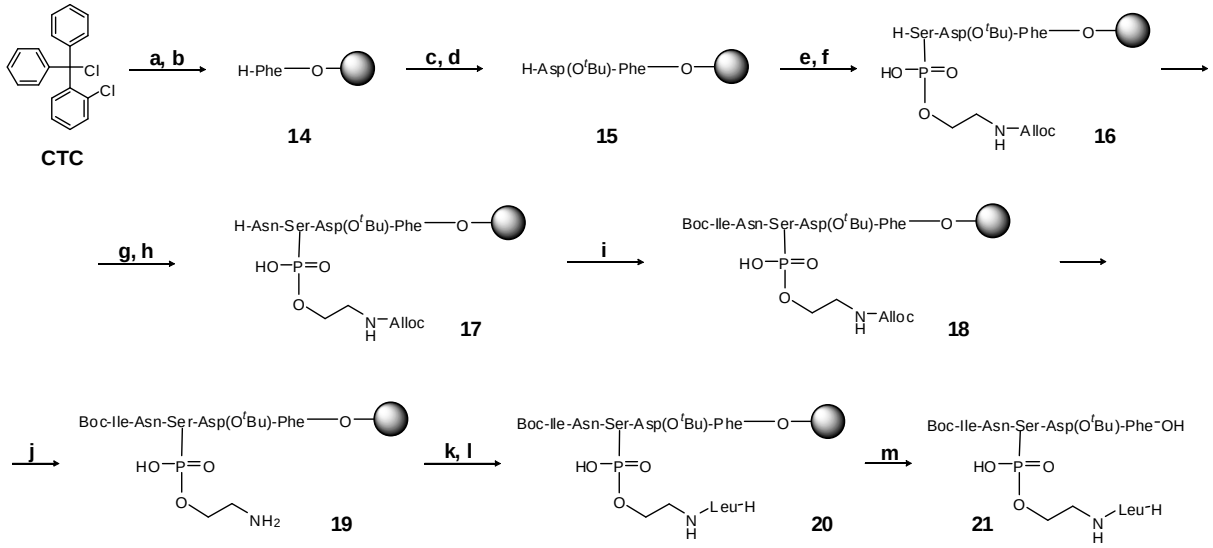
8.3.2 Synthesis of the phosphorus cyclic peptides

Linear and branched solid-phase syntheses of AIPs analogues using different orthogonal protecting schemes have been described in literature.[51,68] In most of them the cyclization and final deprotection are performed in solution.[5,51,52] We decided to adapt these strategies to the synthesis of phosphorus analogues of **AIP-III**. Our strategy is divided in two main parts: 1) Fmoc/*t*-Bu solid-phase synthesis of branched peptides (**scheme S.4**); 2) Cyclization and final deprotection in solution (**scheme S.5**). In the latter case,

the final cyclization is carried out through the formation of an amide bond between L-leucine and L-phenylalanine and there is no risk of a five-member ring formation.

8.3.2.1 Fmoc/*t*-Bu solid-phase synthesis of the branched peptides

Solid-phase synthesis of protected peptides requires a resin that facilitates the cleavage of the peptide without removing the side-chain protecting groups.[69,70] The resin of choice was the commercially available super-acid-labile chlorotrityl chloride polystyrene (CTC) resin (**scheme S.4**) which allows the release of peptides with 1-2 % of TFA in CH_2Cl_2 or even with trifluoroethanol solutions.[71,72] An additional advantage of the CTC resin is that its hindered structure minimizes the formation of diketopiperazines (DKP) during removal of the temporary protecting group of the second amino acid.[73,74] The Fmoc/*t*-Bu solid-phase strategy involves the use of the base labile Fmoc group for α-amino protection and *t*-Bu-based protecting groups for side chain protection. *t*-Bu-based protecting groups are highly convenient because they are removed with high concentrations of trifluoroacetic acid (TFA) in the presence of scavengers, being stable to piperidine, used to remove the Fmoc group, and to low concentrations of TFA, used to cleave the peptide from the CTC resin. In addition, they are also stable to the Pd(0) treatment required to remove the Alloc group. The branched approach depicted in **scheme S.4** was chosen in order to avoid the risk of cyclization on the free phosphate group which would lead to a stable five-member ring.



Scheme S.4. Reagents and conditions: a) Fmoc-L-Phe-OH (0.7 equiv), DIPEA (2.1 + 4.2 equiv), CH_2Cl_2, rt, 45 min; **b) Fmoc-removal:** piperidine-DMF (2:8, v/v) (1 x 2 min, 2 x 10 min); **c)** Fmoc-L-Asp-(O-*t*-Bu) (4 equiv), ethylcyanoglyoxylate-2-oxime (4 equiv), DIC (4 equiv), DMF, rt, 1.5 h; **d)** Fmoc-removal; **e) 12** (3 equiv), PyAOP (3 equiv), DIPEA (3 + 6 equiv), DMF, rt, 2 h; **f)** Fmoc-removal; **g)** Fmoc-L-Asn-OH (4 equiv), ethylcyano-glyoxylate-2-oxime (4 equiv), DIC (4 equiv), DMF, rt, 1.5 h; **h)** Fmoc-removal; **i)** Boc-L-Ile-OH (4 equiv), ethylcyanoglyoxylate-2-oxime (4 equiv), DIC (4 equiv), DMF, rt, 1.5 h; **j) Alloc-removal:** $Pd(PPh_3)_4$ (0.1 equiv), $PhSiH_3$ (10 equiv), CH_2Cl_2 (3 x 15 min); **k)** Alloc-L-Leu-OH (**5**) (4 equiv), ethylcyanoglyoxylate-2-oxime (4 equiv), DIC (4 equiv), DMF, rt, 1.5 h; **l)** Alloc-removal; **m)** TFA:Et_3SiH:CH_2Cl_2 (1:1:98), rt, 1h.

The first coupling of Fmoc-L-Phe-OH on the resin was carried out using DIPEA as a base. The following amino acid-couplings were performed using diisopropylcarbodiimide (DIC), to activate the carboxylic group, and ethylcyanoglyoxylate-2-oxime, to minimize the epimerization. This new *"anti-epimerization agent"* has recently been reported to be a more efficient non-explosive alternative to the commonly used HOBt or HOAt.[75]

The couplings of the phosphorus building blocks **12** and **13** (**scheme S.3**) were performed using PyAOP and DIPEA (**scheme S.4**). Phosphonium derivatives such as PyAOP are more convenient for slow couplings compared with aminium/uronium reagents such as HATU, since this latter can terminate the peptide chain through a guanidination reaction.[76] Furthermore, PyAOP contains HOAt, which is the most reactive benzotriazole.[77] Unfortunately, the coupling reaction worked fine only for building block **12** but not for the more hindered **13**.

The Chloranil test for detection of primary and secondary amines was performed after each coupling to confirm the coupling completion.[78-80] When the test was negative (absence of free amines) the Fmoc group was removed with piperidine-DMF (2:8, v/v). Otherwise the corresponding Fmoc-amino acid was re-coupled in the same conditions. It should be noted that the phosphate only loses the β-cyanoethyl protecting group (**scheme S.3** and **S.4**) after the piperidine-induced Fmoc removal.[66,81] This α-elimination removal of the cyanoethyl group allowed the solid phase synthesis of the remaining phosphoserine peptide using Fmoc-based chemistry.[82-84] Washings between deprotection, coupling and again deprotection steps were performed with DMF and CH_2Cl_2. The Alloc group was removed by Pd(0), using a catalytic amount of Tetrakis(triphenylphosphine)-palladium(0), in rather neutral conditions and in the presence of phenylsilane as a scavenger of the allyl carbocations (**scheme S.4**).[85,86] Washings after deprotection steps were performed using CH_2Cl_2, DMF and a solution 0.02 M of sodium diethyldithiocarbamate in DMF. Peptide synthesis transformations and washings were always done at 25 °C. Synthesis carried out on solid-phase were controlled by HPLC of the intermediates obtained after cleaving an aliquot (approximately 2 mg) of the peptidyl-resin with TFA/Et_3SiH/CH_2Cl_2 (1:1:98) for 1 hour (**scheme S.4**). The required polymer-bound branched protected peptide **22** obtained via standard Fmoc/*t*-Bu solid-phase peptide assembly was subsequently treated with TFA/Et_3SiH/CH_2Cl_2 (1:1:98 v/v) for 1 hour. This step resulted in the chemoselective acidolysis of the 2-chlorotrityl ester resin linkage and afforded the partially protected heptapeptide **21** in typically quantitative yields. Partial purification of **21** was accomplished by filtration of the acidolytic resin suspension into pyridine-methanol (1.5:75 v/v) and evaporation of the filtrate to dryness in vacuo. A small fraction of the dried partially protected peptide **21** was exposed to TFA/H_2O/Et_3SiH (90:5:5) for 1 h to remove the protecting groups, followed by HPLC-UV analysis, which revealed a peptide purity >80%.

8.3.2.2 Macrocyclization

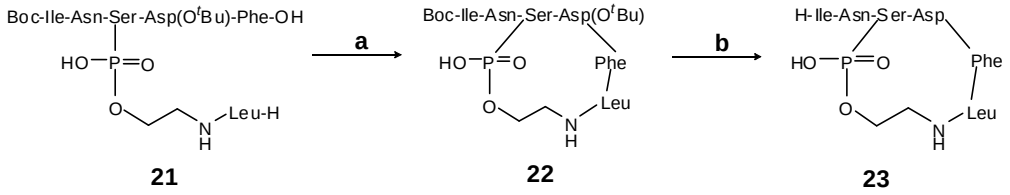
The cyclization of the branched peptide **21** was the limiting step of this synthetic strategy. As shown in **table 8.1**, several macrocyclization conditions were screened. Even when the desired cyclic product was detected, it was part of very complex mixtures.

