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ELINA JARHO

Synthesis, Structure-Activity Relationships and Physico-Chemical Properties of Novel Prolyl Oligopeptidase Inhibitors

Doctoral dissertation

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ABSTRACT

Prolyl oligopeptidase (POP) is a widely distributed and highly conserved serine endopeptidase. It is ubiquitous in the human body and one of the most abundant peptidases in the human brain. POP degrades oligopeptides after internal prolyl residues including a prolinamide terminus. POP inhibitors have been shown to ameliorate amnesia in several animal models. This was first explained by the elevation of the brain levels of certain neuropeptides, which are *in vitro* substrates of POP. However, the results from the *in vivo* studies have been contradictory. Later, an inverse relationship between the concentration of the intracellular inositol-1,4,5-trisphosphate and POP was found. It was proposed that the effect of POP on the phosphatidylinositol pathway might explain the effects of POP inhibitors. POP inhibitors have also been shown to prevent *in vitro* the host-cell invasion of *Trypanosoma cruzi*, which causes Chagas disease.

A series of symmetrical dicarboxylic acid *bis*(L-prolyl-pyrrolidine) amides was modified at the P3 position and beyond it, resulting in dicarboxylic acid azacycle L-prolyl pyrrolidine amides. The novel compounds had lower molecular weights but their POP inhibitory activities did not differ from the parent compounds. A comparative molecular similarity indices analysis model was created in order to explain the structure-activity relationships (SAR) of these atypical POP inhibitors. Two different binding modes were found: one that favors lipophilic structures and one that favors nonhydrophobic structures. In the typical POP inhibitor structure, there is a phenyl-alkanoyl group at the P3 position. The phenyl group was replaced by pyridyl groups in order to introduce an ionizable group into the structure. The 3-pyridyl group gave compounds that were equipotent with their phenyl analogs. The 3-pyridyl group increased the water solubility but decreased the lipophilicity. However, when it was combined with the 5-*tert*-butyl-L-prolyl group at the P2 position, the theoretical optimum for lipophilicity was reached. An L-prolyl group is the favored structure at the P2 position of POP inhibitors and it forms an amide bond with the P3 aryl-alkanoyl group. The P2 L-prolyl residue was replaced by a cyclopent-2-enecarbonyl group in order to replace the P3-P2 amide bond. The α,β-unsaturated carbonyl group was a good mimetic for the amide bond. The novel compounds were equipotent but more lipophilic than their parent compounds. A pyrrolidine group is the favored structure at the P1 position of POP inhibitors and it forms an amide bond with the P2 L-prolyl group. Different 1-cycloalkenyl and aryl groups were studied in the place of the P1 pyrrolidine group. The 1-cyclopentenyl and the 2-thienyl groups were able to mimic the P1 pyrrolidine group and proved that the P1 nitrogen can be removed.

The present study provided new information of the SAR of POP inhibitors. Novel groups that were able to mimic their parent structures were found for all studied positions. The physico-chemical properties were determined for the selected compounds and it was shown that the studied groups can be used to modify these properties. However, pharmacological studies are needed to prove the usefulness of these novel groups *in vivo*.

National Library of Medicine Classification: QU 34, QU 136, QU 143, QV 744
Medical Subject Headings: Serine Endopeptidases; Enzyme Inhibitors; Serine Proteinase Inhibitors; Amides; Structure-Activity Relationship; Memory Disorders; Amnesia; Neurodegerative Diseases; Trypanosoma cruzi; Phosphatidylinositols; Drug Therapy

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Kuopio, January 2007

Elina Jarho

ABBREVIATIONS

AD	Alzheimer's disease
ADMET	absorption, distribution, metabolism, excretion and toxicity
AVP	arginine-vasopressin
BBB	blood-brain barrier
ClogP	calculated logarithm of the partition coefficient
CNS	central nervous system
CoMSIA	comparative molecular similarity indices analysis
3D QSAR	three dimensional quantitative structure-activity relationship
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DMAP	4-dimethylaminopyridine
DMP	Dess-Martin periodinane
DMSO	dimethyl sulfoxide
DPP IV	dipeptidyl-peptidase IV
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
EI	enzyme-inhibitor
ESI-MS	electrospray ionization mass spectrometry
FID	free-induction decay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GOF	goodness-of-fit
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
IC_{50}	inhibitor concentration at which the enzyme reaction velocity is 50% of the uninhibited reaction
IP_2	inositol-4,5-bisphosphate
IP_3	inositol-1,4,5-trisphosphate
IP_5	inositol-1,3,4,5,6-pentakisphosphate
ISA	ionic strength adjusted
K_i	inhibition constant
log P	logarithm of the partition coefficient
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
M_W	molecular weight
m/z	mass-to-charge ratio
NCBI	National Center for Biotechnology Information
NMR	nuclear magnetic resonance (spectroscopy)

P1...Pn	amino acid residues of substrates (or the corresponding structures of substrate-like inhibitors) which bind to the S1...Sn binding sites of the enzyme counting from the scissile bond towards the N-terminus
P1'...Pn'	amino acid residues of substrates (or the corresponding structures of substrate-like inhibitors) which bind to the S1'...Sn' binding sites of the enzyme counting from the scissile bond towards the C-terminus
PDB	protein data bank
PE	petroleum ether
PI	phosphatidylinositol
PIP_2	phosphatidylinositol-4,5-bisphosphate
pK_a	negative logarithm of the ionization constant
PLC	phospholipase C
POP	prolyl oligopeptidase
ppm	parts per million
rt	room temperature
S1...Sn	binding sites of the enzyme for the P1...Pn residues of the substrates or the substrate-like inhibitors
S1'...Sn'	binding sites of the enzyme for the P1'...Pn' residues of the substrates or the substrate-like inhibitors
SAR	structure-activity relationship
SP	substance P
Suc-Gly-Pro-AMC	*N*-succinyl-glycyl-L-prolyl-7-amino-4-methylcoumarin
Tc80	80 kDa protein of *Trypanosoma cruzi*
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMS	tetramethylsilane
TRH	thyroliberin, thyrotropin-releasing hormone
UV	ultraviolet
Z	benzyloxycarbonyl
Z-Gly-Pro-AMC	*N*-benzyloxycarbonyl-glycyl-L-prolyl-7-amino-4-methylcoumarin
ZIP	Z-Pro-prolinal insensitive peptidase

LIST OF ORIGINAL PUBLICATIONS

The present doctoral dissertation is based on the following papers:

I Jarho EM, Wallén EAA, Christiaans JAM, Forsberg MM, Venäläinen JI, Männistö PT, Gynther J, Poso A:
Dicarboxylic acid azacycle L-prolyl-pyrrolidine amides as prolyl oligopeptidase inhibitors and three-dimensional quantitative structure-activity relationship of the enzyme-inhibitor interactions.
J Med Chem 48: 4772-4782, 2005

II Jarho EM, Venäläinen JI, Huuskonen J, Christiaans JAM, Garcia-Horsman JA, Forsberg MM, Järvinen T, Gynther J, Männistö PT, Wallén EAA:
A cyclopent-2-enecarbonyl group mimics proline at the P2 position of prolyl oligopeptidase inhibitors.
J Med Chem 47: 5605-5607, 2004

III Jarho EM, Venäläinen JI, Juntunen J, Yli-Kokko AL, Vepsäläinen J, Christiaans JAM, Forsberg MM, Järvinen T, Männistö PT, Wallén EAA:
An introduction of a pyridine group into the structure of prolyl oligopeptidase inhibitors.
Bioorg Med Chem Lett 16: 5590-5593, 2006

IV Jarho EM, Venäläinen JI, Poutiainen S, Leskinen H, Vepsäläinen J, Christiaans JAM, Forsberg MM, Männistö PT, Wallén EAA:
2(*S*)-(Cycloalk-1-enecarbonyl)-1-(4-phenylbutanoyl)pyrrolidines and 2(*S*)-(aroyl)-1-(4-phenylbutanoyl)pyrrolidines as prolyl oligopeptidase inhibitors.
Bioorg Med Chem 15: 2024–2031, 2007

CONTENTS

1 INTRODUCTION

Prolyl oligopeptidase (POP) is a proline-specific peptidase that was found in the beginning of the 1970's (Walter *et al.* 1971). After that, the enzyme has been well-characterized and the crystal structure was published in 1998 (Fülöp *et al.* 1998). Several studies with specific POP inhibitors in different animal models have associated POP with memory impairments and cognitive disturbances. POP has also been implicated in mood disorders, Chagas disease and celiac disease. However, the full biological role and the different mechanisms of actions still need further studies. Some POP inhibitors have entered clinical studies but none of them has entered the market. Thus, the suggested indication(s) for the prolyl oligopeptidase inhibitors needs to be confirmed.

At the starting point of this work, a lot was known about the structure-activity relationships (SAR) of POP inhibitors. Many potent inhibitors had been published in the literature and some had also been developed by our own group. The best inhibitors had subnanomolar IC_{50} values and the focus was shifted to other issues. The aim was to introduce replacements in the typical POP inhibitor backbone that would make the molecular structure more drug-like and could be used to modify the physico-chemical properties of the inhibitors. The replacements were planned to mimic the replaced group in the POP inhibitor structure in order to maintain the POP inhibitory activity.

The first part of the dissertation is a literature review that summarizes the current knowledge of POP and its biological relevance. The amino acid proline and the properties of drug-like compounds are also briefly discussed. A more detailed summary is given about the SAR of POP inhibitors. After the literature review, each of the four articles that are included in this dissertation has its own chapter. The first and the third of the articles deal with the modifications of POP inhibitors at the P3 position and beyond it, where the structural variation is explored. The second and the fourth of the articles deal with the replacement of the P3-P2 and P2-P1 amide groups.

2 LITERATURE REVIEW

2.1 Nomenclature and classification

Prolyl oligopeptidase (EC 3.4.21.26) is classified as a serine endopeptidase. The name and the classification describe the ability of POP to degrade oligopeptides after internal prolyl residues. POP is a member of peptidase family S9, which belongs to clan C of serine peptidases. Peptidase family S9 is often referred to as prolyl oligopeptidase family and other representative members of this family are oligopeptidase B (EC 3.4.21.83), dipeptidyl-peptidase IV (DPP IV, EC 3.4.14.5) and acylaminoacyl-peptidase (EC 3.4.19.1). POP is also known as prolyl endopeptidase and post-proline cleaving enzyme in older literature (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology 2006, Rawlings *et al*. 2006).

2.2 Distribution of prolyl oligopeptidase

POP is a highly conserved enzyme that is widely distributed. It has been found in all three domains of life; Archaea, Bacteria and Eukarya. Among Eukarya, the sequences of POP have been resolved for many animals and plants (Venäläinen *et al*. 2004a). Among fungi, the existence of POP has been unclear. A similar enzyme to POP has been purified from a fungus *Agaricus bisporus* (Yoshimoto *et al*. 1990). Recently, sequences from fungi with significant alignments with human POP could also be retrieved from the NCBI protein database (National Center for Biotechnology Information 2006).

When over 20 human tissues were studied, POP activity was found in all of them (Kato *et al*. 1980). The highest activity was found in the skeletal muscle. In the brain, the highest enzyme activity was found in the frontal cortex where the activity was one sixth of that in the skeletal muscle. In another study, the highest POP activity in the human brain was found in the cortices (Irazusta *et al*. 2002). In rat, which is often used as the model animal in different performance tasks, the highest POP activity was found in the brain and the activity was distributed more homogenously throughout the brain.

POP has mainly been described as a cytosolic enzyme but a membrane bound form has also been reported (O'Leary *et al*. 1996). The membrane bound form was purified from the bovine brain and it had many characteristics similar to the cytosolic POP although some differences were also found. However, the sequence of this enzyme has not been resolved. When 12 eukaryotic POP sequences were studied, transmembraneous regions or lipid anchors that would indicate about the membrane

bound form were not found (Venäläinen *et al*. 2004a). Furthermore, it seems that a secreted form of POP also exists (Siviter and Cockle 1995, Venäläinen *et al*. 2004a).

2.3 Biological relevance of prolyl oligopeptidase

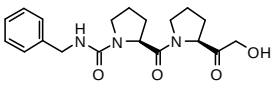
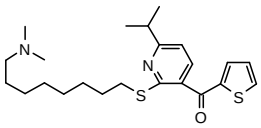
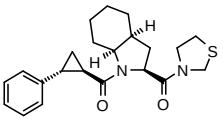
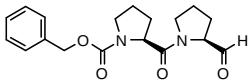
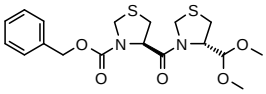
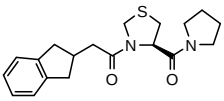
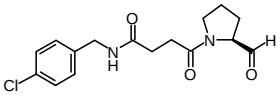
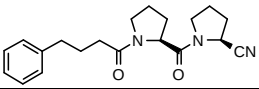
2.3.1 Memory impairments and neurodegenerative disorders

Several studies suggest that POP could be related to neurodegeneration and disturbances in memory and cognition. The expression of the POP gene was up-regulated 11-fold in the hypothalamus (Jiang *et al*. 2001) and the POP protein level was 1.3 times higher in the hippocampus (Roßner *et al*. 2005) of aged mice as compared to young and adult mice, respectively. The results of the cortex were contradictory. Furthermore, a stimulating environment decreased POP expression in the cortex of adult mice (Rampon *et al*. 2000).

Amyloid β plaques are often found in the brains of Alzheimer's disease (AD) patients. The expression of POP was studied in adult and aged transgenic mice, which express amyloid precursor protein (Roßner *et al*. 2005). In the hippocampus of the adult mice, POP expression was increased in parallel with memory deficits but before appearance of the amyloid β plaques. In contrast, the aged mice with the amyloid β deposits did not have altered enzymatic activity, which was confirmed with the AD patients. Instead, negative correlation of POP activity with the scores of senile/neuritic plaques and neurofibrillary tangles was found in the AD patients, which was suggested to reflect the extent of neuronal damage (Laitinen *et al*. 2001). Furthermore, individual neurons with strong immunohistochemical staining of POP were found to have shrunken appearance (Roßner *et al*. 2005). Two specific POP inhibitors, JTP-4819 **1** and Y-29794 **2** (Table 1), prevented accumulation of amyloid β protein *in vitro* and *in vivo*, respectively (Kato *et al*. 1997, Shinoda *et al*. 1997), although another POP inhibitor S 17092 **3** (Table 1) failed to affect amyloid β formation *in vitro* (Petit *et al*. 2000). Instead, S 17092 **3** and another POP inhibitor Z-Pro-prolinal **4** (Table 1) were able to protect cells from death in cell cultures (Shishido *et al*. 1999, Odaka *et al*. 2002).

Several POP inhibitors (Table 1) have prevented amnesia and improved performance in different memory tasks. JTP-4819 **1** improved performance of aged and adult rats, which suffered from scopolamine- or ischemia-induced amnesia (Toide *et al*. 1997). S 17092 **3** alleviated scopolamine-induced amnesia in rats and mice and improved performance of aged mice (Morain *et al*. 2002). S 17092 **3** also improved cognition of the MPTP-treated monkeys, which serve as a model for the early Parkinsonism. Some enhancement was also seen in verbal memory tests in healthy

Table 1. The structures and the potencies of POP inhibitors, which have undergone in vivo studies.

Compound	Name	Structure	Inhibitory activity Source of POP	Reference
1	JTP-4819		IC_{50} = 0.83 nM rat brain	Toide *et al*. 1995
2	Y-29794		K_i = 0.95 nM rat brain	Nakajima *et al*. 1992
3	S 17092		K_i = 1.5 nM human	Barelli *et al*. 1999
4	Z-Pro-prolinal		K_i = 0.5 nM human brain	Bakker *et al*. 1990
5	ZTTA		K_i = 2.9 μM rat brain	Shishido *et al*. 1996
6	Z-321		IC_{50} = 10 nM canine brain	Tanaka *et al*. 1994
7	ONO-1603		K_i = 12 nM rat brain	Katsube *et al*. 1999
8	KYP-2047		K_i = 0.023 nM porcine brain	Venäläinen *et al*. 2006

humans during the phase I study (Morain *et al*. 2000). ZTTA **5** improved performance of rats after cerebral ischemia (Shishido *et al*. 1996) and basal forebrain lesion (Shishido *et al*. 1998). Z-Pro-prolinal **4** prevented scopolamine-induced amnesia in rats (Yoshimoto *et al*.1987) and KYP-2047 **8** improved performance of young but not old scopolamine-treated rats (Jalkanen *et al*. 2006).

Taken together, several studies suggest that POP inhibitors might be beneficial in the treatment of age-related memory disorders or neurodegenerative diseases, like

Alzheimer's disease. Indeed, four POP inhibitors, JTP-4819 **1**, S 17092 **3**, Z-321 **6** and ONO-1603 **7**, have entered clinical trials (Umemura *et al*. 1997, Katsube *et al*. 1999, Umemura *et al*. 1999, Morain *et al*. 2000). However, their mechanism(s) of action is not fully understood, i.e. the secondary effects of POP inhibition are not known for sure. At the moment, there are two main theories for the memory-improving effects of POP inhibitors: elevation of the neuropeptide levels in the central nervous system (CNS) and elevation of the intracellular inositol-1,4,5-trisphosphate (IP_3) level.