	Coupling reagents	solvent	time	work up	HPLC-UV-MS results
1	*N*-polystyrene methyl-*N'*-cyclohexylcarbo-diimide (e equiv), DMAP (0.1 equiv), HOAt (5 equiv)	$CHCl_3$	2 d	filtration and evaporation	CM*. No branched (SM[§]) and no cyclic peptide (**22**)
2	*N*-polystyrene methyl-*N'*-cyclohexylcarbo-diimide (5 equiv), DMAP (0.1 equiv), Ethylcyanoglyoxylate-2-oxime (5 equiv)	$CHCl_3$	2 d	filtration and evaporation	CM. No branched peptide (SM). The peak of **22** is too weak.
3	*N*-polystyrene methyl-*N'*-cyclohexylcarbo-diimide (5 equiv), DMAP (0.1 equiv)	$CHCl_3$	3d	filtration and evaporation	CM. No branched peptide (SM). The peak (+ Na) of **22** is big enough to be detected.
4	*N*-polystyrene methyl-*N'*-cyclohexylcarbo-diimide (5 equiv), DMAP (0.1 equiv), HOAt(5 equiv)	CH_2Cl_2	1 d	filtration and evaporation	CM. No cyclic peptide (**22**). The SM is still in a high amount.
5	*N*-polystyrene methyl-*N'*-cyclohexylcarbo-diimide (5 equiv), DMAP (0.1 equiv)	CH_2Cl_2	1 d	filtration and evaporation	CM. No branched (SM) and no cyclic peptide (**22**)
6	*N*-polystyrene methyl-*N'*-cyclohexylcarbo-diimide (5 equiv), HOAt (5 equiv)	CH_2Cl_2	1 d	filtration and evaporation	CM. No branched (SM) and no cyclic peptide (**22**)
7	EDC (5 equiv), DMAP (0.1 equiv), HOAt (5 equiv)	CH_2Cl_2	1 d	Extraction with citric acid and H_2O, evaporation	CM. The SM is still in a high amount.
8	EDC (5 equiv), DMAP (0.1 equiv)	CH_2Cl_2	1 d	Extraction with citric acid and H_2O, evaporation	CM. No branched (SM) and no cyclic peptide (**22**)
9	EDC (5 equiv), DMAP (0.1 equiv)	$CHCl_3$	2 d	Extraction with citric acid and H_2O, evaporation	CM. No branched (SM) and no cyclic peptide (**22**)
10	PyAOP (1 + 1 equiv), DIEA (2 equiv)	CH_2Cl_2	1 d	Extraction with H_2O, evaporation	CM. No branched (SM) and no cyclic peptide (**22**)
11	DIC (5 equiv), HOAt (5 equiv)	CH_2Cl_2	1 d	evaporation	CM. The peak of **22** is big enough to be detected.

Table 8.1. Macrocyclization conditions screened for the synthesis of transition state mimetic **23** [* CM = complex mixture; [§] SM = starting material (**21**)].

On the basis of the screening, we concluded that the methods based on *N*-polystyrene methyl-*N'*-cyclohexylcarbodiimide/DMAP (**entry 3**, **table 8.1**) or DIC/HOAt (**entry 11**, **table 8.1**) as coupling reagents gave better results because, although we had always a very complex crude mixture to purify, it was possible to detect the peak of the right cyclic peptide, after hydrolysis of the protecting groups (Boc and *t*-Bu), by UV and mass spectrometry HPLC analysis. Therefore, for the purpose of the this dissertation, only these cyclization conditions will be described in detail. In the first case, macrocyclization was achieved by exposing a dilute solution of **21** in $CHCl_3$ (2 mM) to *N*-polystyrene methyl-*N'*-cyclohexylcarbodiimide, a commercially available polymer-supported carbodiimide analogue

of *N*-polystyrene methyl-*N'*-isopropylcarbodiimide,[87,88] in the presence of 4-dimethylaminopyridine (DMAP) for 3 days at room temperature (**scheme S.5**). In the second case macrocyclization was carried out always by exposing a dilute solution of **21** in CH_2Cl_2 to DIC in the presence of 7-aza-1-hydroxybenzotriazole (HOAt) (**scheme S.5**).[89]

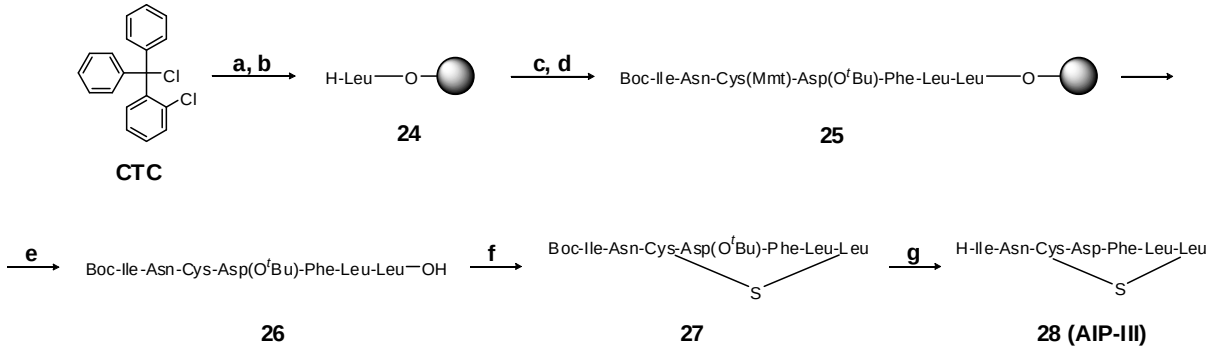


Scheme S.5. Reagents and conditions: a) see **table 8.1**; **b)** TFA:Et_3SiH:H_2O (90:5:5), rt, 1-1.5 h.

In both cyclization rections, after a standard workup, 90% TFA-mediated acidolytic treatment of the crude protected macrocyclic peptide **22** afforded a complex mixture containing the desired phophorus-peptide analogue **23**. Significantly, HPLC analysis showed virtually absence of the starting branched peptide. Unfortunately, after purification by semi-preparative HPLC the cyclic heptapeptide **23** was not obtained as a pure compound.

8.3.3 Total synthesis of the AIP-III

The natural substrate **AIP-III**, involved in the quorum sensing system of *S. aureus*, had been already obtained through different synthetic strategies based on either Fmoc/*t*-Bu or Boc/Bn chemistry.[5,52,68] We used a synthetic pathway described in literature for a similar peptide,[51] based on the Fmoc/*t*-Bu solid-phase synthesis of a linear peptide followed by macrocyclization in solution.[90-93] For this linear approach the macrocyclization was carried out through the formation of a thioester bond between the L-Cys sulfhydryl and the C-terminus carboxyl group of the L-Leu (**scheme S.6**).[94-96]



Scheme S.6. Reagents and conditions: a) Fmoc-L-Leu-OH (0.7 equiv), DIPEA (2.1 + 4.2 equiv), CH_2Cl_2, rt, 45 min; **b) Fmoc-removal:** piperidine-DMF (2:8, v/v) (1 x 2 min, 2 x 10 min); **c)** Fmoc-L-aa-OH or Boc-L-Ile-OH (4 equiv), ethylcyano glyoxylate-2-oxime (4 equiv), DIC (4 equiv), DMF, rt, 1.5-2 h; **d)** Fmoc-removal (for deprotection of all the aa used with the exception of Boc-L-Ile-OH); **e)**TFA:Et_3SiH:CH_2Cl_2 (1:1:98), rt, 1h; **f)** see **table 8.2**; **g)** TFA:Et_3SiH:H_2O (90:5:5), rt, 1.5 h.

Solid-phase synthesis was undertaken using a *tetra-orthogonal protecting scheme* similar to that previously used to obtain the phosphorus cyclic peptide **23** (**scheme S.4** and **S.5**) and it was performed in

polypropylene syringes (12 mL) fitted with a polyethylene porous disc. Solvents and soluble reagents were removed by suction. CTC, protected Fmoc amino acids and all the coupling reagents used during the synthesis are commercially available. After the first coupling of Fmoc-L-leucine on the CTC, carried out using DIEA as a base, all the following amino acid couplings were performed using diisopropylcarbodiimide, to activate the carboxylic group, and ethylcyanoglyoxylate-2-oxime to prevent or minimize epimerization. After each coupling the reaction was checked always by the previous mentioned Chloranil test,[78-80] and the Fmoc group was removed using a piperidine/DMF solution (2:8 v/v). The following treatment of the resin-bonded peptide **25** with TFA/Et_3SiH/CH_2Cl_2 (1:1:98, v/v), raise concomitantly chemoselective unmasking of the Cys sulfhydryl group and release from the solid support. Partial purification of **26** was accomplished by filtration of the acidolytic resin suspension into pyridine-methanol (1.5:75 v/v) and evaporation of the filtrate to dryness in vacuo. After trituration of the residue with ice-water and filtration, the dried partially protected peptide **26** was macrocyclized using a commercially available dialkylcarbodiimide reagent, followed by TFA-mediated global deprotection to afford the desired thiolactone peptide **28** (**AIP-III**). As shown in **table 8.2**, we tried the cyclization of the partially protected peptide screening several coupling methods. A range of results were obtained depending on the carbodiimide involved in the reaction.

	Coupling reagents	solvent	time	work up	HPLC-UV-MS results
1	*N*-polystyrene methyl-*N'*-cyclohexylcarbo-diimide (5 equiv), DMAP (0.1 equiv), HOAt (5 equiv)	$CHCl_3$	3 d	filtration and evaporation	CM. No linear peptide (SM). The peak of **27** is too weak.
2	*N*-polystyrene methyl-*N'*-cyclohexylcarbo-diimide (5 equiv), DMAP (0.1 equiv), ethylcyano-glyoxylate-2-oxime (5 equiv)	$CHCl_3$	3 d	filtration and evaporation	CM. No linear peptide (SM). The peak of **27** is too weak.
3	*N*-polystyrene methyl-*N'*-cyclohexylcarbo-diimide (5 equiv), DMAP (0.1 equiv)	$CHCl_3$	4 d	filtration and evaporation	CM. The SM is still in a high amount. It is possible to detect the peak of **27**
4	EDC (5 equiv), DMAP (0.1 equiv), ethylcyano-glyoxylate-2-oxime (5 equiv)	$CHCl_3$	1 d	extraction with citric acid and H_2O, evaporation	CM. No linear (SM) and no cyclic peptide (**27**)
5	EDC (5 equiv), DMAP (0.1 equiv)	$CHCl_3$	4 d	extraction with citric acid and H_2O, evaporation	CM. No linear peptide (SM). The peak of **27** is detected in a relatively high amount.
6	DCC (5 equiv), DMAP (0.1 equiv)	$CHCl_3$	4 d	filtration and evaporation	CM. The SM is still in a high amount. It is possible to detect the peak of **27**

Table 8.2. Coupling methods screened to carry out the cyclization for the synthesis of the **AIP-III** (**28**).

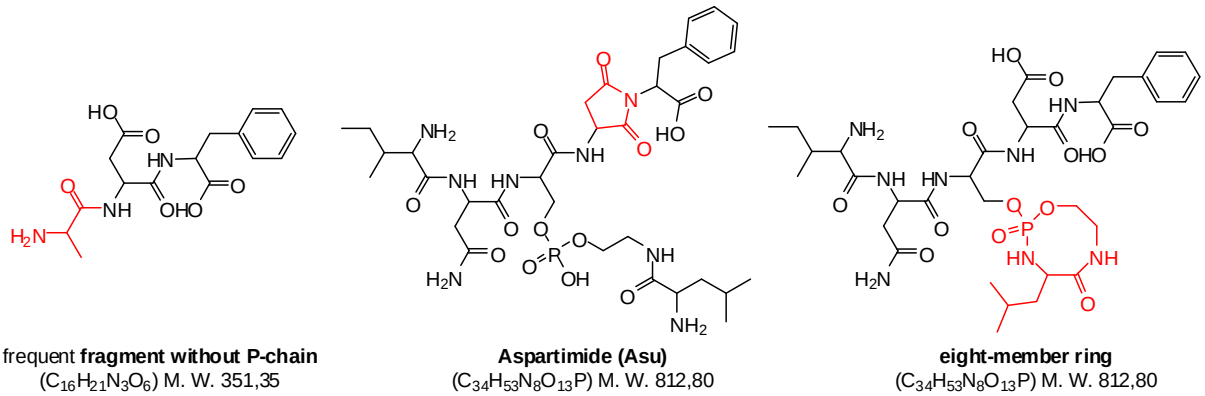
On the basis of this screening, considering the conversion of the linear peptide **26** in the cyclic one **27**, we concluded that the method based on EDC and DMAP gave the best results (**entry 5**, **table 8.2**) in terms of

yields and purity, estimated from the absence of side-products in the HPLC chromatograms. Therefore, a new macrocyclization was carried out following the **entry 5** in **table 8.2** conditions: a dilute solution of **26** in chloroform (2 mM solution) was treated with EDC, in the presence of DMAP for 3 days at room temperature. The crude mixture was washed with a 5 % citric acid solution and water, to remove the excess of carbodiimide and of urea formed during the reaction. The crude protected macrocyclic peptide **27** was treated with TFA/H_2O/Et_3SiH (90:5:5) to afford the desired natural cyclic peptide **28** (**AIP-III**) in a complex mixture (**scheme S.6**). Significantly, HPLC analysis showed virtually absence of the starting linear peptide. After purification by semi-preparative HPLC, the thiolacton heptapeptide **28** was obtained with 10-15% yield, as stated in literature.[50,58,62]

8.4 RESULTS AND CONCLUSIONS

The initial aim of this project was the synthesis of two transition state mimetics of the natural oligopeptide **AIP-III** involved in quorum sensing-system of *S. aureus*. The synthetic strategy involved the use of a Fmoc/*t*-Bu solid-phase methodology and serine phosphodiesters **12** and **13** as the key building-blocks. This required the development of an efficient synthetic route for **12** and **13** (**scheme S.3**), which was achieved after preliminary protection of the starting amino acids and aminoalcohols (**schemes S.1** and **S.2**). The synthetic route outlined in **section 8.3.1** is relatively simple and high-yielding and has been proven to be suitable for scaling-up. The availability of these building blocks is of vital importance to allow us the synthesis of a peptide sequence using standard Fmoc-based chemistry. Unexpectedly, during the solid phase synthesis of the branched peptide (**scheme S.4**) it was possible to introduce in the peptide sequence just the *"serine building block"* **12**. In contrast, the coupling reaction of phosphodiester **13** in the peptide chain didn't work, probably because **13** is more sterically hindered than **12**. However, our data show that the Fmoc/*t*-Bu solid-phase strategy is an efficient way to obtain the branched phospho-peptide **21** (**scheme S.4**) in good yield. The cyclization process was much more difficult to understand. Several coupling methods were screened and different coupling reagents were used (**table 8.1**) to obtain the phosphorus cyclic peptide. Even if the cyclization reaction involved simply the formation of an amide bond (**scheme S.5**), some coupling reagents resulted quite inefficient and when the cyclic product was present, it was part of a complex mixture. On the basis of the screening, *N*-polystyrene methyl-*N'*-cyclohexylcarbodiimide or DIC gave better results as coupling reagent. Although very complex mixtures were always obtained, it was possible to detect the peak of the right cyclic peptide by UV and mass spectrometry. In addition, HPLC analysis showed virtually absence of the starting branched peptide **21**. The main problems for the cyclization were obviously the side reactions and the instability of the

phosphate group. One of the most frequent fragment and two possible side products that could occur during the cyclization, confirmed by mass spectrometry analysis, are shown in the **figure 8.7**.



frequent **fragment without P-chain** ($C_{16}H_{21}N_3O_6$) M. W. 351,35

Aspartimide (Asu) ($C_{34}H_{53}N_8O_{13}P$) M. W. 812,80

eight-member ring ($C_{34}H_{53}N_8O_{13}P$) M. W. 812,80

Figure 8.7. A frequent fragment detected by mass spectrometry due to the instability of the phosphate group and two possible side products that could occur during the cyclization.

It is not completely sure that the two presumed side products (**figure 8.7**) could be formed during the reaction. Considering a difficult event the formation of an eight-member ring, perhaps the aspartimide or aminosuccinimide (3-amino-pyrrolidine-2,5-dione, Asu) formation is a more realistic hypothesis.[97] In fact it is the first step of the well-known degradation, at alkaline, neutral and acidic pH, of peptides and proteins containing aspartic acids/ asparagines.[98-100] The reaction results in a variety of rearranged and racemized products and it is especially problematic in Fmoc/*t*-Bu SPPS because strong base, such as piperidine, promotes Asu formation.[101] Semi-preparative HPLC did not afford the cyclic heptapeptide **23** (**scheme S.4**) as a pure compound due to the high complexity of the crude mixture. Work is underway to better understand the reasons of this evident degradation and formation of side products. The imminent future work will involve a previous purification of the branched peptide **21** by semipreparative HPLC, in order to obtain a crude mixture less complex and easier to purify after cyclization. Moreover, alternative coupling reagents and conditions will be screened in order to improve the yield of the final cyclization for the achievement of **23**.

The total synthesis of the **AIP-III** was the ultimate goal of this project. On the basis of the literature results, the Fmoc/*t*-Bu solid-phase strategy was always the selected methodology for the synthesis of the linear peptide. After cyclization carried out in solution, the **AIP-III** (**28**) has been obtained and it will be useful as reference compound in the biological tests.

In conclusion, this work presents a facile and efficient synthesis of caged phospho-amino acids suitable for Fmoc-based SPPS. Moreover, the total synthesis of the natural substrate **AIP-III** had been successfully carried out and the transition state mimetic analogue **23** had been obtained, but its yield and purity must be improved in order to start the biological assays.

8.5 EXPERIMENTAL CHEMISTRY

8.5.1 Materials and Methods

Reactions were performed using oven-dried glassware apparatus (130 °C) under an atmosphere of nitrogen with anhydrous, freshly distilled solvents unless otherwise stated. Anhydrous reactions were carried out under nitrogen atmosphere. Solvents were distilled prior to use. Reagents/solvents for anhydrous reactions were dried as follows: THF was dried over wire Na and distilled from a mixture of CaH_2 and $LiAlH_4$ with triphenylmethane as indicator; diethyl ether was distilled over a mixture of CaH_2 and $LiAlH_4$; petroleum ether was distilled before use and refers to the fraction between 30-40 °C; dichloromethane, methanol, *n*-hexane, acetonitrile and toluene were distilled from CaH_2. All other reagents were purified in accordance with the instructions in "Purification of Laboratory Chemicals",[102] or obtained from commercial sources.

Room temperature (rt) refers to ambient temperature. Temperatures of 0 °C were maintained using an ice-water bath.