Certain neuropeptides, like substance P (SP), arginine-vasopressin (AVP) and thyroliberin (TRH) (Figure 1), are *in vitro* substrates of POP (Knisatschek and Bauer 1979, Blumberg *et al*. 1980, Yoshimoto *et al*. 1981). These neuropeptides have been shown to improve learning and memory (Griffiths 1987, Kovacs and De Wied 1994, Huston and Hasenöhrl 1995). Consequently, it was concluded that inhibition of POP might increase concentrations of these peptides in the CNS and thus, counteract memory disturbances. Several studies have been performed to verify this theory. A 21-day treatment with JTP-4819 **1** increased the SP and the TRH level in the cortex and the hippocampus of aged rats, while the AVP level was unaffected (Toide *et al*. 1997). In humans, a 7-day treatment with JTP-4819 **1** did not affect the plasma concentration of SP, although a small increase was seen after a single dose (Umemura *et al*. 1997). A single dose of S 17092 **3** increased the SP level in the frontal cortex and the hypothalamus of rats while an 8-day treatment did not have any effect (Bellemère *et al*. 2003). Also the TRH level in the cerebral cortex and the AVP level in the hippocampus of rats were increased after the single dose of S 17092 **3** but only the TRH level in the cerebral cortex was increased after the 8-day treatment (Bellemère *et al*. 2005). A single dose of Z-321 **6** or Z-Pro-prolinal **4** increased the AVP level in the septum of rats (Miura *et al*. 1995). Instead, a 7-day treatment with Z-321 **6** did not affect the plasma levels of SP, TRH or AVP in humans (Umemura *et al*. 1999). A single dose or a 10-day treatment with KYP-2047 **8** or JTP-4819 **1** did not affect the SP level in the cortex, hippocampus or hypothalamus of rats (Jalkanen *et al*. 2006). The single dose of

(a) Arg-Pro-Lys-**Pro-Gln**-Gln-Phe-Phe-Gly-Leu-Met

(b) Cys-Tyr-Phe-Gln-Asn-Cys-**Pro-Arg**-Gly

(c) (pyro)Glu-His-**Pro-NH_2**

Figure 1. The sequences of three neuropeptide-substrates of POP. The scissile bonds are shown in bold. (a) Substance P; (b) Arginine-vasopressin; (c) Thyroliberin.

KYP-2047 **8** slightly increased the neurotensin level in the hypothalamus of rats but not dose-dependently. The chronic administration instead, decreased the neurotensin concentration. JTP-4819 **1** did not affect the neurotensin level in the hypothalamus of rats. The expression of the POP gene was also reported to overlap with the receptors of SP, TRH and AVP in the rat brain (Bellemère *et al*. 2004).

However, the different location of POP, which is mainly described as the cytosolic enzyme, and the neuropeptides, which are located in transcellular space, has questioned the role of POP in neuropeptide metabolism. The membrane bound form of POP might offer an answer to this discrepancy, and indeed, it has been reported to have a higher affinity towards neuropeptides than the cytosolic POP has (O'Leary *et al*. 1996). However, this approach does not explain the role of the cytosolic POP and also ignores the effect of other peptidases.

At the end of the 1990's some light was shed on the intracellular functions of POP when it was linked to the phosphatidylinositol (PI) pathway. Williams *et al*. (1999) studied the molecular basis of the properties of Li^+, which is used in bipolar affective disorder. They screened through the Li^+ resistant mutants of *Dictyostelium* and found that one mutant was lacking the POP gene. Further studies revealed that the lack of the POP gene or inhibition of POP by specific inhibitors elevated the IP_3 concentration by dephosphorylation of inositol-1,3,4,5,6-pentakisphosphate (IP_5) and thus, counteracted the effect of Li^+, which inhibits the PI pathway (Figure 2). Later it was verified that POP is linked to the PI pathway also in mammalian cells (Williams *et al*. 2002). The mechanism behind the inverse relationship of the IP_3 concentration and POP activity is

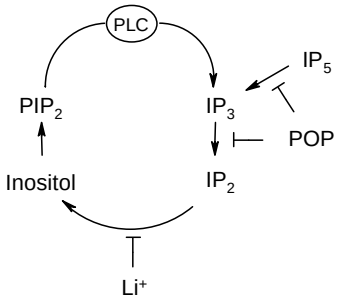


Figure 2. The interaction of POP and lithium with the phosphatidylinositol pathway. Phospholipase C (PLC) cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3). POP inhibits the formation of IP_3 from inositol-1,3,4,5,6-pentakisphosphate (IP_5) and the formation of inositol-4,5-bisphosphate (IP_2) from IP_3. Lithium inhibits the formation of inositol from IP_2. Modified from Williams *et al*. 1999.

still unknown. However, the increase of the IP_3 concentration was delayed after the inhibition of POP and it was suggested that the proteolytic activity of POP is behind this phenomenon, although IP_5 is not a substrate of POP (Schulz *et al*. 2002). Furthermore, Z-Pro-prolinal **4** (Table 1) prevented the translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) into the nucleus of monkey fibroblasts, which had been exposed to 6-hydroxydopamine (Puttonen *et al*. 2006) and ONO-1603 **7** (Table 1) suppressed the overexpression of GAPDH in cultured neurons (Katsube *et al*. 1999). The GAPDH translocation leads to apoptosis in some circumstances and is dependent on PI-3-kinase, which is a phosphorylating enzyme in the PI pathway. In addition, Z-Pro-prolinal **4** prevented the 6-hydroxydopamine-induced formation of reactive oxygen species in monkey fibroblasts (Puttonen *et al*. 2006). However, the IP_3 concentration in the hippocampus or the cortex of rats was not elevated *in vivo* after treatment with JTP-4819 **1**. Recently, POP was also found to be closely associated with tubulin, and was proposed to have a role in protein trafficking and secretion (Schulz *et al*. 2005). Indeed, inhibition of POP and antisense mRNA expression of POP both increased protein secretion, the latter having more evident effect. This led to the conclusion, that the effect of POP on protein secretion may be independent of its peptidase function.

2.3.2 Psychiatric disorders

Altered serum POP activity has been reported in many psychiatric disorders. Lowered serum POP activity was found in anorexia nervosa and bulimia nervosa patients (Maes *et al*. 2001), as well as in unmedicated depressed patients as compared to normal volunteers. In the depressed patients, antidepressants increased POP activity. Increased serum POP activity was found in patients with bipolar disorder (manic) who had been deprived from any medication for at least seven days, but short valproate treatment decreased activity. Increased serum POP activity was also found in schizophrenic patients (Maes *et al*. 1995) and in persons with stress-induced anxiety (Maes *et al*. 1998). It is somewhat unclear how the serum POP activity correlates with the POP activity in the brain and whether the altered POP activity is the cause or the consequence of the disorders. However, the criticism towards these results has mainly originated from the substrate, *N*-Z-Gly-Pro-7-amino-4-methylcoumarin, which was used to determine the serum POP activity in these studies. It has been shown that two distinct enzymes are responsible for the *N*-Z-Gly-Pro-7-amino-4-methylcoumarin-hydrolyzing activity in the bovine serum. One enzyme is prolyl oligopeptidase and the other was

originally named as Z-Pro-prolinal insensitive peptidase (ZIP), because it could not be inhibited by the specific POP inhibitor Z-Pro-prolinal **4** (Cunningham and O'Connor 1997). ZIP was later identified as a seprase with the properties of a proline specific serine protease (Collins *et al*. 2004). However, when an assay that distinguishes between POP and ZIP activities was used, it was found that the activities of the both enzymes were lowered in the serum of bipolar disorder patients that were receiving lithium-treatment and had euthymic mood. On the other hand, no changes were observed in the POP or ZIP activity in the serum of schizophrenic patients (Breen *et al*. 2004).

Interestingly, valproate decreases POP activity in manic patients (Maes *et al*. 1995). The effect of valproate on the phosphatidylinositol pathway has been studied and a dual action was proposed: valproate directly inhibits POP and thus increases PI signalling but in other conditions it inhibits PI signalling by an unknown mechanism. This theory of dual action was used to explain why valproate can prevent swings to both mania and depression. It was suggested that POP inhibitors could be used in the treatment of bipolar disorders. However, it is contradictory that PI signalling is high in manic persons and a decrease in POP activity would still increase it (Cheng *et al*. 2005).

2.3.3 Other disorders

Trypanosoma cruzi is a parasitic protozoan that causes Chagas disease, a disease that is prevalent in Latin America. In 1997, *T. cruzi* was found to secrete an 80 kDa protein that was able to degrade collagen and had a high specificity for proline residues. The enzyme was mainly secreted by trypomastigotes, the infective form of *T. cruzi*. It was suggested that this enzyme might participate in the host-cell infection by degradation of the extracellular collagen matrix (Santana *et al*. 1997). Later, this enzyme was verified to be the POP of *T. cruzi* (POP Tc80), even though it was able to degrade proteins in contrast to mammalian POP (see section 2.4.2). The POP Tc80 had a 43% identity with the porcine POP. The inhibitors of the POP Tc80 prevented the actual invasion phase of trypomastigotes into host-cells but not the parasite attachment to the host-cell membrane (Bastos *et al*. 2005). These results suggest that the inhibitors of the POP Tc80 have potential as antiprotozoal drugs.

Celiac disease is an autoimmune disease that is caused by the ingestion of gluten proteins, gliadins and glutenins. Celiac disease leads to the destruction of the small intestine villi and the only effective therapy at the moment is a gluten-free diet. When α-gliadin was exposed to digestive enzymes, a proline-rich peptide was one of the products (Shan *et al*. 2002). This peptide was resistant to further hydrolyzation and it

was stated to have "an exceptional toxic potency". However, the peptide could be hydrolyzed by bacterial POP and it was suggested that celiac disease patients might benefit from peptidase therapy.

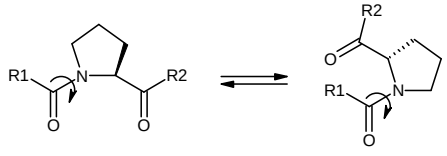
2.4 Substrates

2.4.1 Proline

Proline is often referred to as an imino acid because its side chain is bonded, not only to the α-carbon, but also to the α-amino group (Yaron and Naider 1993, Vanhoof *et al*. 1995, Cunningham and O'Connor 1997). Consequently, proline has a cyclic structure, which prevents free rotation around the φ-bond and, in a peptide sequence proline does not have a main chain NH proton that could form intramolecular hydrogen bonds. These features make proline unique amongst the 20 natural α-amino acids. Proline causes bends in the peptide or protein chains and often breaks repeated structures. It does not participate in hydrogen bonding and can also prevent hydrogen bonding of the neighbouring residues. Peptide bonds are mainly in *trans* conformation but the bond preceding a prolyl residue makes an exception. This bond can be both in *cis* and *trans* conformations (Figure 3), which are separated by an energy barrier of 20 kcal/mol. The isomerization of this bond is thought to be a rate-limiting step in protein folding and degradation. Another important function of prolyl residues in peptide sequences is the protection of biologically active peptides from non-specific degradation. The ability of prolyl residues to protect peptides has led to the evolution of proline specific peptidases, like prolyl oligopeptidase.

2.4.2 Substrate specificity

POP was first recognized as a human uterine enzyme that hydrolyzes the prolyl-leucyl bond in oxytocin (Walter *et al*. 1971). Since then, different mammalian POPs have been



trans Xaa-Pro *cis* Xaa-Pro

Figure 3. *Cis* and *trans* conformations of the peptide bond preceding a prolyl residue. R1 = peptide sequence in the amino terminus; R2 = peptide sequence in the carboxyl terminus.

shown to hydrolyze oligopeptides up to about 30 amino acids at the carboxyl side of a prolyl (or a prolinamide) residue (Wilk 1983, Cunningham and O'Connor 1997). In some cases, POP can also hydrolyze oligopeptides after an alanine residue but not as efficiently as after a proline residue. Recently, a post-cysteine cleavage site was also reported (Bär *et al*. 2006). There are some restrictions too; POP does not hydrolyze a bond between two prolyl residues or the first two bonds of the N-terminus (Wilk 1983, Cunningham and O'Connor 1997). L-Amino acids are also required at the P1 and P2 positions.

2.5 Structure and substrate binding

The sequence analysis of the POP gene of human lymphocytes revealed that the gene codes for an 80 kDa protein that consists of 710 amino acids (Vanhoof *et al*. 1994). Human POP shares 97% identity with the porcine brain POP (Rennex *et al*. 1991) and 95% identity with the rat POP (Kimura and Takahashi 2000), which are often used to determine *in vitro* and *in vivo* effects of POP inhibitors. Furthermore, all 19 differences in the sequences of the porcine and the human POPs are far from the active site (Fülöp *et al*. 1998) and thus, porcine POP can be considered as a good model for the human POP. Instead, the POPs of *Flavobacterium meningosepticum* and *Trypanosoma cruzi* share only about 40% identity with the mammalian POPs (Yoshimoto *et al*. 1991a, Bastos *et al*. 2005).

The crystal structures of the porcine muscle POP and its complex with a transition state analog inhibitor, Z-Pro-prolinal **4**, have been resolved (Fülöp *et al*. 1998, Figures 4 and 5). No differences between the porcine muscle and the porcine brain POP were found. The enzyme has two domains. The catalytic domain consists of residues 1-72 and 428-710 and the C-terminal part of it forms an α/β-hydrolase fold. The noncatalytic β-propeller domain consists of residues 73-427. The β-propeller is 7-bladed and each blade is formed by a four-stranded antiparallel β-sheet. The blades are arranged around the central tunnel and the whole structure is stabilized by hydrophobic interactions.

The substrates of the mammalian POP are restricted to peptides up to about 30 amino acid residues. The mechanism of the restriction of the substrate size is probably related to the entrance of the substrate because the cavity inside POP could accommodate larger substrates. At the moment it seems that the entrance is between the two domains and not through the β-propeller as was originally suggested (Fülöp *et al*. 1998, Szeltner *et al*. 2004, Fuxreiter *et al*. 2005, Juhász *et al*. 2005). A crystallized open

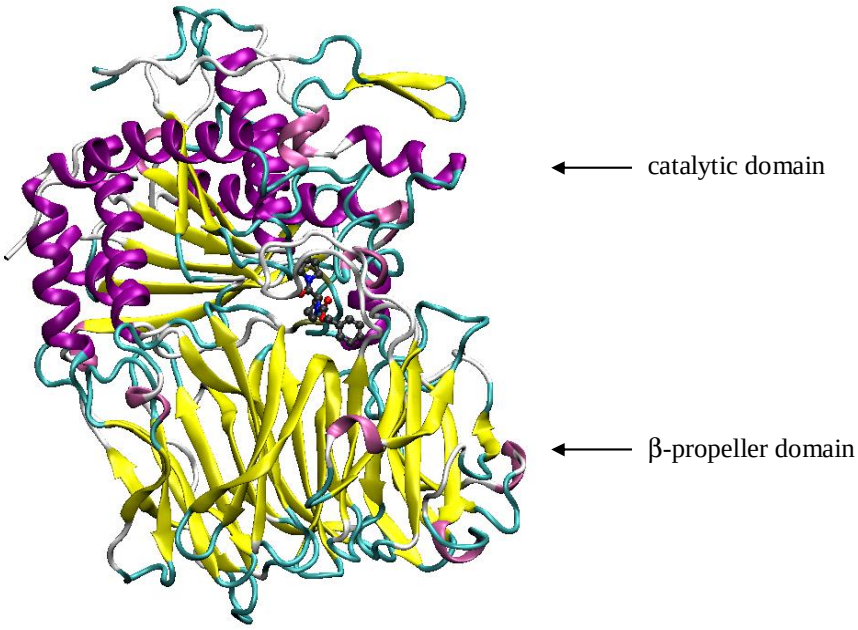


Figure 4. The crystal structure of the porcine muscle POP with the transition state analog inhibitor Z-L-Pro-L-prolinal bound to its active site at the interface of the catalytic domain and the β-propeller domain. The picture was kindly created by Dr. Maija Lahtela-Kakkonen.

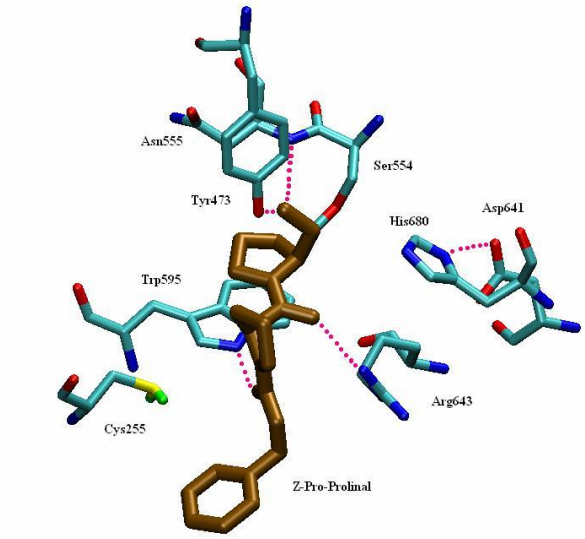


Figure 5. The binding of Z-L-Pro-L-prolinal to the active site of POP. The picture was kindly created by Dr. Maija Lahtela-Kakkonen.

form of the unbound POP of *Sphingomonas capsulata* was recently reported (Shan *et al*. 2005). It was suggested that the substrate causes conformational changes which open the domain interface and that the substrate-protein interactions stabilize the open form. A limited surface area created by the domain opening was postulated to restrict the substrate size. However, care should be taken when extrapolating these results to mammalian enzymes, and indeed, it has been pointed out that the role of the β-propeller domain may have changed during evolution (Venäläinen *et al*. 2004a).

The active site of POP is at the interface of the two domains and contains the catalytic triad, Ser554, Asp641 and His680 (Fülöp *et al*. 1998, Figure 5). Ser554 is easily accessible and it is surrounded by small residues. The other carboxylate oxygen of Asp641 is hydrogen bonded to His680 and a water molecule. The other oxygen is hydrogen bonded to two main chain NH groups of POP. The nucleophilic hydroxyl group of Ser554 attacks the carbonyl group of the scissile bond (or the aldehyde carbonyl group of the transition state analog inhibitor) and a covalent bond is formed. The carbonyl oxygen forms an oxyanion, which is stabilized by two hydrogen bonds; one to a main chain NH group and one to the phenolic hydroxyl group of Tyr473. The S1 subsite has evolved to fit the five-membered ring of the prolyl residue and further stabilization is gained from ring stacking between the P1 prolyl residue and the indole group of Trp595. The S2 subsite is less specific and the P2 residue is stabilized by a hydrogen bond between the P2 carbonyl oxygen and the guanidine group of Arg643. The S3 subsite is located at a large cavity and the S3-P3 hydrogen bond is formed between the P3 carbonyl oxygen and the side chain of Trp595. Also the subsites P4, P1' and P2' have been reported for longer substrates (Fülöp *et al*. 2001).

2.6 Prolyl oligopeptidase inhibitors

A wide variety of POP inhibitors has been described in scientific publications and patents. The aim of this chapter is not to cover all of them but to give an overview of the structure-activity relationships of POP inhibitors. Most of the published inhibitors are substrate-like inhibitors that are based on the *N*-acyl-L-prolyl-pyrrolidine structure and their binding mode is known (Figure 6). A number of atypical POP inhibitors which have been extracted from different plant sources have also been published. Their inhibitory activities are usually rather low and their binding modes unknown. These compounds are not discussed herein. Direct comparisons will be made only between inhibitors that have been tested in the same test system.

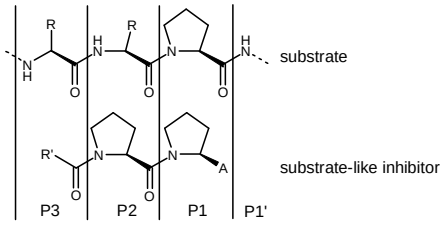


Figure 6. The structures and the binding modes of substrates and substrate-like inhibitors of POP. R = amino acid side chain; R'CO = an *N*-acyl group; A = generally H, CHO, CN or $COCH_2OH$ (other groups are discussed in section 2.6.1.).

2.6.1 The P1 site

The S1 binding site of prolyl oligopeptidase is a specific pocket for the five-membered ring of a prolyl residue (Fülöp *et al*. 1998) and therefore most of the POP inhibitors have a pyrrolidine or a substituted pyrrolidine ring at the P1 site (Table 2). If the ring size of pyrrolidine is decreased (Goossens *et al*. 1997) or increased (Tsuru *et al*. 1988) by one methylene group the inhibitory activity decreases, as is exemplified by compounds **9**-**11**. Pyrrolidine can be replaced by other five-membered rings as thiazolidine (as in compound **3**), isoxazolidine, pyrrole, 2,3-dihydropyrrole and

Table 2. The effect of the ring size and ring opening at the P1 position on the potency of POP inhibitors.

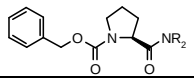
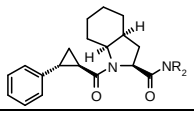
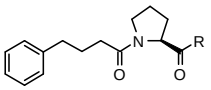
Number	NR_2	IC_{50} (nM)	Source of POP	Reference
				
9		600 540	human platelets bovine brain	Goossens *et al*. 1997 Tsuru *et al*. 1988
10		3000	human platelets	Goossens *et al*. 1997
11		1000000	bovine brain	Tsuru *et al*. 1988
				
12		1.2	rat cortex	Portevin *et al*. 1996
3 (S 17092)		1.3	rat cortex	Portevin *et al*. 1996
13		> 2000	rat cortex	Portevin *et al*. 1996

Table 3. The effect of the carbon rings at the P1 position on potency of POP inhibitors.



Compound	R	IC_{50} (nM)	Source of POP	Reference
14 (SUAM-1221)		2.0	porcine brain	Wallén *et al*. 2002b
15		30		
16		1100		
17		23		

2,5-dihydropyrrole (Portevin *et al*. 1996). However, ring opening (as in compound **13**) abolishes the inhibitory activity.

The evolution of the specific S1 pocket is explained by the lack of the main chain NH proton. Consequently, the amide nitrogen of the P1 prolyl residue is not stabilized by hydrogen bonding to the surroundings (Fülöp *et al*. 1998) and the P1 pyrrolidine nitrogen of POP inhibitors can be removed (Table 3). The pyrrolidine ring of the well-known POP inhibitor SUAM-1221 **14** has been replaced by a cyclopentane ring in compound **15** (Wallén *et al*. 2002b). The inhibitory activity decreased to some extent, which may result from the replacement of the amide nitrogen, which is sp^2 hybridized, by an sp^3 hybridized carbon. The replacement with a cyclohexane ring (compound **16**) decreased the inhibitory activity significantly but the replacement with a benzene ring gave compound **17**, which was equipotent with the cyclopentane derivative **15**.

Substituents at the 3-position of the P1 pyrrolidine ring are rare. Fluorine is the only substituent at this position that has been shown to improve potency (Goossens *et al*. 1997). On the other hand, substituents at the 2-position are not rare and provide the most efficient method to improve the potency of POP inhibitors. Three most studied substituents are the electrophilic 2(*S*)-formyl, 2(*S*)-cyano and 2(*S*)-hydroxyacetyl groups.

Z-Pro-prolinal **4** has the 2(*S*)-formyl group at the 2-position of the P1 pyrrolidine ring. It was originally synthesized as a transition state analog inhibitor (Wilk and Orlowski 1983) and the crystal structure confirmed that the 2(*S*)-formyl group forms a hemiacetal adduct with Ser554 of POP (Fülöp *et al*. 1998, Figure 7a). Later it was

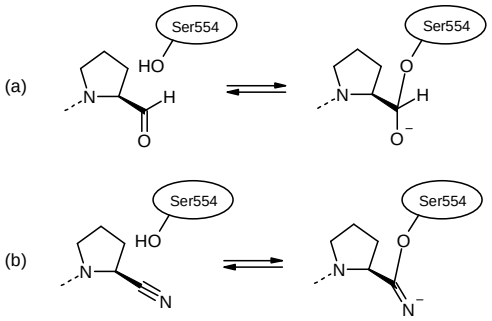
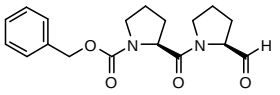


Figure 7. (a) Hemiacetal formation of a 2(*S*)-formyl group and (b) a suggested imidate formation of a 2(*S*)-cyano group with Ser554 of POP.

shown that Z-Pro-prolinal is a slow- and tight-binding competitive inhibitor of POP (Bakker *et al*. 1990). It was 40 times more potent inhibitor of canine POP than Z-L-Pro-pyrrolidine **9** (Tanaka *et al*. 1994).



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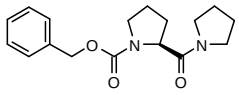
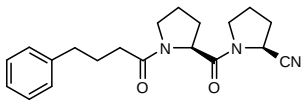


9

The 2(*S*)-cyano group increases the potency of POP inhibitors (Tanaka *et al*. 1994). The cyano group also increases the potency of DPP IV inhibitors, although it is generally considered to give poor serine protease inhibitors (Li *et al*. 1995). KYP-2047 **8** was 40 times more potent than SUAM-1221 **14**, the K_i values were 0.023 and 0.97, respectively (Venäläinen *et al*. 2006). The inhibition of cystein proteases by nitriles has been studied. Cystein proteases have a similar catalytic mechanism to serine proteases and the cyano group forms a covalent, reversible thioimidate adduct with the thiol group of the active site cysteine (Dufour *et al*. 1995). There is most likely a similar interaction between the hydroxyl group of the catalytic Ser554 of POP and a cyano group (Figure 7b).



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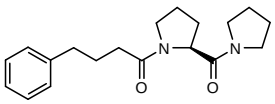
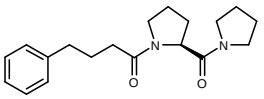


14

The 2(*S*)-hydroxyacetyl group can be considered to be an activated ketone. While the reported IC_{50} value for SUAM-1221 **14** was 2.0 nM, the corresponding analog **18** with the 2(*S*)-hydroxyacetyl group was over 10 times more potent (Wallén *et al*. 2002b). However, the removal of the hydroxyl group resulted in compound **19** with a

significantly decreased potency, the IC_{50} value was 170 nM. This series of compounds shows that the activation of the ketone is crucial for the inhibitory activity. The interaction of the ketone function with POP has not been studied but the 2(*S*)-hydroxyacetyl group might form a hemiketal adduct with the active site serine 554, analogously to the hemiacetal adduct of the 2(*S*)-formyl group.



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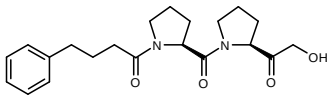

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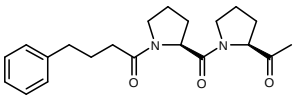
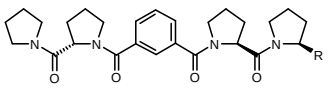


19

Recently, the binding kinetics of a series of isophthalic acid *bis*-(L-prolyl-pyrrolidine) amides **20**-**23** to POP was studied (Venäläinen *et al*. 2004b, Table 4). The formyl, the cyano and the hydroxyacetyl groups increased the potency by two orders of magnitude as compared to an unsubstituted pyrrolidine. They also caused a slow-binding inhibition by reversible covalent binding in contrast to the fast binding of compound **20**. However, differences between compounds **21**-**23** were found in dissociation half-lives. While compound **21** with the 2(*S*)-cyano group had a dissociation half-live of 25 minutes, the corresponding value for compounds **22** and **23** was over 5 hours. It was suggested that these groups can be used to optimize the duration of POP inhibition. Later it was shown that the duration of POP inhibition in rat tissues can be estimated from the dissociation half-lives of the enzyme-inhibitor (EI) complexes (Venäläinen *et al*. 2006).

Table 4. The kinetic parameters of the binding of isophthalic acid *bis*-(L-prolyl-pyrrolidine) amides to POP.[a]



Compound	R	K_i (nM)[b]	EI half-life[b]
20	H	11.8 ± 0.5	-
21	CN	0.39 ± 0.03	25 ± 3 min
22	CHO	0.15 ± 0.01	5.4 ± 0.5 h
23	$COCH_2OH$	0.079 ± 0.010	5.2 ± 0.7 h

[a] Modified from Venäläinen *et al*. 2004b; [b] Determined with the purified porcine POP.

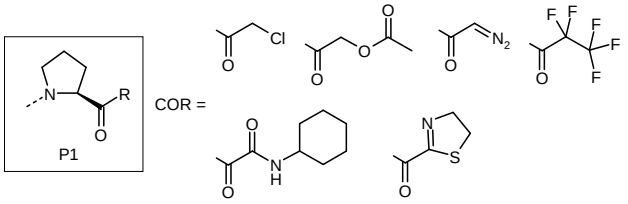
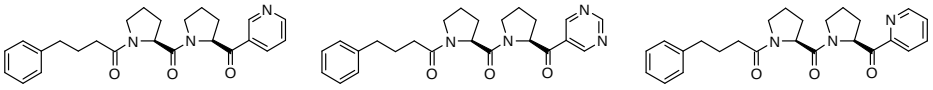


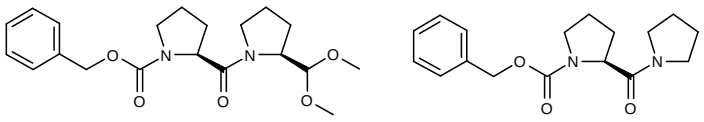
Figure 8. Different groups that have been used to activate the P1 ketone carbonyl of POP inhibitors.

In addition to the 2(*S*)-hydroxyacetyl group, many other activated ketones have also been studied (Figure 8). These are for example 2(*S*)-chloroacetyl, 2(*S*)-acetoxyacetyl (Vendeville *et al*. 2002), 2(*S*)-diazoacetyl (Stone *et al*. 1992), and 2(*S*)-pentafluoropropanoyl groups (De Nanteuil *et al*. 1998), different *N*-cycloalkylated amino-oxo-acetyl groups (Tsuda *et al*. 1996a) and heterocyclic ketones (Tsutsumi *et al* 1994). The activation of the ketone may be caused by an inductive effect because all groups have an electronegative atom near the ketone carbonyl function. Furthermore, in the series of heterocyclic ketones, improvement in potency was observed when compounds **24**-**26** were studied (Tsutsumi *et al*. 1994). One nitrogen at the γ-position from the carbonyl group gave only weak inhibitory activity (IC_{50} of **24** was 2290 nM) but the addition of the second nitrogen improved activity significantly (IC_{50} of **25** was 61 nM). However, the most potent inhibitor was obtained when the nitrogen atom was changed to the β-position from the ketone carbonyl group (IC_{50} of **26** was 6.9 nM).



24 **25** **26**

It has been suggested that the aldehydes and the activated ketones may be too reactive to be used as drugs and a series of acetals was synthesized to overcome this problem (Augustyns *et al*. 1995). These acetals proved to be stable and did not act as prodrugs of aldehydes. The dimethyl acetal **27** was the most potent compound. It was 5 times more potent than the corresponding compound **9** with an unsubstituted pyrrolidine; the IC_{50} values against human POP were 120 nM and 600 nM, respectively.



27 **9**

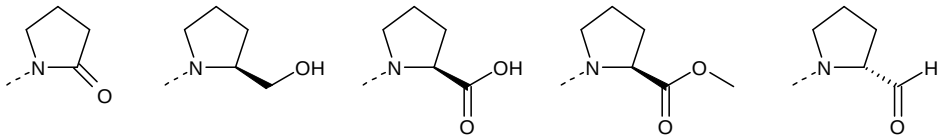
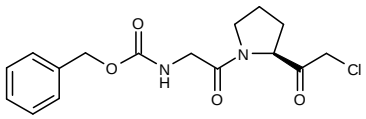


Figure 9. The P1 groups that resulted in loss of POP inhibitory activity.

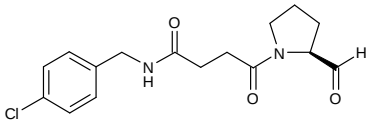
Other structures that have been studied at the P1 position are presented in Figure 9. These were pyrrolidin-2-one (Arai *et al*. 1993), L-prolinol, L-proline (Wilk and Orlowski, 1983), L-prolyl methylester (Tsuru *et al*. 1988) and D-prolinal (Nishikata *et al*. 1986). All these groups resulted in significant decrease in potency.

2.6.2 The P2 site

The S2 binding site of POP is not as specific as the S1 pocket (Fülöp *et al*. 1998) and consequently, much more structural variation is tolerated at the P2 position of POP inhibitors. Among the natural α-amino acids, the cyclic L-proline is preferred (Saito *et al*. 1991). L-Proline is found at the P2 position of many typical POP inhibitors like Z-Pro-prolinal **4** or SUAM-1221 **14**. The achiral glycine gives poor POP inhibitors while the other studied amino acids fall between these two extremes. However, it is intriguing to notice that the change of the amide nitrogen of glycine to an sp^3 hybridized carbon, gives quite potent compounds. Compound **28** was reported to have a K_i value of 70 000 nM against bovine POP, while the reported K_i value for ONO-1603 **7** was 12 nM against rat POP (Yoshimoto *et al*. 1987, Katsube *et al*. 1999). Although the compounds cannot be directly compared, it is unlikely that this large difference in *in vitro* activities could only be explained by different source of POP or other structural differences.



28

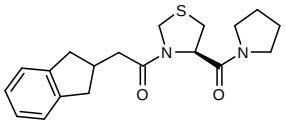


7

It is important that the P2 prolyl residue has an L-configuration, because the replacement with a D-prolyl residue strongly reduced the inhibitory activity. The replacement of the P2 carbonyl group by a methylene group also strongly reduces the inhibitory activity (Yoshimoto *et al*. 1991b). This is understandable because the P2 carbonyl oxygen is needed for hydrogen bonding (Fülöp *et al*. 1998).