Yields refer to chromatographically and spectroscopically pure compounds. All reactions were monitored by thin layer chromatography (TLC) using glass plates precoated with Merck silica gel 60 F254 or aluminum oxide 60 F254. Visualization was performed by the quenching of UV fluorescence (λ_{max} = 254 nm) or by staining with ceric ammonium molybdate or potassium permanganate or Dragendorff's reagent (0.08% w/v bismuth subnitrate and 2% w/v KI in 3M aq. AcOH). Flash column chromatography was performed using slurry-packed Merck 9325 Kieselgel 60 silica gel (230-400 mesh) unless otherwise stated, eluting with distilled solvents as described.

Melting points were obtained using a Reichert hot plate microscope with a digital thermometer attachment and are uncorrected.

Proton magnetic resonance (^{1}H NMR) spectra were recorded using an internal deuterium lock at ambient probe temperatures (unless otherwise stated) on the following instruments: Bruker DPX-400 (400 MHz), Bruker Avance 400 QNP (400 MHz), Bruker Avance 500 BB ATM (500 MHz) and Bruker Avance 500 Cryo Ultrashield (500 MHz), Bruker Avance 700 MHz Fourier transform spectrometers in deuterochloroform or deuterodimethyl sulfoxide operating at 400, 500 and 700 MHz respectively and using an internal deuterium lock at ambient probe temperatures (unless otherwise stated). Chemical shifts (δ) are quoted in ppm, to the nearest 0.01 ppm and are referred to the residual non-deuterated solvent peak. Coupling constants (*J* values) are given in Hz and were calculated using 'Mestre-C 2.3a' software rounded to the nearest 0.5 Hz.[103] Data are reported as follows: chemical shift, multiplicity [exch, exchange; br, broad; s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; sept, septet; m, multiplet; or as a combination of these (e.g. dd, dt *etc*.)], integration, assignment and coupling constant(s). The

numbering/lettering on selected structures does not follow the IUPAC naming system and is used for the assignment of the ^{1}H NMR. Proton assignments were determined either on the basis of unambiguous chemical shift or coupling pattern, by analogy to fully interpreted spectra for related compounds. Diastereotopic protons are assigned as H and *H*.

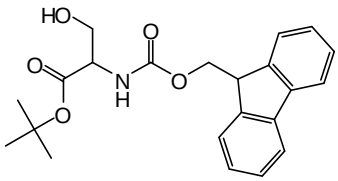
Analytical LC-MS spectra were recorded on an HP/Agilent MSD LC-MS APCI 120-1000 full gradient ACq T = 1 min 1µL. High resolution mass measurements were made using a Micromass Quadrapole-Time of Flight (Q-ToF) spectrometer and reported mass values are within the error limits of ±5 ppm mass units. The ionisation technique used is indicated by the following abbreviations: CI = chemical ionisation; EI = electron ionisation; ESI = electrospray ionisation; FAB (LSIMIS) = fast atom bombardment (liquid secondary ion mass spectrometry); MALDI = matrix-assisted laser desorption /ionisation.

Semi-preparative HPLC putifications were performed on an Agilent HP 1100 series chromatograph (Supelco ABZ+PLUS, 10cm x 2.1mm, 5µm) attached to a HP MSD mass spectrometer with a multimode ESI/APCI ionisation source in ESI/APCI mode. Elution was carried out at a flow rate of 1.0 mL/min using a reverse phase gradient of acetonitrile and water containing 0.1 % formic acid and detection was with diode array detection (λ = 195, 210, 215, 220 and 254 nm). Retention times (in min) are reported below as t_R. MALDI-TOF and MS (ESI) analysis of peptide samples were performed in a PerSeptive Biosystems Voyager DE RP, using ACH matrix, and in a Waters Micromass ZQ spectrometer and in an Agilent Ion Trap 1100 Series LC/MSDTrap.

The "&" symbol is used in the nomenclature for cyclic peptides and precursors.[104] The appearance of "&" in a given position of the one-line formula indicates the location of one end of a chemical bond and the second "&" the point to which this bond is attached. Thus, "&" represents the start or the end of a chemical bond, which is 'cut' with the aim to facilitate the view of a complex formula. In this way, two "&" symbols indicate one chemical bond.

8.5.2 Protection of amino acids and amino alcohols

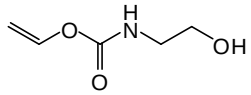
8.5.2.1 *tert*-Butyl-2-{[(9H-fluoren-9-yl)methoxy]carbonylamino}-3-hydroxy propanoate, "Fmoc-Ser-O-*t*-Bu" (1)



To a solution of Fmoc-Ser-OH (1.0 g, 3.10 mmol, 1 equiv) in EtOAc (30 ml), a solution of *t*-Butyltrichloro-acetimidate (2.6 g, 12.20 mmol, 4 equiv) in CHX (10 ml) was added at rt. The mixture was stirred at rt until TLC analysis (Hex/EtOAc 2:1) showed absence of Fmoc-L-Ser-OH (12 h). The solvents

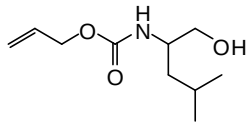
were removed under reduced pressure and the crude residue was purified by flash column chromatography (Hex/EtOAc 2:1) to afford the desired compound as a white solid (1.1 g, 2.88 mmol, 94 % yield). mp = 102-04 °C. ^{1}H NMR ($CDCl_3$) δ 7.78 (d, 2H, Ar, J = 7.5 Hz), 7.62 (d, 2H, Ar, J = 7.5 Hz), 7.42 (t, 2H, Ar, J = 7.5 Hz), 7.33 (t, 2H, Ar, J = 7.5 Hz), 6.37-6.68 (exch br s, 1H, OH) 5.79 (exch br d, 2H, NH, J = 6.5 Hz), 4.43 (d, 2H, CH_2OCO, J = 7.0 Hz), 4.32-4.38 (m, 1H, *CH*CH_2OCO), 4.24 (t, 1H, *CH*CH_2OH, J = 7.0 Hz), 3.92-3.98 (m, 2H, *CH*$_2$OH), 1.51 (s, 9H, 3 CH_3 *t*-Bu).

8.5.2.2 Allyl 2-hydroxyethylcarbamate, “Alloc-ethanolamine” (2)



A solution of ethanolamine (1.0 ml, 16.40 mmol, 1 equiv) in dioxane/H_2O (1:1, 25.0 ml) was cooled to 0 °C. $NaHCO_3$ (1.5 g, 18.00 mmol, 1.1 equiv) and allylchloroformate (1.9 ml, 18.00 mmol, 1.1 equiv) were added. The mixture was allowed to warm to rt and stirred until TLC analysis (Hex/EtOAc, 1:2) showed absence of ethanolamine (3 h). The organic solvent was removed under reduced pressure and the aqueous phase was extracted with EtOAc (3 times). The organic layers were then collected, dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was purified by flash column chromatography (Hex/EtOAc , 1:2) to yield **2** as a colourless oil (2.3 g, 15.70 mmol, 96 % yield). ^{1}H NMR ($CDCl_3$) δ 5.80-5.90 (m, 1H, *CH*CH_2O), 5.23 (dq, 1H, CH-*H*$CHCH_2$ allyl, J = 12.5 Hz, J = 1.5 Hz), 5.14 (dt, 1H, CH-*H*$CHCH_2$ allyl, J = 1.5 Hz, J = 1.0 Hz), 4.51 (d, 2H, CH_2OCO, J = 5.5 Hz), 3.65 (t, 2H, *CH*$_2$OH, J = 6.0 Hz), 3.28 (t, 2H, *CH*$_2$NH, J = 6.0 Hz).

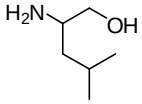
8.5.2.3 Allyl 1-hydroxy-4-methylpentan-2-ylcarbamate, “Alloc-L-leucinol” (3)



A solution of L-leucinol (**4**) (0.9 mg, 7.60 mmol, 1 equiv) in dioxane/H_2O (1:1, 25.0 ml) was cooled to 0 °C. $NaHCO_3$ (0.7 mg, 8.40 mmol, 1.1 equiv) and allylchloroformate (0.9 ml, 8.40 mmol, 1.1 equiv) were added. The mixture was allowed to warm to rt and stirred until TLC analysis (Hex/EtOAc, 1:2) showed absence of L-leucinol (3 h). The organic solvent was removed under reduced pressure and the aqueous phase was extracted with EtOAc (3 times). The organic layers were then collected, dried ($MgSO_4$) and concentrated under reduced pressure. The resulting colourless oil was purified by flash column chromatography (Hex/EtOAc 1:2) to yield **3** as a colourless oil (1.3 g, 6.56 mmol, 86 % yield). ^{1}H NMR ($CDCl_3$) δ 5.80-5.90 (m, 1H, *CH*CH_2O), 5.24 (dt, 1H, CH-*H*$CHCH_2$ allyl, J = 12.5 Hz, J = 1.5 Hz), 5.14 (dd, 1H, CH-*H*$CHCH_2$ allyl, J = 9.0 Hz, J = 1.5 Hz), 4.80 (exch br d, 1H, NH, J = 8.0 Hz), 4.49 (d, 2H, CH_2OCO, J = 5.0 Hz), 3.66-3.75 (m, 1H, *CH*NH), 3.59-3.63 (m, 1H, CH-*H*OH), 3.44-3.48 (m, 1H, CH-

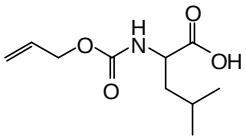
*H*OH), 2.41 (exch br s, 1H, OH), 1.57-1.64 (m, 1H, *CH*(CH_3)$_2$), 1.17-1.34 (m, 2H, *CH*$_2$CH(CH_3)$_2$), 0.87 (dd, 6H, 2 CH_3, *J* = 5.0 Hz, *J* = 1.5 Hz).