Only few other 5-membered rings besides the L-prolyl group have been studied at the P2 position. The L-prolyl group could be replaced by an L-thioprolyl group as in

Z-321 **6** (Tanaka *et al*. 1994). The replacement by a cyclopentane ring was also tolerated; the IC_{50} value of compound **29** against bovine POP was 14 nM (Ohno *et al*. 1988a). 4-Oxo-L-proline, 5-oxo-L-proline, *trans*- and *cis*-4-hydroxy-L-proline decreased the potency (Yoshimoto *et al*. 1987, Saito *et al*. 1991).



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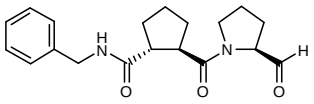
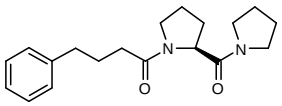
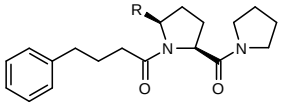


29

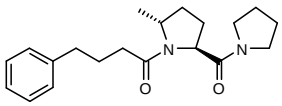
On the other hand, 5-alkyl-L-prolines gave compounds that were slightly more potent than the corresponding L-prolyl analogs (Wallén *et al*. 2003). The IC_{50} values of compounds **14** and **30**-**32** against porcine POP were 2.2 nM, 1.2 nM, 0.71 nM and 1.4 nM, respectively. Later it was shown that compound **30** had a 10 times longer dissociation half-life than compound **14** (Venäläinen *et al*. 2006).



14

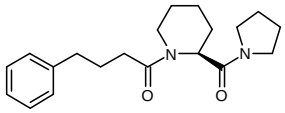


30; R = *tert*-Bu
31; R = Me

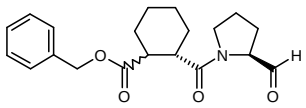


32

Unlike at the P1 position, a 6-membered ring was tolerated at the P2 position, although it slightly decreased the potency (Saito *et al*.1991). Compound **33** has a piperidine group at the P2 site and it was reported to be 5 times less active than SUAM-1221 **14** against bovine POP. *Trans*- and *cis*-cyclohexyl rings were also tolerated, the K_i value was 3.0 nM for both compounds **34** and **35** against mouse POP (Bakker *et al*. 1991). It remained unclear whether the cyclohexyl groups were mixtures of *trans*- and *cis*- compounds or had they pure *S,S*- and *R,S*-stereocenters as drawn below.

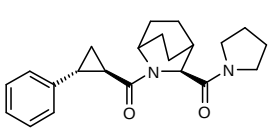


33



34 (*trans*)
35 (*cis*)

Different kinds of bridged and fused rings have also been studied at the P2 position. Azabicyclo[2.2.2]octane (**36**), azabicyclo[2.2.1]heptane (**37**) and perhydroindole (**38**) gave potent compounds, the last two were slightly better than the first one. The IC_{50} values against rat POP were 5, 1.4 and 1.2 nM, respectively (Portevin *et al*. 1996).



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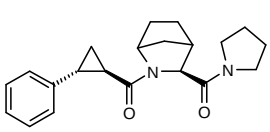

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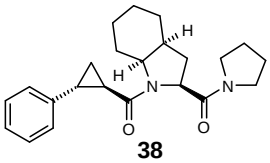
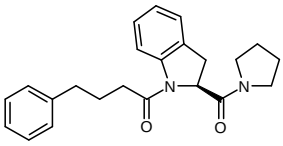
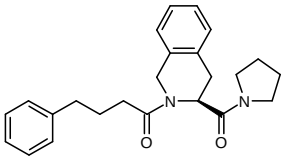


38

Compound **39** with 2,3-dihydroindole at the P2 position was over 200 times less potent than SUAM-1221 **14** against bovine POP (Saito *et al*. 1991). 1,2,3,4-Tetrahydroisoquinoline has been utilized to give the selective inhibition of *T. cruzi* POP (Vendeville *et al*. 2002). Compound **40** was 78 times more potent against *T. cruzi* POP that human POP. However, the selectivity decreased when 1,2,3,4-tetrahydroisoquinoline was combined with other types of P3 structures.



39

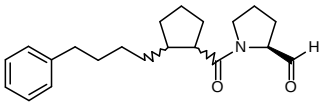


40

2.6.3 The P3 site and beyond it

The S3 subsite is the least specific binding site of POP. A wide variety of groups has been studied at the P3 position of POP inhibitors and some large structures reach even beyond the P3 site. This chapter covers only a small part of the studied structures to give a general overview of the SAR of the P3 site.

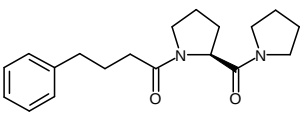
The P3 carbonyl oxygen is important for good inhibitory activity. The IC_{50} value of compound **41** was only 130 nM despite the 2(*S*)-formyl substituent at the P1 ring (Ohno 1988b). Practically all POP inhibitors have an *N*-acyl group attached to the P2 nitrogen and they can be roughly divided in two groups: compounds with an aliphatic acyl group and compounds with an aryl-alkanoyl group. The latter group is preferred.



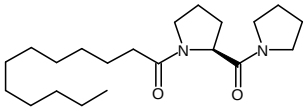
41

The aliphatic chain can be straight, branched, cyclic, saturated or unsaturated. The relative potency of aliphatic acyl groups in relation to aryl-alkanoyls is difficult to define. In the study of Saito *et al*. (1991) aliphatic alkanoyls and alkenoyls, whose lengths ranged between 6 and 18 carbons, were all less potent than SUAM-1221 **14**. The best compound was compound **42**; the IC_{50} value was 420 nM while the IC_{50} of

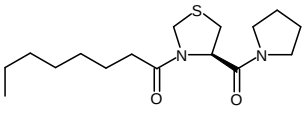
SUAM-1221 **14** was 190 nM against bovine POP. However, Kánai *et al*. (1997) tested a series of alkanoyl-L-thioprolyl-pyrrolidines against rat POP. The lengths of the alkanoyl chains ranged between 4 and 20 carbons. In their study, the best compound **43** was more potent that SUAM-1221 **14**; the IC_{50} values were 11 and 30 nM, respectively.



14

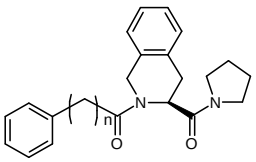


42

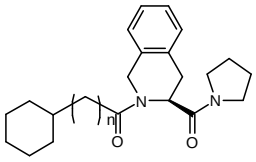


43

In the study of Vendeville *et al*. (2002) compound **40** with an aryl-alkanoyl group was less potent against human POP and more potent against *T. cruzi* POP than compound **44** with an aliphatic group. When the chain was extended by one methylene group, the situation changed. Compound **45** was more potent against both human and *T. cruzi* POP than compound **46**.



40; n = 3
45; n = 4

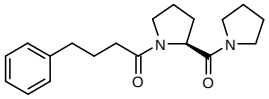


44; n = 3
46; n = 4

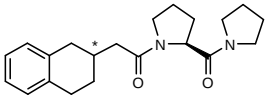
Amongst the aryl-alkanoyl groups, 4-phenylbutanoyl and benzyloxycarbonyl are probably the two most utilized groups and are found for example in SUAM-1221 **14** and Z-Pro-prolinal **4**, respectively. The effect of the chain length has been studied but the comparison suffers from that POPs from different species have been used. In general, it can be said that the 4-phenylbutanoyl or 5-phenylvaleryl groups give more potent compounds that the shorter aryl-alkanoyl groups (Arai *et al*. 1993, Saito *et al*. 1990, Vendeville *et al*.2002, Yoshimoto *et al*. 1991b). There is also some discrepancy about the effect of heteroatoms in the chain. According to Yoshimoto *et al*. (1991b) the Z-group is superior to the 3-phenylpropionyl group and superior or equally good with the 4-phenylbutanoyl group. However, in the study of Tanaka *et al*. (1994) the 4-phenylbutanoyl group was superior to the Z-group. According to our own studies, neither an oxygen nor a nitrogen atom next to the P3 carbonyl group has a significant effect on POP inhibitory activity.

In an attempt to increase the potency of POP inhibitors, the P3 chain has been made more rigid. This approach has sometimes been successful and compound **47** was over 10 times more potent than SUAM-1221 **14** (Tanaka *et al*. 1994). However, also the

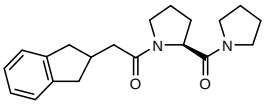
stereochemistry plays a role here; the other diastereomer **48** was equipotent with SUAM-1221. Compound **49** was almost 3 times more potent than SUAM-1221 **14**.



14

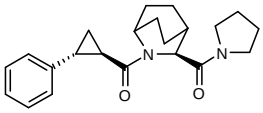


47; *S,S*
48; *R,S*

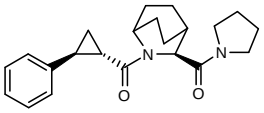


49

The incorporation of a cyclopropane ring into the P3 chain gave also potent compounds but the stereochemistry was crucial also in this case. The *R,R,S*-diastereomer **36** was shown to inhibit rat POP *in vivo* while the *S,S,S*-diastereomer **50** was totally inactive (Portevin *et al*. 1996).



36

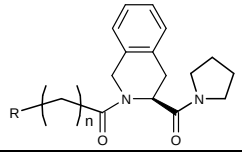


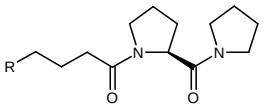
50

Differently substituted phenyl moieties have also been studied at the P3 position. A series of *para*-substituted 4-phenylbutanoyls were studied against canine POP (Arai *et al*. 1993). All substituents decreased the potency (1.5-24 times) and the inhibitory activities decreased in the order: $-CH_3$, -Cl, -OH > $-OCH_3$, $-NO_2$ >>> $-NH_2$. A series of phenyl-cyclopropanoyls was studied against rat POP (Portevin *et al*. 1996). The *p*-fluoro and *p*-methyl substituted compounds had comparable potencies to their unsubstituted counterparts while the *m*-trifluoromethyl group slightly increased and *p*-methoxy group decreased potency. In the study against human and *T. cruzi* POPs, both *p*-nitro and *p*-methoxy substituted 4-phenylbutanoyls increased the potency against the former and decreased the potency against the latter enzyme (Vendeville *et al*. 2002).

Besides the phenyl group, also other aromatic structures have been studied at the P3 position and beyond it (Table 5). The 2-thienyl and 1-pyrenyl groups improved potency one order of magnitude and the 3-pyridyl group slightly decreased potency when compared to the phenyl group (Vendeville *et al*. 2002). The replacement of the phenyl group by the phenoxy group decreased potency. However, the larger 2-styrylphenoxy and 2-benzylphenoxy groups gave compounds with comparable IC_{50} values to SUAM-1221 **14**. The 5-isoquinolinoxy group was comparable to the phenoxy group (Saito *et al*. 1991).

Table 5. Aromatic groups at the P3 position.

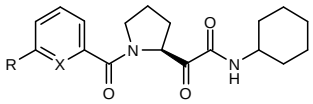




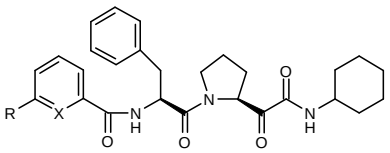
Compound	R	n	IC_{50} (nM)[a]	Compound	R	IC_{50} (nM)[b]
40	Phenyl	3	550	**14**	Phenyl	190
51	2-Thienyl	3	55	**55**	Phenoxy	450
52	1-Pyrenyl	3	50	**56**	2-Styrylphenoxy	140
53	Phenyl	1	400	**57**	2-Benzylphenoxy	210
54	3-Pyridyl	1	600	**58**	5-Isoquinolinoxy	400

[a] The IC_{50} values were determined against human POP and were published in Vendeville *et al*. 2002; [b] The IC_{50} values were determined against bovine POP and were published in Saito *et al*. 1991.

Compounds **59**-**61** and related structures are an exceptional series of POP inhibitors because they do not have any P3 group (assuming that the prolyl group binds to the S1 subsite). They were relatively potent but the elongated compounds **62**-**64** with the P3 structure were 10-100 times more potent against porcine POP (Tsuda *et al*. 1996a, Tsuda *et al*. 1996b).



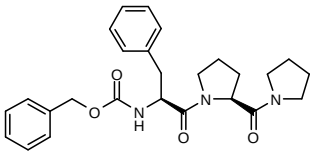
59; X = CH, R = H
60; X = N, R = H
61; X = CH, R = Phenoxy



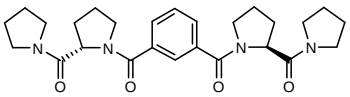
62; X = CH, R = H
63; X = N, R = H
64; X = CH, R = Phenoxy

POP inhibitors which reach beyond the P3 site have also been made. One group of compounds is represented by compound **65**. These compounds had an L-phenylalanine or L-β-alanine moiety at the P3 position and a Z-group reaching beyond that. These inhibitors had strongly reduced inhibitory activities as compared to compounds where the L-phenylalanine or the L-β-alanine was removed and the Z-group was directly attached to the P2 residue (Tsuru *et al*. 1988). Another relatively potent group of compounds was based on a symmetrical structure and is represented by compound **20**

(Wallén *et al*. 2002a). These compounds had a dicarboxylic acid spacer at P3 site connecting two L-Pro-pyrrolidine groups. Concequently, the direction of the peptide bonds was reversed beyond the P3 position. These inhibitors are included in the CoMSIA model described in Chapter 5.



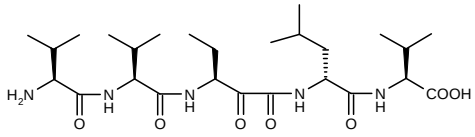
65



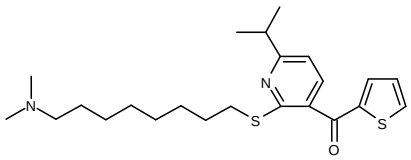
20

2.6.4 Atypical POP inhibitors

There are two interesting atypical POP inhibitors. The first is poststatin **66**, which was isolated from the fermentation broth of *Streptomyces viridochromogenes* (Aoyagi *et al*. 1991). It is a relatively potent natural product and the IC_{50} value against porcine POP was reported to be 0.03 μg/ml which makes 55 nM. The design of the previously described compounds **59**-**64** was based on poststatin. It can be assumed that the *N*-terminal L-valine binds to the S3 subsite and the α-ketoamide group reacts with Ser554 of POP. The second compound is Y-29794 **2**, which at the first glance does not resemble typical POP inhibitors. Its pharmacological properties were reviewed in section 2.3.1. and the reported K_i value against rat POP was 0.95 nM (Nakajima *et al*. 1992). It is probable that the thiophene ring forms the P1 site and the pyridine ring the P2 site. However, this is one of the few POP inhibitors which have an ionizable group included in the structure.



66



2

2.7 Drug-like properties

The chemical space is vast and it has raised the question; how can we differentiate between drug-like and non-drug compounds? Lipinski suggested that drug-like compounds form small clusters in the chemical space (Lipinski 2000). To make a drug we need high affinity to the target protein and also suitable absorption, distribution, excretion, metabolism and toxicity (ADMET) properties. The further suggestion of

Lipinski was that “ADME chemistry space is much simpler than pharmacological target chemistry space” (Lipinski 2000). In the past, several drug development projects have failed because pharmacokinetic and safety properties have been ignored. Nowadays, ADMET properties are considered, alongside the potency, from the early stages of drug development (Kerns and Di 2003).

What are these drug-like properties that we can use to discriminate between a drug-like and a non-drug compound? A set of molecular properties are size and shape, lipophilicity, polarity, hydrogen bonding and charge (Lipinski 2000, Kerns and Di 2003, van de Waterbeemd 2003). These define structural and physico-chemical properties like, solubility, permeability and stability which in turn define ADMET properties. Lipinski suggested that simple rules can be used for ADME (Lipinski 2000) and one of the simplest is the rule of 5 (Lipinski *et al*. 1997). This thumb-rule tells that the compound is likely to have poor absorption or permeation if two of the following rules are exceeded; ≤ 5 hydrogen bond donors, ≤10 hydrogen bond acceptors, $M_W \leq 500$, ClogP ≤ 5. Several other filters have also been developed (Walters and Murcko 2002).

For a CNS targeted drug, the limits are narrower because the blood-brain barrier (BBB) protects the brain from xenobiotics. BBB is formed by endothelial tight junction in the brain capillary, efflux proteins and enzymatic activity. The tight junctions block the paracellular route to the brain. In several studies, different molecular properties have been used to predict passive penetration through the BBB. Some limits have also been set. The log P for the optimal brain penetration was found to be 1.5-2.7 while the mean was 2.1 (Pajouhesh and Lenz 2005). The molecular weight should be below 400-600 and the number of rotatable bonds should be 5 or less. High hydrogen bonding potential decreases brain penetration and polar surface area should be less than about 90 $Å^2$. The pK_a value should fall between 4 and 10.

In conclusion, the most potent compound *in vitro* may not be the best candidate for further development. All the described properties need to be optimized and a compromise in one property may be a gain in another. Drug design is like the dance on a trapeze - you must not loose the balance.

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3 THE AIMS OF THE STUDY

The aim of the study was to design and synthesize new, low molecular weight POP inhibitors, which have an inhibitory activity in the low nanomolar range and to increase the knowledge of the structure-activity relationships and physico-chemical properties of POP inhibitors. More specifically:

1. To explore different replacements at the P3 position and beyond it.
2. To study amide bond replacements of the P3-P2 and P2-P1 amide bonds.
3. To study the effect of the structural changes on physico-chemical properties of the compounds with the focus on drug-like properties.

4 EXPERIMENTAL PROCEDURES

4.1 General synthetic procedures

This section describes only the general synthetic procedures. The detailed synthetic procedures are given in Chapters 5-8.

Reagents and solvents were of the commercial high purity quality and did not need purification prior to use. When needed, the solvents were dried over 3 Å or 4 Å molecular sieves or dried over the sieves and then distilled. Ethers for the reactions with organometallic reagents or in low temperatures were dried over sodium and distilled under argon or nitrogen. For the moisture sensitive reactions, the glassware was dried at 140 °C and cooled in a desiccator or under argon and the reactions were performed under argon or nitrogen.

When possible, the reactions and the purifications were first monitored by thin layer chromatography (Silica gel 60 F_{254}, aluminium sheets, Merck 1.05554.0001). The spots were visualized by UV light (254 nm), iodine, heating, ninhydrin, anisaldehyde or potassium permanganate. Purifications by flash chromatography were performed on J. T. Bakers silica gel for chromatography (pore size 60 Å, particle size 50 μm). The chromatotron plates were made of Merck silica gel 60 PF_{254}-containing gypsum (particle size 5-40 μm).

4.2 Analytical procedures

Nuclear magnetic resonance (NMR) spectroscopy. ^{1}H and decoupled ^{13}C NMR spectra were recorded on a Bruker AM 400 WB (400.1 MHz and 100.6, respectively) or a Bruker Avance 500 spectrometer (500.1 MHz and 125.1, respectively). $CDCl_3$ was used as solvent and tetramethylsilane (TMS) as an internal standard, unless otherwise stated. When the solvent was used as an internal standard, its chemical shift was set according to Gottlieb *et al*. 1997. The FID files were processed with MestRe-C (version 4.3.6.0 or earlier, Mestrelab Research) or TopSpin software (version 1.3, Bruker Biospin) to obtain NMR spectra. Chemical shifts (δ) are given in ppm downfield from TMS (δ 0.00).

The *N*-amide bond of a proline residue or a 2-substituted pyrrolidine moiety has energetically similar *cis* and *trans* isomers (rotamers). These rotamers have slightly different shifts. Minor rotamers that are less than 20% of the major rotamers are not reported for ^{13}C NMR spectra.

Electrospray ionization mass spectrometry (ESI-MS). ESI-MS spectra were obtained on a LCQ ion trap mass spectrometer equipped with an ESI source (Finnigan MAT, San Jose, CA, USA).

Elemental analysis. Elemental analyses (C, H, N) were carried out with a Thermo Quest CE Instruments EA 1110 CHNS-O elemental analyzer at the University of Kuopio or a comparable equipment at the University of Oulu. For the analyses which agree calculated data within ±0.4 percentage units the results are reported "Anal. (molecular formula) C, H, N". For the analyses which do not agree the calculated data within ±0.4 percentage units the calculated and the found results are reported separately.

4.3 *In vitro* assay for POP activity

The whole porcine brains, excluding cerebellum and most of the brain stem, were placed in liquid nitrogen within 30 min after the animals were killed and stored at -80 °C until homogenized. The brains were homogenized in 3 volumes (w/v) of ice-cold 0.1 M sodium-potassium phosphate buffer (pH 7.0), and the homogenates were centrifuged for 20 min at 4 °C at 10000 *g*. The supernatants were pooled and stored in small aliquots at -80 °C until used. The supernatant was thawed in ice just before it was used in the activity assay and diluted in a ratio 1:2 with homogenization buffer.

In the microplate assay, 10 µl of the enzyme preparation was preincubated with 460 µl of 0.1 M sodium-potassium phosphate buffer (pH 7.0) and 5 µl of a solution of the compound dissolved in dimethyl sulfoxide (DMSO) and diluted with 0.1 M sodium-potassium phosphate buffer at 30 °C for 30 min (final DMSO concentration was less than 0.1%). The controls contained 10 µl of enzyme preparation and 465 µl of 0.1 M sodium-potassium phosphate buffer (pH 7.0). The reaction was initiated by adding 25 µl of 4 mM Suc-Gly-Pro-AMC dissolved in 0.1 M sodium-potassium phosphate buffer (pH 7.0), and the mixture was incubated at 30 °C for 60 min. The reaction was terminated by adding 500 µl of 1 M sodium acetate buffer (pH 4.2).

The formation of 7-amido-4-methylcoumarin was determined fluorometrically with microplate fluorescence reader (excitation at 360 nm and emission at 460 nm). In the uninhibited reaction, the specific POP activity was 3.8 nmol/min/mg protein. Five to seven inhibitor concentrations were used to determine the IC_{50} values and the final concentration of the compounds in the assay mixture varied from 10^{-12} to 10^{-4} M. Two to four independent measurements were made for each inhibitor. The inhibitory activities (percent of control) were plotted against the log concentration of the

compound, and the IC_{50} value was determined by nonlinear regression utilizing GraphPad Prism 3.0 software.

4.4 Determination of the K_i values

Expression and purification of porcine prolyl oligopeptidase. Porcine brain RNA was obtained by the Trizol reagent protocol (Life technologies). POP cDNA was generated by the reverse transcriptase (AMV) reaction using the primer 5'-GATAAAAATCCCCGAGGCAGT-3'. The entire coding region, including the stop codon, was amplified by PCR (*Pfu* DNA polymerase) and *Nco*I/*Hin*dIII sites were introduced at the 3'- and 5'-ends respectively. Primers were: sense, 5'-CCCCAGCCATGGTGTCCTTCC-3'; antisense, 5'-TTAAGCTTATGGAATCC-3'. The *Nco*I/*Hin*dIII digested PCR product was then cloned into the *Nco*I/*Hin*dIII site of pBAD/myc-HisA (Invitrogen) obtaining pBADPigPOP. The recombinant plasmid was used to transform *E. coli* TOP10 competent cells. For expression, a colony of *E. coli* TOP10 harboring pBADPigPOP was grown 12 h in 50 ml of Luria broth containing ampicillin 50 µg/ml. A total of 30 ml of this culture were used to inoculate 1 l of Luria broth supplemented with ampicillin 50 µg/ml, and grown until an OD_{600} ~0.5, then arabinose was added to 0.02% and the growth continued for additional 4–6 h. Cells were harvested and the pellet resuspended in 280 ml 10 mM Tris, 100 mM NaCl, 0.5 mM EDTA, pH 8. Cells were treated with lysozyme (0.5 mg/ml) for 30 min at 37 °C. Sphaeroplasts were centrifuged and the pellets frozen at -70 °C. Pellet was resuspended in 30 ml 10 mM Tris, pH 8 by sonication (three times for 5 s), and the debris was removed by centrifugation (5 min, 5,000 *g*). Supernatant was then used immediately for POP purification. POP was purified from cell suspension by following the procedure of Szeltner *et al*. (2000). Practically pure POP was obtained after DEAE and Blue Sepharose chromatography with yield of 68%. The active enzyme concentration was determined by utilizing Morrison equation (Copeland 2000).

Determination of the K_i values. Purified porcine POP (0.57 nM) was incubated in 465 µl of 0.1 M sodium-potassium buffer (pH 7.0) containing 0.1 mM of dithiothreitol for 2 h in the presence of various concentrations of inhibitors at room temperature. The reaction was started with 25 µl of 200 µM Z-Gly-Pro-AMC and the reaction was monitored every 1 min for 30 min. Over that time scale, the product formations were linear, indicating that the inhibitors did not dissociate markedly from the enzyme. K_i values were calculated using the Morrison equation that takes the tight binding inhibition into account (Copeland 2000):

$$\frac{v_i}{v_0} = 1 - \frac{(E+I+K_i) - \sqrt{(E+I+K_i)^2 - 4E \cdot I}}{2E}$$

where v_0 and v_i are reaction velocities in the absence and presence of the inhibitor (I), respectively, K_i is the inhibition constant of the inhibitor and E is the active enzyme concentration in the reaction medium. Since the inhibitor did not dissociate from the enzyme during the measurement, competition of binding between substrate and inhibitor did not occur and hence the calculated K_i value is the real dissociation constant of the inhibitor (Gutheil and Bachovchin 1993).

4.5 Determination of the log P values by the shake-flask method

A known concentration of an inhibitor in phosphate buffer, pH 7.4 (saturated with 1-octanol) was shaken with a suitable volume of 1-octanol (saturated with phosphate buffer, pH 7.4) for 60 min at room temperature. The phases were separated by centrifugation for 5 min at 2000 rpm, and the aqueous phase was analyzed with HPLC. In the HPLC methods, the mobile phase consisted of 20 mM KH_2PO_4 (pH 7) and 90% acetonitrile aq. The methods were tested for linearity and repeatability. The partition coefficient was calculated in relation to a control that was treated as the samples but without 1-octanol. Each partition coefficient was determined at least in triplicate. The Merck Hitachi HPLC system consisted of a UV detector (L-7400), an interface module (D-7000), a pump (L-7100), an autosampler (L-7250), and a Purospher RP-C18e column (125 × 4 mm, 5 µm).

4.6 Determination of the pK_a values and the log P values by the Sirius PCA200 automatic titrator

The titrations were performed according to the Sirius PCA instruction manual at 25 °C (Sirius 2000). 0.15 M KCl aq. solution was used as the ionic strength adjusted (ISA) matrix in all titrations. The blank titrations were done every day to test the proper function of the electrode and the Four-Plus parameters were checked to fall into the acceptable limits. The reported pK_a and log P values were obtained from the Multisets as described below.

Determination of the pK_a values. 2-5 mg of the sample was placed in a beaker and dissolved in 10 or 12 ml of ISA water. The sample was titrated two or three times (Multititration) from pH 1.8 to pH 10.0 with the Sirius PCA200 automatic titrator (Sirius, Forest Row, UK). The individual titrations were refined with the Refine200

software (Sirius, Forest Row, UK). The Goodness-of-Fit (GOF) values ranged from 0.23 to 1.45. The individual titrations of each sample were combined into a Multiset that was refined with the Refine200 software to give the final pK_a value. The GOF values of the Multisets ranged from 0.37 to 1.07.

Determination of the log P values. 2-6 mg of the sample was placed in a beaker and dissolved in 5-10 ml of ISA water. The same sample was multititrated 2-3 times or 2-3 individual samples were each titrated once. In all titrations, the volume of 1-octanol was chosen on the basis of the expected log P values according to the Sirius PCA instruction manual. The individual titrations were refined and the GOF values ranged from 0.37 to 1.35. The individual titrations of each sample were combined into a Multiset that was refined to give the final log P value. The GOF values of the Multisets ranged from 0.49 to 2.08.

4.7 Determination of water solubilities

The Merck Hitachi HPLC system consisted of a UV detector (L-7400), an interface module (D-7000), a pump (L-7100), an autosampler (L-7250), and a Supelcogel ODP50 column (150 × 4 mm, 5 µm). In the HPLC methods, the mobile phase consisted of 20 mM KH_2PO_4 (pH 7) and 90% acetonitrile aq., and the flow rate was 1 ml/min. The methods were tested for linearity and the calibration curves were obtained. The methods were also tested for repeatability.

To three samples of a compound was added 1000 µl of 50 mM phosphate buffer (pH 7.4, ion strength 0.15). The samples were shaken 72 h and filtered through 0.45 µm filter. Compounds were checked not to be adsorbed by the filter. The filtered samples were diluted with the HPLC-eluent and analyzed with the HPLC. The concentrations were calculated from the equations of the calibration curves.

4.8 Preparation of the CoMSIA model

Unless noted otherwise, molecular modeling was performed using Sybyl 6.9.0 and 6.9.1 (Tripos Inc. *SYBYL,* S. H. R., St. Louis, MO) with default options. To prepare the enzyme structure, the POP structure was derived from PDB (ID code 1QFS, resolution 2.0 Å). Prior to docking, the cocrystallized inhibitor was removed from the structure, and the hydrogen atoms were added using Sybyl Biopolymer module. Because the X-ray structure may also contain energetic tensions, an energetically favorable conformation of the suggested active site was searched by carrying out a molecular mechanics minimization using the MMFF94 force field (Halgren 1990). Nitrogen NE2

of histidine 680 was not protonated while ND1 was protonated thus allowing interaction with Asp641.

The inhibitor structures (total number 52) were created using automatic translation from in-house ISIS database to mol2-files, using CONCORD software (Pearlman 2004). After that, it was verified that the inhibitors had right configurations. The resulting structures were minimized using the MMFF94S force field as implemented in Sybyl. The following structures were docked into the active site of POP model using the GOLD program (Jones *et al*. 1995, Jones *et al*. 1997), version 2.2, with standard default settings unless otherwise mentioned. The docking area was defined using NE atom of Arg643 as a center, with a radius of 20 Å. Because the current docking programs cannot automatically take into account a possible covalent interaction between a ligand and a host and several of the compounds possibly have a covalent interaction, two violations for steric clashes were allowed. The docking procedure was repeated 10 times for each inhibitor using default parameters. All dockings, except for compound **32** in Chapter 5, were ranked using GOLD score, and the best alternatives were visually inspected. For compound **32** in Chapter 5, a CSCORE calculation within Sybyl was carried out.

The selected docking modes were further analyzed using the CoMSIA method. The CoMSIA descriptor fields (steric, electrostatic, hydrophobic, acceptor, donor) were calculated in a 3D cubic box extending 4 Å beyond the aligned inhibitors. Atomic point charges for inhibitors were calculated using the Gasteiger-Hückel model as implemented in Sybyl. pIC_{50} values were used as dependent variables in the PLS statistical analysis of the resulting data. The predictive value of the PLS model and the optimum number of the PLS components were evaluated by the progressive scrambling method (Clark and Fox 2004). The final noncross-validated model was developed using the optimal number of components that had both the highest scrambled q^2 value and the smallest value of the estimated crossvalidated standard error with the slope of q^2 under 1.1.

4.9 References

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5 DICARBOXYLIC ACID AZACYCLE L-PROLYL-PYRROLIDINE AMIDES AS PROLYL OLIGOPEPTIDASE INHIBITORS AND THREE-DIMENSIONAL QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP OF THE ENZYME-INHIBITOR INTERACTIONS*

Abstract. A series of dicarboxylic acid azacycle L-prolyl-pyrrolidine amides was synthesized, and their inhibitory activity against prolyl oligopeptidase (POP) from porcine brain was tested. Three different azacycles were tested beyond the P3 position and six different dicarboxylic acids at the P3 position. L-Prolyl-pyrrolidine and L-prolyl-2(*S*)-cyanopyrrolidine were used at the P2-P1 positions. The IC_{50} values ranged from 0.39 to 19000 nM. The most potent inhibitor was the 3,3-dimethylglutaric acid azepane L-prolyl-2(*S*)-cyanopyrrolidine amide. Molecular docking (GOLD) was used to analyze binding interactions between different POP inhibitors of this type and the POP enzyme. The data set consisted of the novel inhibitors, inhibitors published previously by our group, and well-known reference compounds. The alignments were further analyzed using comparative molecular similarity indices analysis. The binding of the inhibitors was consistent at the P1-P3 positions. Beyond the P3 position, two different binding modes were found, one that favors lipophilic structures and one that favors nonhydrophobic structures.

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5.1 Introduction

The novel inhibitors that are described in this chapter were developed from the series of large, symmetrical POP inhibitors (**I** in Figure 1) that were published by Wallén *et al*. (2002a). These large inhibitors were very potent, and another series of large inhibitors were later developed from the first set of compounds (Wallén *et al*. 2003a). To diversify the compound set for the three-dimensional (3D) quantitative structure-activity relationship (QSAR) study, two series of smaller compounds were synthesized. The removal of the pyrrolidine-1-carbonyl group from both ends (**II** in Figure 1, not published) decreased strongly the potency. The succinic acid dipyrrolidine amide was the only compound with a weak inhibitory activity and it had an IC_{50} value of 13 μM against POP from rat brain. (Inhibitory activities against POP from rat brain and porcine brain are similar in our test system.) These compounds were not included in the 3D QSAR study. However, removal of the pyrrolidine-1-carbonyl group from only one end resulted in the novel inhibitors, most of them having the IC_{50} value in the nanomolar range (**III** in Figure 1).

The 3D QSAR models were created using the comparative molecular similarity indices analysis (CoMSIA) method, which produces information of the favored and disfavored properties of the examined compound set (Klebe *et al*. 1994). The compound set consisted of the novel inhibitors (Table 1) and the previously published inhibitors and reference compounds (Table 2). These data are useful to explain and predict activities of different POP inhibitors and to find out how different types of POP inhibitors may bind to the active site of the enzyme. To our knowledge, this is the first time when the systematic molecular docking and 3D QSAR studies have been described for POP inhibitors.