8.5.2.4 2-Amino-4-methylpentan-1-ol, "L-leucinol" (4)



Trimethylsylilchloride (3.9 ml, 30.50 mmol, 4 equiv) was added to a cold (0 °C) solution of lithium borohydride (0.3 mg, 15.30 mmol, 2 equiv) in 3 ml of anhydrous THF under N_2. The cooling ice/water bath was removed and the mixture was stirred at rt for 15 min. The mixture was cooled to 0 °C and a solution of L-Leu (1.0 g, 7.62 mmol, 1 equiv) in 3 ml of anhydrous THF was added. The cooling bath was removed and the reaction mixture was stirred at rt for 16 h. The mixture was cooled again to 0 °C and methanol (7.5 ml) was added dropwise followed by NaOH (4.1 ml, 2.5 N aqueous solution). The organic solvents were removed under reduced pressure and the resulting residue was extracted with chloroform (3 times). The combined organic extracts were dried ($MgSO_4$) and concentrated under reduced pressure to furnish **4** as a colourless oil (0.9 g, 7.59 mmol, quantitative yield). The product was analytically pure and was used in subsequent reactions without further purification. ^{1}H NMR ($CDCl_3$) δ 3.88 (dd, 1H, CH-*H*OH, *J* = 8.0 Hz, *J* = 3.0 Hz), 3.41-3.47 (m, 1H, CH-*H*OH), 3.30 (exch br s, 1H, OH), 3.11-3.16 (m, 1H, *CH*NH_2), 2.47 (exch br s, 2H, NH_2), 1.43-1.60 (m, 1H, *CH*(CH_3)$_2$), 1.05-1.15 (m, 2H, *CH*$_2$CH(CH_3)$_2$), 0.75-0.81 (m, 6H, 2 CH_3).

8.5.2.5 2-(Allyloxycarbonylamino)-4-methylpentanoic acid, "Alloc-L-Leu-OH" (5)

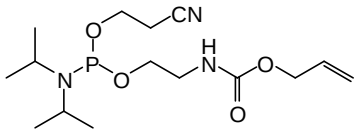


Allyl-chloroformate (0.7 ml, 6.35 mmol, 1 equiv) was dissolved in dioxane (4.0 ml) and a solution of NaN_3 (0.6 g, 9.52 mmol, 1.5 equiv) in H_2O (3.0 ml) was added at rt. The reaction mixture was stirred at rt for 1 h whereupon a solution of L-Leu (1.0 g, 7.62 mmol, 1.2 equiv) in 1% aqueous Na_2CO_3/dioxane 1:1 (20.0 ml) was added. The pH of the reaction was monitored periodically and 10% aqueous Na_2CO_3 was added when necessary to keep the pH between 8-10. The reaction was stirred at rt until TLC analysis (Hex/EtOAc, 1:4) showed absence of the allyl-chloroformate (24 h). Then the mixture was poured into water (100.0 mL), 10% aqueous Na_2CO_3 was added to keep the pH between 9 and 10 and the aqueous phase was extracted several times with *tert*-butyl methyl ether to remove the by-products (e.g. azidoformate, protected dipeptides). The aqueous solution was acidified to pH 2.0 with 2 N aqueous HCl and extracted with EtOAc (3 times). The organic phase was dried ($MgSO_4$) and concentrated under reduced pressure to yield a colourless oil (1.1 g, 4.8 mmol, 77% yield). The purity of the colorless oil was measured by NMR and it was > 95 %. The product was used for the synthesis of **20** without further

purification. ^{1}H NMR ($CDCl_3$) δ 10.34 (exch br s, 1H, OH), 5.88-6.02 (m, 1H, *CH*CH_2O), 5.32 (dd, 1H, CH-*H*$CHCH_2$ allyl, J = 17.0 Hz, J = 1.0 Hz), 5.23 (dd, 1H, CH-*H*$CHCH_2$ allyl, J = 9.0 Hz, J = 1.29 Hz), 5.19 (exch br s, 1H, NH), 4.60 (d, 2H, CH_2OCO, J = 5.5 Hz), 4.37-4.44 (m, 1H, *CH*NH), 1.68-1.81 (m, 2H, *CH*$_2$CH$(CH_3)_2$), 1.53-1.62 (m, 1H, *CH*$(CH_3)_2$), 0.98 (d, 6H, 2 CH_3, J = 6.5 Hz).

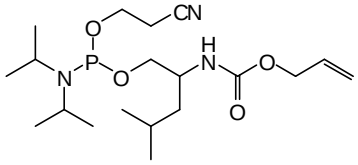
8.5.3 Phosphorus couplings

8.5.3.1 Allyl-2-(2-cyanoethoxydiisopropylaminophosphinooxy)ethyl carbamate (6)



To a solution of **2** (0.3 g, 1.80 mmol, 1 equiv) in anhydrous CH_2Cl_2 (8.0 ml) under N_2, DIPEA (2.1 ml, 12.60 mmol, 7 equiv) and 2-cyanoethyl-N,N-diisopropyl-chloro-phosphoramidite (0.6 ml, 2.70 mmol, 1.5 equiv) were added at rt. The reaction mixture was stirred at rt until TLC analysis (Hex/EtOAc 1:1) showed absence of **2** (1.5 h). The solvent was removed under reduced pressure. The residue was then dissolved in EtOAc and washed with brine (3 times). The organic phase was dried ($MgSO_4$) and concentrated under reduce pressure. The resulting colourless oil was purified by flash column chromatography (Hex/EtOAc 1:1) to furnish **6** as a colourless oil (0.5 g, 1.49 mmol, 83 %). ^{1}H NMR ($CDCl_3$) δ 5.81-5.90 (m, 1H, *CH*CH_2O), 5.23 (dd, 1H, CH-*H*$CHCH_2$ allyl, J = 5.5 Hz, J = 1.5 Hz), 5.14 (dd, 1H, CH-*H*$CHCH_2$ allyl, J = 10.45 Hz, J = 1.5 Hz), 5.09 (exch br s, 1H, NH), 4.50 (d, 2H, CH_2OCO, J = 5.0 Hz), 3.49-3.83 (m, 6H, *CH*$_2$CH$_2$CN + *CH*$_2$*CH*$_2$NH), 3.33 (q, 2H, 2 *CH*$(CH_3)_2$, J = 10.5 Hz), 2.58 (t, 2H, *CH*$_2$CN, J = 6.5 Hz), 1.12 (dd, 6H, 2 CH_3, J = 6.5 Hz, J = 5.5 Hz).

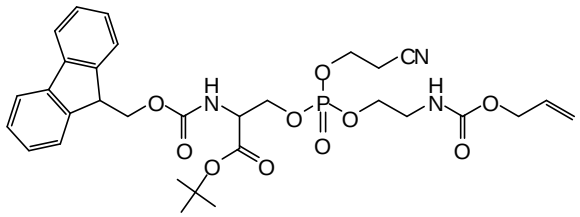
8.5.3.2 Allyl-1-(2-cyanoethoxydiisopropylaminophosphinooxy)-4-methyl pentan-2-yl-carbamate (7)



To a solution of **3** (0.6 g, 2.82 mmol, 1 equiv) in anhydrous CH_2Cl_2 (10.0 ml) under N_2, DIPEA (3.3 ml, 19.76 mmol, 7 equiv) and 2-cyanoethyl-N,N-diisopropyl-chlorophosphoramidite (0.9 ml, 4.23 mmol, 1.5 equiv) were added at rt. The reaction mixture was stirred at rt until TLC analysis (Hex/EtOAc 1:1) showed absence of **3** (1.5 h). The solvent was removed under reduced pressure. The residue was then dissolved in EtOAc and washed with brine (3 times). The organic phase was dried ($MgSO_4$) and concentrated under reduce pressure. The resulting colourless oil was purified by flash column chromatography (Hex/EtOAc 1:1) to furnish **7** as a colourless oil (1.0 g, 2.55 mmol, 90 % yield). ^{1}H NMR ($CDCl_3$) δ 9.19 (exch br s, 1H, NH), 5.80-5.96 (m, 1H, *CH*CH_2O), 5.23 (dq, 1H, CH-*H*$CHCH_2$

allyl, *J* = 12.5 Hz, *J* = 1.5 Hz), 5.17-5.12 (m, 1H, CH-*H*CHCH$_2$ allyl), 4.47-4.53 (m, 2H, CH$_2$OCO), 4.21-4.27 (m, 1H, *CH*NH), 3.96-4.12 (m, 2H, *CH$_2$*CH$_2$CN), 3.71-3.89 (m, 2H, *CH$_2$*CHNH), 3.50-3.57 (m, 2H, 2 *CH*(CH$_3$)$_2$), 2.63-2.72 (m, 1H, *CH*-H-CN), 2.53-2.60 (m, 1H, *CH*-H-CN), 1.54-1.66 (m, 1H, CH$_2$*CH*(CH$_3$)$_2$), 1.17-1.19 (m, 2H, CH*CH$_2$*CH), 1.11 (dd, 12H, 2 x *(CH$_3$)$_2$*CHN, *J* = 4.0 Hz, *J* = 3.0 Hz), 0.86 (dt, 6H, 2 x *CH$_3$*CHCH$_2$, *J* = 3.5 Hz, *J* = 3.0 Hz).