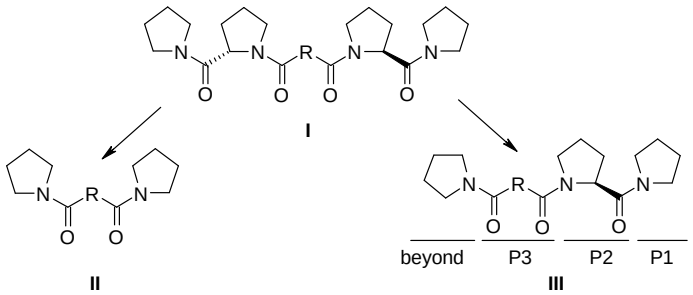


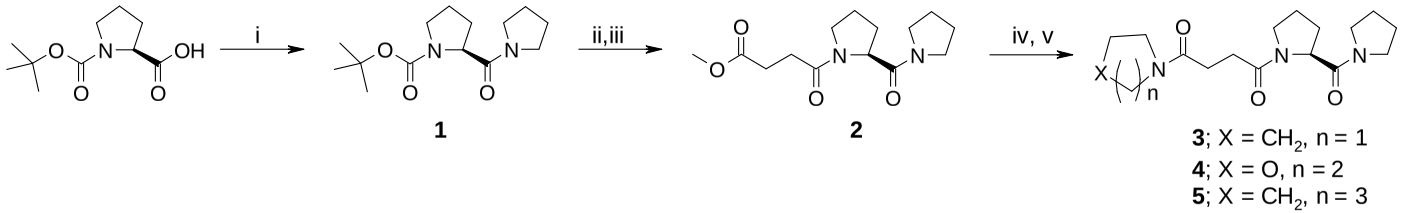
Figure 1. Rationale behind the novel inhibitors. (I) A series of large symmetrical POP inhibitors (Wallén *et al*. 2002a). (II) A series of small symmetrical compounds, which were very poor POP inhibitors. (III) A series of the novel POP inhibitors.

5.2 Methods

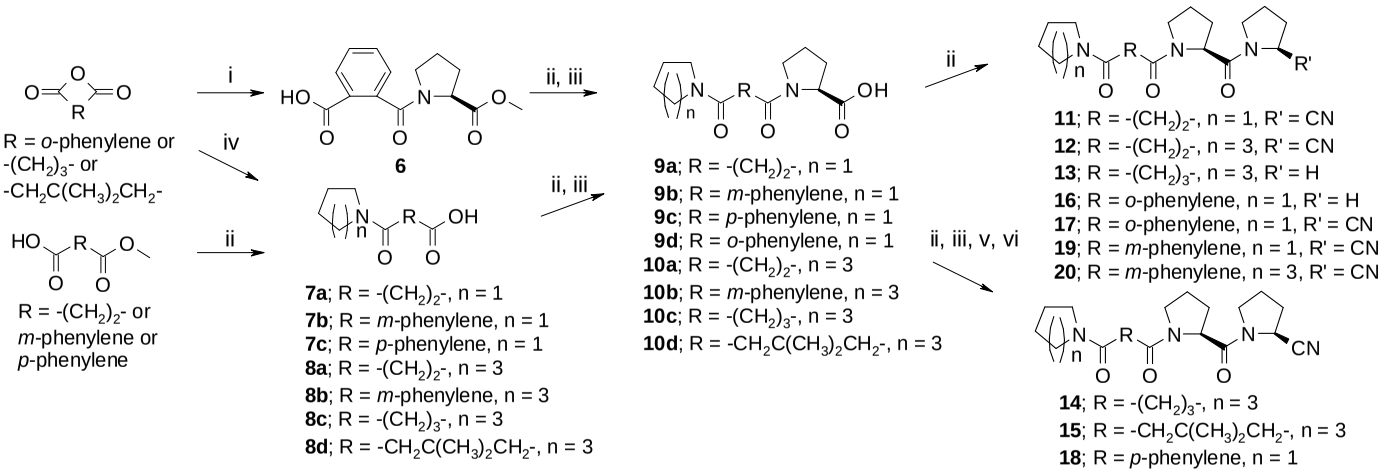
Synthetic chemistry. A series of dicarboxylic acid azacycle L-prolyl-pyrrolidine amides was synthesized (Table 1). L-Pro-pyrrolidine and L-Pro-2(*S*)-cyanopyrrolidine were used at the P2-P1 positions and different dicarboxylic acids as coupled to different azacycles were used at the positions P3 and beyond P3. The inhibitors were synthesized via the synthetic routes described in Schemes 1 and 2 using a similar set of chemical reactions. The building blocks were coupled to each other by amide bonds, which were synthesized in two different ways: the carboxylic acid was activated with trimethylacetyl chloride and reacted with the amine, or the dicarboxylic anhydride was reacted with the amine. The only problematic step was the deprotection of Boc-2(*S*)-cyanopyrrolidine. The cyano group reacted to some extent during the deprotection and, consequently, the yields of these coupling reactions were low, ranging from 10% to 45% for compounds **11**, **12**, **17**, **19** and **20**. Therefore, the cyano group was introduced in the last synthetic step of the compounds that were made in the later part of the study (**14**, **15** and **18**). The compounds in Table 2 were synthesized as described previously (Wallén *et al*. 2002a, 2002b, 2003a, 2003b, Jarho *et al*. 2004).

Molecular modeling. The structure of prolyl oligopeptidase, 1QFS (native, Fülöp *et al*. 1998) was derived from PDB and the co-crystallized inhibitor (Z-L-Pro-L-prolinal) was removed from the crystal structure. The POP inhibitors in Table 1, and the previously published compounds in Table 2 were docked into this crystal structure of POP using GOLD program (Jones *et al*. 1997). The docking area was defined using NE atom of Arg643 as a center, with a radius of 20 Å. Unfortunately current docking programs cannot automatically take into account a possible covalent interaction between a ligand and a host. Since several of the compounds possibly have a covalent interaction, two violations for steric clashes were allowed. With these settings all the compounds were docked succesfully into the active site of POP.

The dockings were further analyzed using the CoMSIA method (Klebe *et al*. 1994) and a statistically valid 3D QSAR model was created. Originally CoMSIA was carried out by using all five different field types, namely steric, electrostatic, hydrophobic, acceptor and donor fields. Even this model was statistically valid (q^2 close to 0.7 with 6 components). However, a closer inspection revealed that most of the model was explained by the acceptor, electrostatic and hydrophobic fields, which were then used in the final models. The selection of the PLS components was based on Scrambling stability test, as proposed by Clark and Fox (2004). This test yields adapted q^2, CSDEP

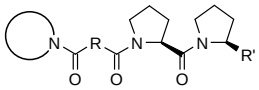


Scheme 1. (i) 1. Et_3N, $(CH_3)_3CCOCl/CH_2Cl_2$, 0 ºC 2. Et_3N, pyrrolidine/CH_2Cl_2; (ii) HCl/EtOAc; (iii) succinic acid monomethyl ester pivalic acid anhydride (prepared from succinic acid monomethyl ester and trimethylacetyl chloride in CH_2Cl_2 at 0 ºC), Et_3N/ CH_2Cl_2; (iv) LiOH/MeOH-H_2O; (v) 1. Et_3N, $(CH_3)_3CCOCl/CH_2Cl_2$, 0 ºC 2. Et_3N, pyrrolidine or morpholine or azepane/CH_2Cl_2



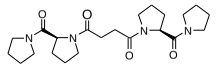
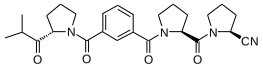
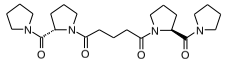
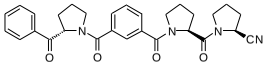
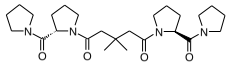
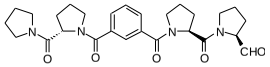
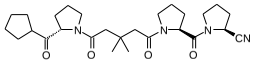
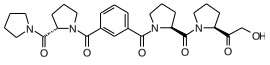
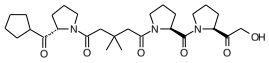
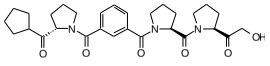
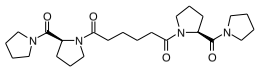
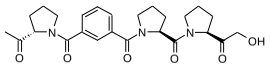
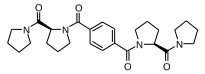
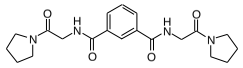
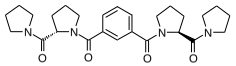
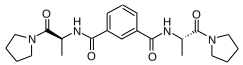
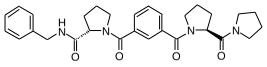
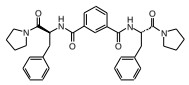
Scheme 2. (i) L-proline methyl ester HCl salt, Et_3N/ CH_2Cl_2; (ii) 1. Et_3N, $(CH_3)_3CCOCl$ /CH_2Cl_2, 0 ºC 2. Et_3N, pyrrolidine or azepane or 2(*S*)-cyanopyrrolidine/CH_2Cl_2; (iii) LiOH/MeOH-H_2O; (iv) azepane/THF; (v) 1. Et_3N, $(CH_3)_3CCOCl/CH_2Cl_2$, 0 ºC 2. Et_3N, L-proline methyl ester HCl salt/CH_2Cl_2; (vi) 1. Et_3N, ethyl chloroformate/THF, -10 ºC 2. NH_3/H_2O; (vii) Et_3N, trifluoroacetic anhydride/THF, 0 ºC.

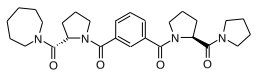
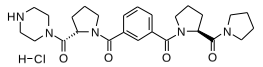
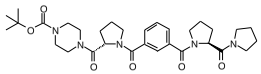
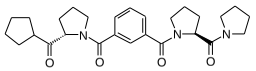
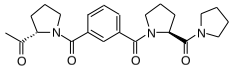
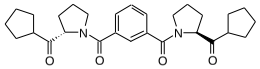
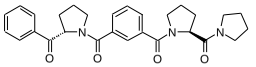
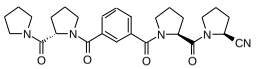
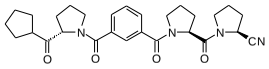
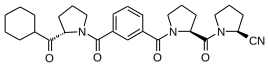
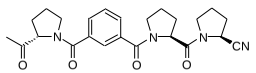
Table 1. Structures and inhibitory activities (95% confidence intervals are presented in parentheses) of the synthesized compounds.

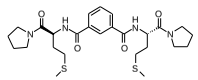
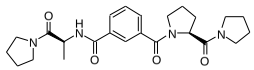
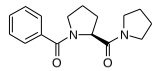
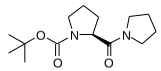
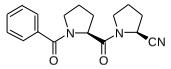
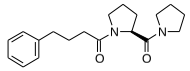
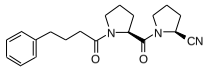
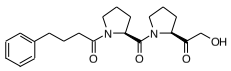
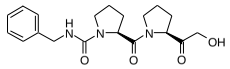
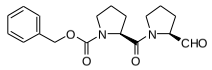


Compd		**R**	**R'**	**IC_{50} (nM)**
3		$-CH_2-CH_2-$	H	110 (110 – 120)
4		$-CH_2-CH_2-$	H	1200 (940 – 1400)
5		$-CH_2-CH_2-$	H	41 (35 – 48)
11		$-CH_2-CH_2-$	CN	2.9 (1.9 – 4.4)
12		$-CH_2-CH_2-$	CN	0.76 (0.50 – 1.2)
13		$-CH_2-CH_2-CH_2-$	H	28 (17 – 44)
14		$-CH_2-CH_2-CH_2-$	CN	1.2 (0.70 – 2.0)
15		$-CH_2-C(CH_3)_2-CH_2-$	CN	0.39 (0.27 – 0.56)
16			H	19000 (14000 – 27000)
17			CN	1500 (1300 – 1700)
18			CN	1.6 (1.3 – 2.0)
19			CN	6.9 (6.2 – 7.8)
20			CN	4.6 (3.4 – 6.2)

Table 2. Structures and inhibitory activities of the previously published compounds and the reference compounds.

Compd	Structure	IC_{50} (nM)	Reference	Compd	Structure	IC_{50} (nM)	Reference
21		77	Wallén *et al.* 2002a	**41**		1.6	Wallén *et al.* 2003a
22		48	Wallén *et al.* 2002a	**42**		1.3	Wallén *et al.* 2003a
23		13	Wallén *et al.* 2002a	**43**		1.3	Wallén *et al.* 2002a
24		0.57	Wallén *et al.* 2003a	**44**		0.39	Wallén *et al.* 2002a
25		0.32	Wallén *et al.* 2003a	**45**		0.61	Wallén *et al.* 2003a
26		68	Wallén *et al.* 2002a	**46**		1.2	Wallén *et al.* 2003a
27		81	Wallén *et al.* 2002a	**47**		640	Wallén *et al.* 2003a
28		26	Wallén *et al.* 2002a	**48**		87	Wallén *et al.* 2003a
29		31	Wallén *et al.* 2003a	**49**		81	Wallén *et al.* 2003a

30		23	Wallén *et al*. 2003a
31		33	Wallén *et al*. 2003a
32		170	Wallén *et al*. 2003a
33		14	Wallén *et al*. 2003a
34		65	Wallén *et al*. 2003a
35		78	Wallén *et al*. 2003a
36		18	Wallén *et al*. 2003a
37		1.5	Wallén *et al*. 2002a
38		1.1	Wallén *et al*. 2003a
39		0.72	Wallén *et al*. 2003a
40		4.2	Wallén *et al*. 2003a

50		110	Wallén *et al*. 2003a
51		39	Wallén *et al*. 2003a
52		66	Wallén *et al*. 2002a, Arai *et al*. 1993
53		29	Wallén *et al*. 2003b, Portevin *et al*. 1996
54		3.1	Not published before
55 SUAM-1221		2.2	Wallén *et al*. 2002a, Yoshimoto *et al*. 1991
56		0.22	Jarho *et al*. 2004
57		0.22	Wallén *et al*. 2002b
58 JTP-4819		0.20	Wallén *et al*. 2002b, Toide *et al*. 1995
59 Z-Pro-prolinal		0.33	Wallén *et al*. 2002a, Wilk and Orlowski 1983

and dq**2/dr2yy values. CSDEP had the optimum with 3 PLS-components, and the dq**2/dr2yy well below 1 (0.91), indicating low enough PLS component number. The final CoMSIA model had the following leave-one-out PLS statistics: $q^2 = 0.69$, $S_{PRESS} = 0.65$, 3 PLS components. The final non-validated model resulted in $r^2 = 0.91$, F-value = 175 and s-value = 0.34.

5.3 Results and discussion

Molecular Docking. All compounds were successfully docked into the binding cavity of POP. When selected dockings are analyzed together, it is obvious that most of the compounds occupy in general the same binding area. Because GOLD produces usually several alternative binding modes, other binding modes were also detected, but in all cases, the selection was based on the scoring function of GOLD. The only exception was compound **32**, where the GOLD-proposed docking was replaced by the CSCORE-proposed docking. The binding mode that GOLD proposed to be the best for compound **32** differed in the P1-P2 area from the rest of the compounds. To obtain a better mode, a CSCORE calculation within Sybyl was carried out.

The data set consisted of POP inhibitors with varying inhibitory activities. There was not much structural variation at the P1 and P2 positions and without exception, these positions were bound as in the original crystal structure (Fülöp *et al*. 1998). Most of the variation was at the P3 position and beyond it. However, there is a S3 binding site for the P3 position, which is occupied by all ligands. This position is surrounded by the lipophilic residues Ile591, Ala594, Cys259, and Phe173. Beyond the P3 position, conformational freedom increases and one consistent binding site was not found. Instead, two binding modes, which are clearly favored, were found. Each of them is represented by one inhibitor in Figure 2a. The more populated binding mode is called binding mode A (compound **15** in Figure 2a). It is occupied by 60% of the ligands, and it is surrounded by Gly254, Cys255, Arg252, and Met245. The less populated binding mode is called binding mode B (compound **14** in Figure 2a), and it is occupied by 40% of the ligands. Both binding modes resulted in equal inhibitory activities.

The novel inhibitors (Table 1) and most of the previously synthesized compounds (**21**-**51** in Table 2) do not have an aryl-alkanoyl structure, typical to POP inhibitors, at the P3 and beyond P3 positions. Instead, they have rather hydrophilic P3 and beyond P3 positions, due to the amide and ketone carbonyl oxygens and the amide nitrogens. However, their modes of binding do not seem to differ from that of the typical POP inhibitors. For example, SUAM-1221 (compound **55**) occupies the binding mode A.

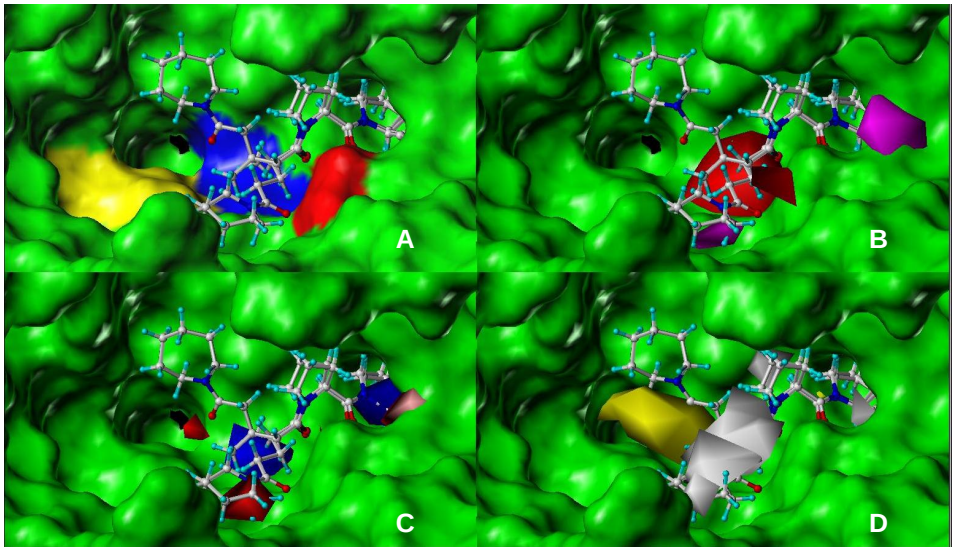


Figure 2. Most favored binding modes and the CoMSIA fields. The model compounds **14** and **15** are docked into the active site of POP. (a) Binding modes A and B are represented by compounds **15** and **14**, respectively. Ile591 and Ala594 are in blue, Met235 is in yellow, and Arg643 is in red. (b) Hydrogen bond acceptor properties. Favored areas are in magenta, and disfavored areas are in red. (c) Electrostatic properties. The partial negative charge is favored in red areas, and the partial positive charge is favored in blue areas. (d) Hydrophobic properties. Hydrophobic properties are favored in yellow areas, and hydrophilic properties are favored in white areas.

CoMSIA. The properties of the CoMSIA model are in agreement with the known SAR of POP inhibitors and the 3D structure of the POP enzyme (Fülöp *et al*. 1998). Hydrogen bond acceptor properties (Figure 2b) are important near the electrophilic 2(*S*)-substituent of the P1 pyrrolidine ring. In the crystal structure, this substituent forms a hemiacetal bond with Ser554 of POP. The thus formed oxyanion is stabilized by two hydrogen bonds to the main chain NH group of Asn555 and to the hydroxyl group of Tyr473 (Fülöp *et al*. 1998). The P2 carbonyl oxygen is hydrogen bonded to Arg643 and the P3 carbonyl oxygen to Trp595. The P2 hydrogen bond has been proven to be crucial for the inhibitory activity (Yoshimoto *et al*. 1991). However, in the model, these hydrogen bonds seem to have only a small contribution to the activity. This misinform arises from the lack of structural variation in these positions within the data set, and consequently, the real contribution to the activity cannot be revealed by the model. The binding mode B includes an area, near Arg128, where hydrogen bond acceptor

properties are also favored. The formation of the hydrogen bond is not probable due to internal hydrogen bonds of POP, but the protein surface is positively charged in this area. The same area also favors partial negative charge.

Hydrogen bond acceptor properties are disfavored in the area located near the residues that do not have any hydrogen bond donors, especially the residues Ile591 (blue in Figure 2a), Ala594 (blue in Figure 2a), and Met235 (yellow in Figure 2a). Any hydrogen bond acceptor atom that points toward this area is entropically nonfavorable, since typically all polar atoms of the bound ligand have a high tendency to interact either with the receptor residues or with the surrounding water molecules.

Partial negative charge (Figure 2c) is favorable near Arg643 (red in Figure 2a), Arg128 and surprisingly near Met235 (yellow in Figure 2a). While the area near Arg643 is explained by the P2 carbonyl oxygen of an inhibitor that forms a hydrogen bond with Arg643, the area near Met235 does not seem to have a clear explanation. It may be an artifact arising from the fact that many potent inhibitors have carbonyl oxygen located in this area even though the oxygen cannot form a hydrogen bond with the enzyme. The tendency to favor partial positive charge over the carbons of the P1 prolyl ring and partial negative charge over the 2(*S*)-substituent (Figure 2c) is easily understood via the polarization between the substituent and the ring. When this polarization is strong, the interaction between the inhibitor and the Ser554 becomes stronger.

Hydrophobic properties (Figure 2d) are clearly divided; the hydrophobic nature of the inhibitors is disfavored at the P1-P2 area, near the residue Arg643. The P3 position favors lipophilic aliphatic structures. Lipophilic aromatic structures with negative π-electrons are not optimal structures but are tolerated. Beyond the P3 position, the binding mode A shows a clear hydrophobic area. The binding mode B, on the other hand, favors nonhydrophobic structures near the residue Arg128.

SARs. Three different azacycles, pyrrolidine, morpholine, and azepane, were studied at the position beyond P3, resulting in compounds **3**, **4**, and **5** respectively. A succinic acid residue was located at the P3 position and L-Pro-pyrrolidine at the P2-P1 positions. The morpholine moiety caused considerably lower potency than the azepane and pyrrolidine moieties. The replacement by the azepane moiety resulted in a more potent inhibitor than the replacement by the pyrrolidine moiety. This difference was also observed in compounds **11** and **12**, which have a 2(*S*)-cyanopyrrolidine moiety at the P1 position. When the flexible succinic acid residue was changed to the rigid

isophthalic acid residue (**19** and **20**), the azepane and pyrrolidine moieties gave equipotent compounds.

The polar oxygen of the morpholine ring is located in an area that favors lipophilic structures. This explains the significant decrease in inhibitory activity caused by morpholine. However, the increased lipophilicity of azepane does not explain the difference between the IC_{50} values of compounds **3** and **5**. In compound **3**, the carbonyl group next to the pyrrolidine moiety is located in an area that disfavors hydrogen bond acceptors. The carbonyl oxygen points toward the enzyme surface, which lacks hydrogen bond donors to form a bond with, and this results in an unfavorable entropic effect. In compound **5**, the larger ring size of azepane distorts the carbonyl oxygen in a different direction, toward an open cavity. The cavity is large enough to include a water molecule that may form a hydrogen bond with the oxygen. In compounds **19** and **20**, the rigid isophthalic acid residue fixes the conformation and the inhibitors bind in a similar way to the enzyme. The phenyl group is located in an area that disfavors both lipophilic structures and negative charge, and this may explain the lower activity of compounds **19** and **20** as compared to compound **12**.

Three aliphatic chains, succinic acid, glutaric acid and 3,3-dimethylglutaric acid, were studied at the P3 position, resulting in compounds **12**, **14**, and **15**, respectively. Azepane was used at the position beyond P3 and L-Pro-2(*S*)-cyanopyrrolidine at the P2-P1 positions. The elongation of the chain from succinic acid to glutaric acid decreased the potency. 3,3-Dimethylglutaric acid, on the other hand, increased the potency slightly.

Compound **14** (orange in Figure 2a) seems to have a different binding mode from compounds **12** and **15** (violet in Figure 2a); the molecule turns toward the open cavity. However, the slight decrease in potency cannot be explained with the CoMSIA model. On the other hand, the increased lipophilicity of compound **15** in comparison to compound **14** may explain the increased potency in this case.

Three different substitution patterns of the phenylene moiety were studied at the P3 position, resulting in compounds **17**, **18**, and **19**. Pyrrolidine was used at the position beyond P3 and an L-Pro-2(*S*)-cyanopyrrolidine group at the P2-P1 positions. The ortho-substituted inhibitor **17** had strikingly 3 orders of magnitude lower activity than the para- and meta-substituted analogues **18** and **19**, respectively. The para-substitution resulted in the best inhibitory activity.

A rigid phenylene ring fixes the conformation, and the inhibitor cannot adjust itself to a more favorable conformation. In compound **17**, the phenylene ring is located in an

area that favors hydrophilic structures. In addition, both carbonyl oxygens of the ortho-phthalic residue are located in the area that disfavors hydrogen bond acceptors.

Pyrrolidine and 2(*S*)-cyanopyrrolidine moieties were studied at the P1 position of the novel inhibitors. Inhibitors containing a 2(*S*)-cyanopyrrolidine group at the P1 position are very potent POP inhibitors (Tanaka *et al*. 1994). Also in this study, the 2(*S*)-cyanopyrrolidine group increased the potency significantly as compared to a pyrrolidine group (**3** vs **11**, **5** vs **12**, **13** vs **14**, and **16** vs **17**). The interaction of the cyano group with the cystein protease papain has been studied. Cystein proteases have a similar catalytic mechanism to serine proteases. The cyano group forms a covalent, reversible thioimidate adduct with the thiol group of the active site cysteine (Brisson *et al*. 1986, Liang and Abeles 1987). A similar interaction between the hydroxyl group of the catalytic Ser554 and a cyano group is possible. Recently, the binding kinetics of compound **37** to POP was studied (Venäläinen *et al*. 2004). It was found to be slow binding, and the inhibition was suggested to be a direct binding process.

The role of the molecular size was studied, and the novel inhibitors were compared to the previously synthesized compounds. The removal of the pyrrolidine-1-carbonyl group from compounds **21** (flexible P3 moiety) and **37** (rigid P3 moiety) resulted in compounds **3** and **19**, respectively. In both cases, the potency decreased slightly. The replacement of the L-Pro-pyrrolidine group beyond the P3 position of compounds **21**, **22** and **37** with azepane resulted in compounds **5**, **13**, and **20**, respectively. Compounds **5** and **13** have flexible P3 moieties, and in both cases, the replacement increased the inhibitory activity. On the other hand, in the case of compound **20** with a rigid P3 moiety, the inhibitory activity decreased. Compound **20** is also less potent than the other large compounds of the same series as compound **37** (**38**-**42**). Interestingly, also, the small compound **54**, having only a benzoyl group at the P3 position, was slightly more active than compound **20**. The differences in the inhibitory activities between the novel compounds and the previously synthesized larger compounds were rather small and could not be explained with the CoMSIA model.

5.4 Conclusions

The P1 and P2 positions of the docked ligands were bound to POP in the same way as Z-L-prolyl-L-prolinal in the crystal structure. A consistent P3 position was also found. This position is surrounded by lipophilic residues, and lipophilic aliphatic structures are favored. Lipophilic aromatic structures with a π-electron cloud can also be used, but then, the substitution pattern becomes crucial. Beyond the P3 position, the docked

ligands divided into two different binding modes A and B, which were occupied by 60 and 40% of the inhibitors, respectively. According to the previously postulated idea of the lipophilic cavity, the binding mode A favors lipophilic structures. The binding mode B instead favors nonhydrophobic structures, and partial negative charge is favored near Arg128, where the protein surface has a partial positive charge. The P3 and beyond P3 area is sufficiently large, and the interactions between POP and the ligands are weak. This area can accommodate several different groups without affecting the inhibitory activity and could be a valuable modification site for the optimization of ADME properties. The docking studies with the 3D QSAR models are helpful in the design of new compounds.

5.5 Synthetic procedures and analytical data

General Synthetic Procedure A: A solution of 1.0 eq trimethylacetyl chloride in dichloromethane (DCM) is added to a solution of 1.0 eq carboxylic acid and 1.1 eq triethylamine in DCM at 0 °C. The reaction mixture is stirred at 0 °C for 1 h. A solution of 1.1 eq triethylamine and 1.0-1.1 eq amine in DCM is slowly added at 0 °C (if the amine is in the form of a trifluoroacetic acid salt or HCl salt, then 3.3 eq triethylamine is used and the triethylamine is added separately before the amine is added). The reaction mixture is stirred 2 h or overnight at rt. The DCM solution is washed with 30% citric acid aq, saturated NaCl aq and saturated $NaHCO_3$ aq, dried over anhydrous Na_2SO_4 and evaporated, yielding the crude product.

General Synthetic Procedure B: 1.0-1.5 eq $LiOH{\cdot}H_2O$ is added to a solution of 1.0 eq carboxylic acid methyl ester in 20-30% water in methanol. The reaction mixture is stirred 2 h or overnight at rt. The solvent (methanol) is evaporated and the residue is dissolved in water. The aqueous phase is washed with DCM. The aqueous phase is made acidic with 2-3 M HCl aq, and the product is extracted with DCM. The organic phase is dried over anhydrous Na_2SO_4 and evaporated, yielding the product.

General Synthetic Procedure C: 2-4 ml ethyl acetate saturated with dry HCl is added to 1.0 mmol Boc protected amine at rt. The reaction mixture is stirred at rt for 30 min. Another way to proceed is to dissolve 1.0 mmol Boc protected amine in 5-10 ml DCM and add 2-4 ml trifluoroacetic acid at 0°C. The reaction is stirred at 0°C for 2-2.5 h. In both cases the solvent is removed and the product is finally evaporated *in vacuo*, yielding the corresponding amine HCl salt or TFA salt.

Boc-L-prolyl-pyrrolidine (1). Boc-L-proline (7.97 g, 37.0 mmol) and pyrrolidine (3.09 ml, 37.0 mmol) were coupled according to procedure A. The product was purified

by flash chromatography (2.5-5% MeOH in DCM). Yield 8.31 g, 84%. ^{1}H NMR δ 1.40 (4.5 H, s), 1.46 (4.5 H, s), 1.77-2.21 (8 H, m), 3.37-3.76 (6 H, m), 4.35 (0.5 H, dd, *J* = 5.0 Hz, 8.0 Hz), 4.48 (0.5 H, dd, *J* = 3.1 Hz, 7.7 Hz).

Succinic acid monomethyl ester L-prolyl-pyrrolidine amide (2). Boc-L-prolyl-pyrrolidine (1.98 g, 7.38 mmol) was deprotected using 30 ml HCl saturated ethyl acetate according to procedure C. Succinic acid monomethyl ester (0.98 g, 7.42 mmol) and the L-prolyl-pyrrolidine HCl salt were coupled according to procedure A. The product was purified by flash chromatography (5-10% MeOH in EtOAc). Yield 1.81 g, 86%. ^{1}H NMR δ 1.79-2.17 (7 H, m), 2.21-2.35 (1 H, m), 2.45-2.63 (2 H, m), 2.68-2.86 (2 H, m), 3.35-3.51 (2 H, m), 3.52-3.63 (2 H, m), 3.68 (3 H, s), 3.70-3.82 (2 H, m), 4.58 (0.1 H, dd, *J* = 3.0 Hz, 8.5 Hz), 4.66 (0.9 H, dd, *J* = 3.6 Hz, 8.3 Hz).

Succinic acid pyrrolidine L-prolyl-pyrrolidine amide (3). The methyl ester group of **2** (750 mg, 2.7 mmol) was hydrolyzed using LiOH·H_2O (170 mg, 4.1 mmol) according to procedure B. Yield 360 mg, 1.3 mmol, 45%. The obtained product was coupled with pyrrolidine (0.11 ml, 1.3 mmol) according to procedure A. The product was purified by flash chromatography (25% MeOH in EtOAc). Yield 170 mg, 41%. ^{1}H NMR δ 1.79-2.18 (10 H, m), 2.23-2.45 (2 H, m), 2.47-2.68 (2 H, m), 2.78-2.92 (2 H, m), 3.39-3.62 (7 H, m), 3.64-3.84 (3 H, m), 4.68 (dd, 0.9 H, *J* = 3.8 Hz, 8.4), 4.77 (dd, 0.1 H, *J* = 3.0 Hz, 8.8 Hz); ^{13}C NMR δ 24.1, 24.4, 24.8, 26.0, 26.2, 28.9, 29.2, 29.3, 45.7, 45.9, 46.2, 46.4, 47.3, 57.8, 170.5, 170.7, 170.8; MS (ESI, +) *m/z* 322 $[M + H]^+$; Anal.($C_{17}H_{27}N_3O_3$) C, H, N.

Succinic acid morpholine L-prolyl-pyrrolidine amide (4). The methyl ester group of **2** (560 mg, 2.0 mmol) was hydrolyzed using LiOH·H_2O (84 mg, 2.0 mmol) in 10 ml 20% water in MeOH at rt. After 4 h the solvent was evaporated and the residue was dissolved in DCM. The organic phase was dried and evaporated. The obtained product was coupled with morpholine (0.18 ml, 2.05 mmol) according to procedure A. The product was purified by flash chromatography (20-25% MeOH in EtOAc). Yield 330 mg, 49%. ^{1}H NMR δ 1.80-2.05 (6 H, m), 2.08-2.35 (2 H, m), 2.39-2.64 (2 H, m), 2.78-2.95 (2 H, m), 3.35-3.81 (14 H, m), 4.64 (0.9 H, dd, *J* = 3.8 Hz, 8.3 Hz), 4.69 (0.1 H, dd, *J* = 3.8 Hz, 8.5 Hz); ^{13}C NMR δ 24.1, 24.8, 26.2, 27.7, 28.9, 29.3, 42.2, 45.8, 46.1, 46.4, 47.4, 58.0, 66.6, 66.9, 170.9, 171.0, 171.1; MS (ESI, +) *m/z* 338 $[M + H]^+$; Anal. ($C_{17}H_{27}N_3O_4$·0.3H_2O) C, H, N.