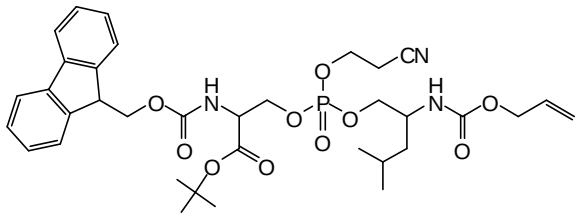
8.5.3.3 *tert*-Butyl-8-(2-cyanoethoxy)-1-(9*H*-fluoren-9-yl)-3,13-dioxo-2,7,9,14-tetraoxa-4,12-diaza-8-phosphoryloxyheptadec-16-ene-5-carboxylate (10)



To a solution of **6** (500.0 mg, 1.50 mmol, 1 equiv) in anhydrous THF (6.0 ml) under N$_2$, a solution of tetrazole (130.0 mg, 1.80 mmol, 1.2 equiv) in anhydrous THF (6.0 ml) was added at rt. The solution was stirred at rt for 15 min and then added dropwise to a previously prepared solution of **1** (580.0 g, 1.50 mmol, 1 equiv) in anhydrous THF (8.0 ml) under N$_2$ at rt. The mixture was stirred at rt until TLC analysis (Hex/EtOAc 1:3) showed absence of **6** (15 h). The solvent was then removed under reduced pressure and the resulting residue was dissolved in EtOAc and washed with 10 % aqueous NaHCO$_3$ (2 times) and brine. The organic layer was dried (MgSO$_4$) and concentrated under reduced pressure to yield the corresponding unstable phosphite **8** (940.0 mg, 1.50 mmol, quantitative yield).

To a solution of **8** (940.0 g, 1.50 mmol, 1 equiv) in anhydrous THF (15.0 ml) under N$_2$, *t*-butylhydroperoxide (0.3 ml, 3.00 mmol, 2 equiv) was added at rt. The reaction was stirred at rt until TLC analysis (Hex/EtOAc 1:3) showed absence of the phosphite (2 h). The solvent was then removed under reduced pressure. The residue was dissolved in EtOAc and washed with 10% aqueous NaHCO$_3$ (2 times) and brine. The organic layer was dried (MgSO$_4$) and concentrated under reduced pressure. The resulting colourless oil was then purified by flash column chromatography (Hex/EtOAc gradient 1:1 to 1:3) to afford **10** as a clear oil (540 mg, 0.84 mmol, 57 % yield over 2 steps from **6**). ^{1}H NMR (CDCl$_3$) δ 7.69 (d, 2H, Ar, *J* = 7.5 Hz), 7.55 (t, 2H, Ar, *J* = 6.0 Hz), 7.33 (t, 2H, Ar, *J* = 7.5 Hz), 7.24 (t, 2H, Ar, *J* = 11.0 Hz), 5.77-5.89 (m, 1H, *CH*CH$_2$O allyl), 5.32 (exch br s, 1H, NH), 5.20 (d, 1H, CH-*H*CHCH$_2$ allyl, *J* = 17.0 Hz), 5.11 (d, 1H, CH-*H*CHCH$_2$ allyl, *J* = 10.5 Hz), 4.48 (d, 2H, CH$_2$OCO allyl, *J* = 4.5 Hz), 4.30-4.42 (m, 4H, CH*CH$_2$*OCO + *CH$_2$*CHCOO), 4.10-4.18 (m, 4H, *CH$_2$*CH$_2$CN + *CH$_2$*CH$_2$NH), 4.02-4.08 (m, 3H, CH$_2$*CH$_2$*NH + *CH*CH$_2$OCO), 3.38 (d, 1H, CH$_2$*CH*COO, *J* = 5.0 Hz), 2.63 (t, 2H, CH$_2$*CH$_2$*CN, *J* = 6.0 Hz), 1.42 (s, 9H, 3 CH$_3$-CO). MS (ESI) calcd. For C$_{31}$H$_{38}$N$_3$O$_{10}$P, 643.621. Found: *m/z* 644.13 [M + H]$^+$, 588.50 [M + H - *t*-Bu]$^+$.

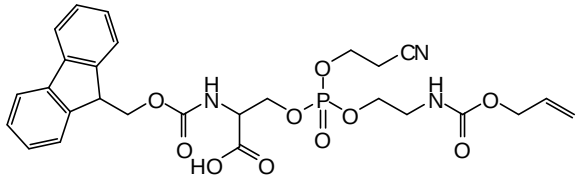
8.5.3.4 *tert*-Butyl-8-(2-cyanoethoxy)-1-(9*H*-fluoren-9-yl)-11-isobutyl-3,13-dioxo-2,7,9,14-tetraoxa-4,12-diaza-8-phosphoryloxyheptadec-16-ene-5-carboxylate (11)



To a solution of **7** (1.0 g, 2.54 mmol, 1 equiv) in anhydrous THF (8.0 ml) under N_2, a solution of tetrazole (0.2 g, 3.05 mmol, 1.2 equiv) in anhydrous THF (8.0 ml) was added at rt. The solution was stirred at rt for 15 min and then added dropwise to a previously prepared solution of **1** (1.0 g, 2.54 mmol, 1 equiv) in anhydrous THF (9.0 ml) under N_2 at rt. The mixture was stirred at rt until TLC analysis (Hex/EtOAc 1:3) showed absence of **7** (15 h). The solvent was then removed under reduced pressure and the resultant residue was dissolved in EtOAc and washed with 10 % aqueous $NaHCO_3$ (2 times) and brine. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The crude mixture of the phosphite **9** was directly used without purification for the following oxidation reaction to get the compound **11**, as already seen for the analogue unstable compound **8** (1.7 g, 2.54 mmol, quantitative yield).

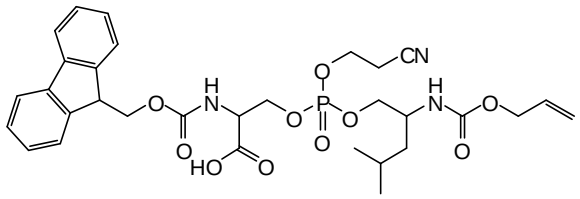
To a solution of **9** (1.7 g, 2.54 mmol, 1 equiv) in anhydrous THF (15.0 ml) under N_2, *t*-butylhydroperoxide (0.5 ml, 5.08 mmol, 2 equiv) was added at rt. The reaction was stirred at rt until TLC analysis (Hex/EtOAc 1:2) showed absence of the phosphite (2.5 h). The solvent was then removed under reduced pressure. The residue was dissolved in EtOAc and washed with 10% aqueous $NaHCO_3$ (2 times) and brine. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The resulting colourless oil was then purified by flash column chromatography (Hex/EtOAc gradient 1:1 to 1:2) to afford **11** as a clear oil (350.0 mg, 0.50 mmol, 20 % yield over 2 steps from **7**). ^{1}H NMR ($CDCl_3$) δ 8.62 (exch br s, 1H, NH), 7.78 (d, 2H, Ar, J = 7.5 Hz), 7.64 (d, 2H, Ar, J = 7.0 Hz), 7.42 (t, 2H, Ar, J = 7.5 Hz), 7.33 (t, 2H, Ar, J = 7.5 Hz), 5.88-5.99 (m, 1H, *CH*CH_2O allyl), 5.30 (dq, 1H, CH-*H*CHCH_2 allyl, J = 5.5 Hz, J = 1.5 Hz), 5.18-5.24 (m, 1H, CH-*H*CHCH_2 allyl), 4.56 (d, 2H, CH_2OCO allyl, J = 5.0 Hz), 4.37-4.45 (m, 2H, *CH*$_2$CHCOO), 4.15-4.29 (m, 2H, CH*CH*$_2$CO), 4.10-4.16 (m, 2H, *CH*$_2$CH_2CN), 3.96 (d, 2H, *CH*$_2$CHNH, J = 5.5 Hz), 3.88-3.97 (m, 1H, *CH*CH_2OCO), 3.74-3.77 (m, 1H, CH_2*CH*COO), 3.39-3.51 (m, 1H, CH_2*CH*NH), 2.61 (t, 2H, CH_2*CH*$_2$CN, J = 6.0 Hz), 1.65-1.72 (m, 1H, *CH*CH_3), 1.52 (s, 9H, 3 CH_3-CO), 1.33-1.44 (m, 2H, CH*CH*$_2$CH), 0.95 (d, 6H, 2 *CH*$_3$CH, J = 6.5 Hz). MS (ESI) calcd. For $C_{35}H_{46}N_3O_{10}P$, 699.73. Found: *m/z* 700.17 $[M + H]^+$, 644.50 $[M + H - t\text{-Bu}]^+$, 616.16 $[M + H - \text{Alloc}]^+$.

8.5.3.5 8-(2-Cyanoethoxy)-1-(9*H*-fluoren-9-yl)-3,13-dioxo-2,7,9,14-tetraoxa-4,12-diaza-8-phosphoryloxyheptadec-16-ene-5-carboxylic acid (12)



TFA (1.3 ml, 16.93 mmol, 20 equiv) was added to a solution of **10** (540 mg, 0.85 mmol, 1 equiv) in CH_2Cl_2 (1.3 ml) at rt. The reaction was stirred at rt until TLC analysis (Hex/EtOAc 1:3) showed absence of **10** (2.5 h). The excess TFA was removed by co-evaporation with CH_2Cl_2 under reduced pressure (5 times). The residue was dried in a vacuum desiccator o/n to yield **12** as a colourless oil (480.0 mg, 0.82 mmol, 97 %). The compound was used in subsequent SPPS without further purification. ^{1}H NMR ($CDCl_3$) δ 8.20 (exch br s, 1H, NH), 7.69 (d, 2H, Ar, J = 7.5 Hz), 7.52 (d, 2H, Ar, J = 4.5 Hz), 7.33 (t, 2H, Ar, J = 7.5 Hz), 7.24 (t, 2H, Ar, J = 7.0 Hz), 5.80-5.84 (m, 1H, *CH*CH_2O allyl), 5.23 (d, 1H, CH-*H*$CHCH_2$ allyl, J = 5.0 Hz), 5.13 (d, 1H, CH-*H*$CHCH_2$ allyl, J = 4.0 Hz), 4.50-4.57 (m, 4H, CH_2OCO allyl, CH*CH_2*OCO), 4.32-4.38 (m, 3H, *CH_2*CH_2CN + *CH*CH_2OCO), 4.04-4.16 (m, 6H, + *CH_2*CHCOO + *CH_2CH_2*NH), 3.28-3.43 (m, 1H, CH_2*CH*COO), 2.65 (t, 2H, CH_2*CH_2*CN, J = 5.5 Hz). MS (ESI) calcd. For $C_{27}H_{30}N_3O_{10}P$, 587.51. Found: *m/z* 588.06 $[M + H]^+$.

8.5.3.6 8-(2-Cyanoethoxy)-1-(9*H*-fluoren-9-yl)-11-isobutyl-3,13-dioxo-2,7,9,14-tetraoxa-4,12-diaza-8-phosphoryloxyheptadec-16-ene-5-carboxylic acid (13)



TFA (0.7 ml, 9.66 mmol, 20 equiv) was added to a solution of **11** (0.3 g, 0.48 mmol, 1 equiv) in CH_2Cl_2 (0.7 ml) at rt. The reaction was stirred at rt until TLC analysis (Hex/EtOAc 1:3) showed absence of **11** (2.5 h). The excess TFA was removed by co-evaporation with CH_2Cl_2 under reduced pressure (5 times). The residue was dried in a vacuum desiccator o/n to yield **13** as a colourless oil (0.3 g, 0.47 mmol, 97 %). The compound was used in subsequent SPPS without further purification. ^{1}H NMR ($CDCl_3$) δ 7.99 (exch br s, 1H, NH), 7.69 (d, 2H, Ar, J = 7.5 Hz), 7.56 (t, 2H, Ar, J = 6.0 Hz), 7.33 (t, 2H, Ar, J = 7.5 Hz), 7.24 (t, 2H, Ar, J = 14.0 Hz) 5.79-5.88 (m, 1H, *CH*CH_2O allyl), 5.23 (d, 1H, CH-*H*$CHCH_2$ allyl, J = 17.0 Hz), 5.13 (d, 1H, CH-*H*$CHCH_2$ allyl, J = 10.0 Hz), 4.47-4.58 (m, 3H, CH_2OCO allyl + *CH*CH_2OCO), 4.26-4.35 (m, 2H, *CH_2*CHCOO), 4.10-4.19 (m, 2H, CH*CH_2*CO), 4.00-4.08 (m, 1H, CH_2*CH*COO), 3.77-3.96 (m, 4H, *CH_2*CHNH + *CH_2*CH_2CN), 3.34-3.43 (m, 1H, CH_2*CH*NH), 2.63-2.70 (m, 2H, CH_2*CH_2*CN),

1.58-1.64 (m, 1H, *CH*CH_3), 1.28 (t, 2H, CH*CH*$_2$CH, *J* = 2.0 Hz), 0.87 (d, 6H, 2 *CH*$_3$CH, *J* = 6.5 Hz). MS (ESI) calcd. For $C_{31}H_{38}N_3O_{10}P$, 643.62. Found: *m/z* 644.14 $[M + H]^+$.

8.5.4 Total synthesis of the TS mimetic

8.5.4.1 H-Phe-O-CTC (14)

Cl-TrtCl-resin (0.2 g, 1.01 mmol/g) was placed in a 20 ml polypropylene syringe fitted with a polyethylene filter disk. The resin was then washed with CH_2Cl_2 (5 x 0.5 min) and a solution of Fmoc-L-Phe-OH (54.2 mg, 0.14 mmol, 0.7 equiv) and DIPEA (70.0 µL, 0.42 mmol, 2.1 equiv) in CH_2Cl_2 (2.5 ml) was added. The mixture was then stirred for 15 min. Extra DIPEA (140.0 µL, 0.85 mmol, 4.2 equiv) was added and the mixture was stirred for an additional 45 min. The reaction was stopped by adding MeOH (320.0 µl) and stirred for 10 min. The aim of this procedure was to obtain a resin loading of 0.7 mmol/g. The Fmoc-L-Phe-O-TrtCl-resin was subjected to the following washings/treatments with CH_2Cl_2 (3 x 0.5 min), DMF (3 x 0.5 min) and piperidine-DMF solution to remove the Fmoc as indicated in the following General Procedure for Fmoc-removal (**section 8.5.4.2**).

8.5.4.2 General Procedure for Fmoc-removal: the Fmoc group was removed by treatment with piperidine/DMF (2:8, v/v) (1 x 2 min, 2 x 10 min). Washings between deprotection, coupling, and again deprotection steps were performed with DMF (5 x 0.5 min) and CH_2Cl_2 (5 x 0.5 min) using 10.0 ml solvent/g resin each time.

8.5.4.3 H-Asp(O-*t*-Bu)-Phe-O-CTC (15)

Fmoc-L-Asp(O-*t*-Bu)-OH (230.4 mg, 0.56 mmol, 4 equiv), DIC (86.8 µL, 0.56 mmol, 4 equiv) and Ethylcyanoglyoxylate-2-oxime (79.6 mg, 0.56 mmol, 4 equiv) in DMF (2.5 ml) were added to the above obtained H-Phe-O-CTC (**14**). After 90 min of coupling, the chloranil test was negative. Removal of Fmoc group and washings were performed as described in General Procedures for Fmoc-removal (**section 8.5.4.2**).

8.5.4.4 H-Ser[P(OC$_2$H$_4$-NH-Alloc)O$_2$H]-Asp(O-*t*-Bu)-Phe-O-CTC (16)

Fmoc-Ser[P(OC$_2$H$_4$CN)(OC$_2$H$_4$-NH-Alloc)O$_2$H] **(12)** (246.8 mg, 0.42 mmol, 3 equiv), PyAOP (218.9 mg, 2.5 mmol, 3 equiv) and DIPEA (308.2 µL, 1.86 mmol, 9 equiv) were added to the above obtained H-Asp(O-*t*-Bu)-Phe-O-CTC (**15**). After 2 h, the peptidyl-resin was subjected to the following washings/treatments with CH_2Cl_2 (3 x 0.5 min), DMF (3 x 0.5 min). Before the piperidine treatment for Fmoc-removal, an aliquot of the peptidyl-resin was treated with TFA/Et_3SiH/CH_2Cl_2 (1:1:98) and TFA/Et_3SiH/H_2O (90:5:5). The HPLC analysis (t_R = 4.34 min) of the crude obtained after evaporation showed a purity of > 95%. MS (ESI) calcd. For $C_{44}H_{51}N_4O_{15}P$, 906.87. Found: *m/z* 850.11 $[M - t\text{-Bu}]^+$. Removal of Fmoc group and washings were performed as described in General Procedures for Fmoc-removal (**section 8.5.4.2**).

8.5.4.5 H-Asn-Ser[P(OC_2H_4-NH-Alloc)O_2H]-Asp(O-*t*-Bu)-Phe-O-CTC (17)

Fmoc-L-Asn-OH (198.4 mg, 0.56 mmol, 4 equiv) was added to the above obtained H-Ser[P(OC_2H_4-NH-Alloc)O_2H]-Asp(O-*t*-Bu)-Phe-O-CTC **(16)** using DIC (86.8 µL, 0.56 mmol, 4 equiv) and Ethylcyanoglyoxylate-2-oxime (79.6 mg, 0.56 mmol, 4 equiv) in DMF (2.5 ml). After 90 min of coupling, the chloranil test was negative. Removal of Fmoc group and washings were performed as described in General Procedures for Fmoc-removal (**section 8.5.4.2**).

8.5.4.6 Boc-Ile-Asn-Ser[P(OC_2H_4-NH-Alloc)O_2H]-Asp(O-*t*-Bu)-Phe-O-CTC (18)

Boc-L-Ile-OH (129.4 mg, 0.56 mmol, 4 equiv) was added to the above obtained H-Asn-Ser[P(OC_2H_4-NH-Alloc)O_2H]-Asp(O-*t*-Bu)-Phe-O-CTC **(17)** using DIC (86.8 µL, 0.56 mmol, 4 equiv) and ethylcyanoglyoxylate-2-oxime (79.6 mg, 0.56 mmol, 4 equiv) in DMF (2.5 ml). After 90 min of coupling, the chloranil test was positive and the coupling was repeated again in the same conditions. An aliquot of the peptidyl-resin was treated with TFA/Et_3SiH/CH_2Cl_2 (1:1:98) and TFA/Et_3SiH/H_2O (90:5:5). The HPLC analysis (t_R = 3.27 min) of the crude obtained after evaporation showed a purity of > 90 %. MS (ESI) calcd. For $C_{40}H_{59}N_6O_{18}P$, 942,90. Found: *m/z* 801.11 [M - H - Boc and *t*-Bu]$^-$.

8.5.4.6 General procedure for Alloc-removal: Boc-Ile-Asn-Ser[P(OC_2H_4-NH_2)O_2H]-Asp(O-*t*-Bu)-Phe-O-CTC (19). The Alloc group of the peptide resin (**18**) was removed with $Pd(PPh_3)_4$ (16.2 mg, 0.04 mmol, 0.1 equiv) in the presence of $PhSiH_3$ (520. µL, 4.21 mmol, 10 equiv) and the resin was washed with CH_2Cl_2 (3 x 0.5 min), DMF (3 x 0.5 min), 0.02 M sodium diethyldithiocarbamate in DMF (3 x 15 min) and DMF (3 X 0.5 min).

8.5.4.7 Boc-Ile-Asn-Ser[P(OC_2H_4-NH-Leu)O_2H]-Asp(O-*t*-Bu)-Phe-O-CTC (20)

Alloc-L-Leu-OH (120.5 mg, 0.56 mmol, 4 equiv) was coupled to the above obtained Boc-Ile-Asn-Ser[P(OC_2H_4-NH_2)O_2H]-Asp(O-*t*-Bu)-Phe-O-CTC **(19)** using DIC (86.8 µL, 0.56 mmol, 4 equiv) and ethylcyanoglyoxylate-2-oxime (79.6 mg, 0.56 mmol, 4 equiv) in DMF (2.5 ml). After 90 min of coupling, the chloranil test was negative. The peptidyl-resin was subjected to the following washings/treatments with CH_2Cl_2 (3 x 0.5 min) and DMF (3 x 0.5 min). Removal of Alloc group and washings were performed as already described in General Procedures for Alloc-removal (**section 8.5.4.6**).

8.5.4.8 Boc-Ile-Asn-Ser[P(OC_2H_4-NH-Leu-NH_2)O_2H]-Asp(O-*t*-Bu)-Phe-OH (21)

The protected and branched polymer-bound peptide (**20**) was cleaved from the resin by treatment with TFA/Et_3SiH/CH_2Cl_2 (1:1:98, 2.0 ml) for 1 h. Partial purification of **21** was accomplished by filtration of the acidic resin suspension in 4.5 ml of pyridine-methanol (1.5:75 v/v) solution and evaporation of the filtrate to dryness in vacuo. A small title of the dried partially protected peptide **21** was exposed to TFA/H_2O/Et_3SiH (90:5:5) for 1 h to remove the protecting groups and the HPLC (t_R = 11.35 min) of the crude obtained after evaporation showed a purity of > 80%. MS (ESI) calcd. For $C_{42}H_{67}N_8O_{16}P$, 971,00.

Found: *m/z* 830.18 [M - Boc and *t*-Bu]$^+$. MS (MALDI) calcd. for $C_{42}H_{67}N_8O_{16}P$, 971,00. Found: *m/z* 831.30 [M + 1 - Boc and *t*-Bu]$^+$. The product was used without further purification.

8.5.4.9 Boc-Ile-Asn-Ser{P[OC$_2$H$_4$-NH-Leu(&)]O$_2$H}-Asp(O-*t*-Bu)-Phe(&) (22)

Procedure A (entry 3 in table 8.1, section 8.3.2.2): The protected peptide (**21**) (90.0 mg, 0.09 mmol, 1 equiv) was dissolved in $CHCl_3$ (45.6 ml, 2 mM solution) and *N*-polystyrene methyl-*N'*-cyclohexylcarbodiimide (197.8 mg, 2.3 mmol/g, 5 equiv) and DMAP (1.10 mg, 0.01 mmol, 0.1 equiv) were added. The mixture was stirred for 3 d and the course of the cyclization was checked by HPLC (t_R = 9.34 min). The solid supported corbodiimide was then filtered and the solvent was removed by evaporation in vacuo.

Procedure B (entry 11 in table 8.1, section 8.3.2.2): The protected peptide (**21**) (25.0 mg, 0.02 mmol, 1 equiv) was dissolved in CH_2Cl_2 (9.0 mL, 2 mM solution) and DIC (15.8 µl, 0.10 mmol, 5 equiv) and HOAt (17.0 mg, 0.12 mmol, 0.1 equiv) were added. The mixture was stirred for 1 d. The course of the cyclization was checked by HPLC (t_R = 9.34 min) and the solvent was removed by evaporation under reduced pressure.

8.5.4.10 H-Ile-Asn-Ser{P[OC$_2$H$_4$-NH-Leu(&)]O$_2$H}-Asp-Phe(&) (23)

In both cyclization reactions the protected cyclic peptide (**22**) (87.0 mg, 0.09 mmol, 1 equiv) was exposed to TFA-mediated acidolytic treatment for 1.5 h with TFA/H_2O/Et_3SiH (90:5:5, 4 ml). The HPLC (t_R = 13.75 min) of the crude obtained after evaporation showed a purity of < 5 %. MS (ESI) calcd. For $C_{34}H_{53}N_8O_{13}P$, 812,80. Found: *m/z* 813.28 [M + 1]$^+$, 825.50 [M + Na]$^+$. MS (MALDI) calcd. for $C_{34}H_{53}N_8O_{13}P$, 812,80. Found: *m/z* 813.29 [M + 1]$^+$. After semipreparative HPLC (t_R = 11.04 min) the product **23** was not pure enough for the biological tests.

8.5.5 Total synthesis of the AIP-III

8.5.5.1 H-Leu-O-CTC (24)

Cl-TrtCl-resin (0.2 g, 1.01 mmol/g) was placed in a 20.0 ml polypropylene syringe fitted with a polyethylene filter disk. The resin was then washed with CH_2Cl_2 (5 x 0.5 min), and a solution of Fmoc-L-Leu-OH (49.4 mg, 0.14 mmol, 0.7 equiv) and DIPEA (70.0 µl, 0.42 mmol, 2.1 equiv) in CH_2Cl_2 (2.5 ml) was added. The mixture was then stirred for 15 min. Extra DIPEA (140.0 µL, 0.85 mmol, 4.2 equiv) was added and the mixture was stirred for an additional 45 min. The reaction was stopped by adding MeOH (320.0 µl) and stirred for 10 min. The aim of this procedure was obtaining a resin loading of 0.7 mmol/g. The Fmoc-L-Leu-O-TrtCl-resin was subjected to the following washings/treatments with CH_2Cl_2 (3 x 0.5 min), DMF (3 x 0.5 min), piperidine-DMF solution to remove the Fmoc as indicated in the General Procedure for Fmoc-removal (**section 8.5.4.2**).

8.5.5.2 Boc-Ile-Asn-Cys(Mmt)-Asp(O-*t*-Bu)-Phe-Leu-Leu-O-CTC (25)

Fmoc-L-Leu-OH (198.00 mg, 0.56 mmol, 4 equiv), Fmoc-L-Phe-OH (217.00 mg, 0.56 mmol, 4 equiv), Fmoc-L-Asp(O-*t*-Bu)-OH (230.40 mg, 0.56 mmol, 4 equiv), Fmoc-L-Cys(Mmt)-OH (344.80 mg, 0.56 mmol, 4 equiv), Fmoc-L-Asn-OH (198.40 mg, 0.56 mmol, 4 equiv) and Boc-L-Ile-OH (129.40 mg, 0.56 mmol, 4 equiv) were added sequentially to the above obtained H-Leu-O-TrtCl-resin (**24**) using DIC (86.80 µL, 0.56 mmol, 4 equiv) and ethylcyanoglyoxylate-2-oxime (79.60 mg, 0.56 mmol, 4 equiv) in DMF (2.5 mL). After 90 min shaking, with the exception for Fmoc-L-Cys(Mmt)-OH (2 h), the chloranil test was negative for all the amino acids coupled. After each coupling removal of Fmoc group and washings were performed as described in General Procedures for Fmoc-removal (**section 8.5.4.2**), with the exception of Boc-L-Ile-OH coupling.

8.5.5.3 Boc-Ile-Asn-Cys-Asp(O-*t*-Bu)-Phe-Leu-Leu-OH (26)

The protected linear polymer-bound peptide (**25**) was cleaved from the resin by treatment with TFA/Et_3SiH/CH_2Cl_2 (1:1:98, 3 ml) for 40 min. Partial purification of **26** was accomplished by filtration of the acidolytic resin suspension in 4.5 ml of pyridine-methanol (1.5:75 v/v). After evaporation of the solvents, the residue was triturated with ice-water, filtrated and dried under reduced pressure. A small title of the dried partially protected peptide **26** was exposed to TFA/H_2O/Et_3SiH (90:5:5) for 1 h to remove the protecting groups and HPLC analysis (t_R = 3.69 min) of the crude obtained after evaporation showed a purity of > 90%. MS (ESI) calcd. For $C_{47}H_{76}N_8O_{13}S$, 993,22. Found: *m/z* 837.27 [M - Boc and *t*-Bu]$^+$. The product was used without further purification.

8.5.5.4 Boc-Ile-Asn-Cys(&)-Asp(O-*t*-Bu)-Phe-Leu-Leu(&) (27)

The protected peptide (**26**) (80.00 mg, 0.08 mmol, 1 equiv) was dissolved in $CHCl_3$ (40.28 mL, 2 mM solution) and EDC (76.70 mg, 0.40 mmol, 5 equiv) and DMAP (1.00 mg, 0.008 mmol, 0.1 equiv) were added. The mixture was stirred for 3 d. The course of the cyclization was checked by HPLC (t_R = 3.88 min). The residue was then extracted twice with 5% citric acid solution to remove the excess of carbodiimide and washed with water. The organic phase was concentrated by evaporation under reduced pressure.

8.5.5.5 H-Ile-Asn-Cys(&)-Asp-Phe-Leu-Leu(&) (28) (AIP-III)

The protected cyclic peptide (**27**) (78.00 mg, 0.08 mmol, 1 equiv) was exposed to TFA-mediated acidolytic treatment for 1.5 h with TFA:H_2O:Et_3SiH (90:5:5, 4 ml). The crude obtained was dissolved in 5 ml of water and washed with $CHCl_3$. After evaporation under reduced pressure of the aqueous phase, the crude residue was purified by semi-preparative HPLC (t_R = 3.97 min) and obtained with 10-15% yield. Following HPLC analysis showed a purity between 80 and 90 %. MS (ESI) calcd. For $C_{38}H_{58}N_8O_{10}S$, 818,98. Found: *m/z* 819.30 [M + 1]$^+$.

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