Succinic acid azepane L-prolyl-pyrrolidine amide (5). The methyl ester group of **2** (560 mg, 2.0 mmol) was hydrolyzed using LiOH·H_2O (84 mg, 2.0 mmol) in 10 ml 20% water in MeOH at rt. After 3 h the solvent was evaporated and the residue was

dissolved in DCM. The organic phase was dried and evaporated. The obtained product was coupled with azepane (0.23 ml, 2.04 mmol) according to procedure A. The product was purified by flash chromatography (20% MeOH in EtOAc). Yield 350 mg, 50%. ^{1}H NMR δ 1.51-1.61 (4 H, m), 1.64-1.77 (4 H, m), 1.81-2.45 (8 H, m), 2.51-2.64 (2 H, m), 2.80-2.88 (2 H, m), 3.36-3.90 (10 H, m), 4.65 (0.9 H, dd, *J* = 3.8 Hz, 8.4 Hz), 4.71 (0.1 H, dd, *J* = 3.1 Hz, 8.6 Hz); ^{13}C NMR δ 24.1, 24.8, 26.2, 26.9, 27.1, 27.6, 28.0, 28.9, 29.0, 29.6, 45.9, 46.0, 46.3, 47.2, 47.7, 57.8, 170.7, 170.9, 171.6; MS (ESI, +) *m/z* 350 $[M + H]^+$; Anal. ($C_{19}H_{31}N_3O_3 \cdot 0.2H_2O$) C, H, N.

Phthalic acid mono-(L-proline methyl ester) amide (6). Phthalic anhydride (7.4 g, 45 mmol) was added to a solution of L-proline methyl ester HCl salt (7.4 g, 45 mmol) and triethylamine (13.8 ml, 99.0 mmol) in DCM at 0°C. The reaction mixture was stirred 4 h at rt. The organic phase was extracted with saturated $NaHCO_3$ aq. The aqueous phase was acidified with 3 M HCl aq. and extracted with chloroform. The chloroform phase was dried and evaporated. Yield 6.53 g, 51%.

Succinic acid mono-pyrrolidine amide (7a). Succinic acid monomethyl ester (1.98 g, 14.9 mmol) and pyrrolidine (1.3 ml, 15 mmol) were coupled according to procedure A. The product was purified by flash chromatography (EtOAc). Yield 1.75 g, 63%. ^{1}H NMR δ 1.82-1.89 (2 H, m), 1.94-2.01 (2 H, m), 2.56-2.59 (2 H, m), 2.67-2.70 (2 H, m), 3.44-3.48 (4 H, m), 3.69 (3 H, s). The methyl ester group of the product was hydrolyzed using $LiOH \cdot H_2O$ (0.44 g, 10.4 mmol) according to procedure B. Yield 1.37 g, 85%.

Isophthalic acid mono-pyrrolidine amide (7b). Isophthalic acid monomethyl ester (2.25 g, 12.5 mmol) and pyrrolidine (1.04 ml, 12.5 mmol) were coupled according to procedure A. The product was purified by flash chromatography with 30% petroleum ether (PE) in EtOAc as the eluent. Yield 1.75 g, 60%. ^{1}H NMR δ 1.83-2.02 (4 H, m), 3.43 (2 H, t, *J* = 6.6 Hz), 3.66 (2 H, t, *J* = 6.9 Hz), 3.93 (3 H, s), 7.50 (1 H, td, *J* = 7.7 Hz, 0.6 Hz), 7.73 (1 H, ddd, *J* = 7.7 Hz, 1.2 Hz, 1.7 Hz), 8.09 (1 H, ddd, *J* = 7.7 Hz, 1.2 Hz, 1.7 Hz), 8.19 (1 H, td, *J* = 1.7 Hz, 0.6 Hz). The methyl ester group of the product was hydrolyzed using $LiOH \cdot H_2O$ (470 mg, 11.3 mmol) according to procedure B. Yield 1.49 g, 91%.

Terephthalic acid mono-pyrrolidine amide (7c). Terephthalic acid monomethyl ester (1.08 g, 5.99 mmol) and pyrrolidine (0.50 ml, 6.0 mmol) were coupled according to procedure A. The product was purified by flash chromatography (EtOAc). Yield 0.50 g, 36%. ^{1}H NMR δ 1.87-1.92 (2 H, m), 1.95-2.00 (2 H, m), 3.38 (2 H, t, *J* = 6.7 Hz), 3.66 (2 H, t, *J* = 7.0 Hz), 3.94 (3 H, s), 7.56-7.57 (1 H, m), 7.58-7.59 (1 H, m), 8.06-8.07 (1 H, m), 8.08-8.09 (1 H, m). The methyl ester group of the product was

hydrolyzed using $LiOH \cdot H_2O$ (135 mg, 3.2 mmol) according to procedure B. Yield 0.41 g, 87%.

Succinic acid mono-azepane amide (8a). Succinic acid monomethyl ester (1.98 g, 15.0 mmol) and azepane (1.7 ml, 15 mmol) were coupled according to procedure A. The product was purified by flash chromatography (30% PE in EtOAc). Yield 2.48 g, 78%. ^{1}H NMR δ 1.52-1.62 (4 H, m), 1.67-1.78 (4 H, m), 2.62-2.70 (4 H, m), 3.46 (2 H, t, *J* = 6.1 Hz), 3.53 (2 H, t, *J* = 6.1 Hz), 3.69 (3 H, s). The methyl ester group of the product was hydrolyzed using $LiOH \cdot H_2O$ (540 mg, 12.9 mmol) according to procedure B. Yield 2.34 g, 100%.

Isophthalic acid mono-azepane amide (8b). Isophthalic acid monomethyl ester (2.25 g, 12.5 mmol) and azepane (1.41 ml, 12.5 mmol) were coupled according to procedure A. The product was purified by flash chromatography (40% PE in EtOAc). Yield 2.02 g, 62%. ^{1}H NMR δ 1.57-1.69 (6 H, m), 1.83-1.89 (2 H, m), 3.35-3.37 (2 H, m), 3.68-3.71 (2 H, m), 3.93 (3 H, s), 7.49 (1 H, dd, J = 7.6 Hz, 8.2 Hz), 7.57-7.60 (1 H, m), 8.05-8.08 (2 H, m). The methyl ester group of the product was hydrolyzed using $LiOH \cdot H_2O$ (490 mg, 11.7 mmol) according to procedure B. Yield 1.89 g, 99%.

Glutaric acid mono-azepane amide (8c). To a solution of azepane (4.5 ml, 40 mmol) in 40 ml tetrahydrofuran (THF) was added glutaric anhydride (2.3 g, 20 mmol) in 15 ml THF dropwise and the solution was stirred for 2 days. The reaction mixture was evaporated, dissolved in 0.025 M NaOH aq., and washed with DCM. The aqueous phase was acidified with 3 M HCl and the product was extracted with DCM. The combined organic phases were dried and evaporated. Yield 1.61 g, 38%. ^{1}H NMR δ 1.52-1.62 (4 H, m), 1.68-1.78 (4 H, m), 1.99 (2 H, qui, *J* = 7.0 Hz), 2.46 (2H, t, *J* = 7.0 Hz), 2.47 (2H, t, *J* = 7 Hz), 3.44 (2 H, t, *J* = 6.1 Hz), 3.54 (2 H, t, *J* = 6.1 Hz).

3,3-Dimethylglutaric acid mono-azepane amide (8d). To a solution of azepane (1.74 ml, 15.4 mmol) in 10 ml THF was added 3,3-dimethylglutaric anhydride (1.0 g, 7.0 mmol) in 10 ml THF dropwise at 0 °C. The reaction mixture was stirred overnight at rt, evaporated and dissolved in 0.025 M NaOH aq. The aqueous phase was washed with DCM, acidified with 3 M HCl aq. and extracted with DCM. The organic phase was dried and evaporated, yielding solid product, 1.53 g, 90%. ^{1}H NMR δ 1.13 (6 H, s), 1.56-1.64 (4 H, m), 1.74-1.79 (4 H, m), 2.45 (2 H, s), 2.50 (2 H, s), 3.56-3.58 (2 H, m), 3.60-3.62 (2 H, m), 13.79 (1 H, br s).

Succinic acid pyrrolidine L-proline amide (9a). 7a (1.37g, 8.00 mmol) was coupled with proline methyl ester HCl salt (1.3 g, 8.0 mmol) according to procedure A. The product was purified by flash chromatography (5% MeOH in DCM). Yield 1.45 g,

64%. The methyl ester group of the product was hydrolyzed using $LiOH \cdot H_2O$ (0.32 g, 7.65 mmol) according to procedure B. Yield 1.32 g, 96%. ^{1}H NMR δ 1.81-2.08 (7 H, m), 2.22-2.40 (1 H, m), 2.53-2.64 (2 H, m), 2.66-2.81 (2 H, m), 3.39-3.70 (6 H, m), 4.52-4.59 (1 H, m).

Isophthalic acid pyrrolidine L-proline amide (9b). 7b (1.49 g, 6.80 mmol) and proline methyl ester HCl salt (1.13 g, 6.82 mmol) were coupled according to procedure A. The product was purified by flash chromatography (10% MeOH in EtOAc). Yield 1.83 g, 81%. ^{1}H NMR δ 1.85-2.11 (7 H, m), 2.19-2.38 (1 H, m), 3.41 (2 H, t, *J* = 6.4 Hz), 3.50-3.56 (1 H, m), 3.60-3.69 (3 H, m), 3,78 (3 H, s), 4.29-4.33 (0.2 H, m), 4.67 (0.8 H, dd, *J* = 5.3 Hz, 8.3 Hz), 7.46 (1 H, t, *J* = 7.8 Hz), 7.58-7.60 (1 H, m), 7.61-7.64 (1 H, m), 7.71-7.73 (1 H, m). The methyl ester group of the product was hydrolyzed using $LiOH \cdot H_2O$ (350 mg, 8.3 mmol) according to procedure B. Yield 1.58 g, 91%.

Terephthalic acid pyrrolidine L-proline amide (9c). 7c (0.41 g, 1.87 mmol) and L-proline methyl ester HCl salt (0.31 g, 1.87 mmol) were coupled according to procedure A. The product was purified by flash chromatography (10% MeOH in EtOAc). Yield 0.54 g, 87%. ^{1}H NMR δ 1.86-2.13 (7 H, m), 2.19-2.36 (1 H, m), 3.39 (2 H, t, *J* = 6.6 Hz), 3.47-3.52 (1 H, m), 3.60-3.67 (3 H, m), 3.79 (3 H, s), 4.29-4.31 (0.2 H, m), 4.68 (0.8 H, dd, *J* = 5.2 Hz, 8.5 Hz), 7.39-7.41 (0.4 H, m), 7.50-7.52 (0.4 H, m), 7.54-7.56 (1.6 H, m), 7.59-7.61 (1.6 H, m). The methyl ester group of the product was hydrolyzed using $LiOH \cdot H_2O$ (103 mg, 2.45 mmol) according to procedure B. Yield 0.50 g, 97%.

Phthalic acid pyrrolidine L-proline amide (9d). 6 (4.25 g, 17.0 mmol) and pyrrolidine (1.6 ml, 18.7 mmol) were coupled according to procedure A. The product was purified by flash chromatography (5-20% MeOH in EtOAc). Yield 4.77 g, 85%. The methyl ester group of the product was hydrolyzed using $LiOH \cdot H_2O$ (906 mg, 21.6 mmol) according to procedure B. Yield 3.42 g, 75%.

Succinic acid azepane L-proline amide (10a). 8a (2.34 g, 11.7 mmol) was coupled with proline methyl ester HCl salt (1.9 g, 11.7 mmol) according to procedure A. The product was purified by flash chromatography (5% MeOH in DCM). Yield 2.22 g, 61%. ^{1}H NMR δ 1.47-1.60 (4 H, m), 1.63-1.78 (4 H, m), 1.83-1.35 (4 H, m), 2.47-2.93 (4 H, m), 3.41-3.71 (6 H, m), 2.72 (2.5 H, s), 3.76 (0.5 H, s), 4.49 (0.8 H, dd, *J* = 3.8 Hz, 8.7 Hz), 4.65 (0.2 H, dd, *J* = 2.6 Hz, 8.5 Hz). The methyl ester group of the product was hydrolyzed using $LiOH \cdot H_2O$ (0.45 g, 10.7 mmol) according to procedure B. Yield 2.01 g, 95%.

Isophthalic acid azepane L-proline amide (10b). 8b (1.89 g, 7.64 mmol) and proline methyl ester HCl salt (1.27 g, 7.67 mmol) were coupled according to procedure A. The product was purified by flash chromatography (10% MeOH in EtOAc). Yield 2.08 g, 76%. ^{1}H NMR δ 1.55-1.67 (4 H, m), 1.81-1.94 (4 H, m), 1.98-2.10 (3 H, m), 2.20-2.37 (1 H, m), 3.35 (2 H, t, *J* = 5.5 Hz), 3.50-3.56 (1 H, m), 3.61-3.74 (3 H, m), 3.78 (3 H, s), 4.29-4.32 (0.1 H, m), 4.66 (0.9 H, dd, *J* = 5.5 Hz, 8.3 Hz), 7.44-7.46 (2 H, m), 7.58-7.61 (2 H, m).The methyl ester group of the product was hydrolyzed using LiOH·H_2O (370 mg, 8.7 mmol) according to procedure B. Yield 1.67 g, 83%.

Glutaric acid azepane L-proline amide (10c). 8c (1.61 g, 7.55 mmol) and L-proline methyl ester HCl salt (1.24 g, 7.49 mmol) were coupled according to procedure A. The product was purified by flash chromatography (5-10% MeOH in EtOAc) to give the product as colourless oil. Yield 1.8 g, 74%. ^{1}H NMR δ 1.50-1.60 (4 H, m), 1.66-1.74 (4 H, m), 1.87-2.34 (6 H, m), 2.35-2.48 (4 H, m), 3.42-3.68 (6 H, m), 3.72 (2.5 H, s), 3.75 (0.5 H, s), 4.45-4.49 (1 H, m). The methyl ester group of the product (1.8 g, 5.6 mmol) was hydrolyzed using LiOH·H_2O (0.35 g, 8.3 mmol) according to procedure B. Yield 1.55 g, 90%.

3,3-Dimethylglutaric acid azepane L-proline amide (10d). 8d (0.50 g, 2.07 mmol) and L-proline methyl ester HCl salt (0.34 g, 2.05 mmol) were coupled according to procedure A. The product was purified by flash chromatography (25-50% EtOAc in PE). Yield 0.55 g, 75%. ^{1}H NMR δ 1.19 (3 H, s), 1.20 (3 H, s), 1.50-1.58 (4 H, m), 1.66-1.74 (4 H, m), 1.84-2.26 (4 H, m), 2.43 (1 H, d, *J* = 15.5 Hz), 2.53 (1 H, d, *J* = 15.0 Hz), 2.57 (1 H, d, *J* = 15.5 Hz), 2.63 (1 H, d, *J* = 15.0 Hz), 3.44-3.70 (6 H, m), 3.71 (2.5 H, s), 3.75 (0.5 H, s), 4.46 (0.8 H, dd, *J* = 4.2 Hz, 8.7 Hz), 4.58 (0.2 H, dd, *J* = 2.4 Hz, 8.5 Hz). The methyl ester group of the product (0.55g, 1.6 mmol) was hydrolyzed using LiOH·H_2O (0.10 g, 2.4 mmol) according to procedure B. Yield 0.52 g, 96%.

Succinic acid pyrrolidine L-prolyl-2(*S*)-cyanopyrrolidine amide (11). Boc-2(*S*)-cyanopyrrolidine (0.96 g, 4.89 mmol) was deprotected using 20 ml trifluoroacetic acid in 50 ml DCM according to procedure C. 2(*S*)-cyanopyrrolidine TFA salt was coupled with **9a** (1.32 g, 4.92 mmol) according to procedure A. The product was purified by flash chromatography (10% MeOH in EtOAc). Yield 170 mg, 10%. ^{1}H NMR δ 1.81-1.86 (2 H, m), 1.91-2.05 (4 H, m), 2.12-2.32 (6 H, m), 2.42-2.54 (2 H, m), 2.73-2.88 (2 H, m), 3.36-3.48 (4 H, m), 3.60-3.63 (1 H, m), 3.66-3.75 (2 H, m), 3.82-3.86 (1 H, m), 4.59 (1 H, dd, *J* = 4.0 Hz, 8.4 Hz), 4.78-4.82 (1 H, m); ^{13}C NMR δ 24.4, 24.8, 25.4, 26.0, 27.7, 29.1, 29.3, 29.7, 45.7, 46.3, 46.4, 46.5, 47.3, 57.5, 118.6, 170.3, 171.2,

171.4; MS (ESI, +) *m/z* 369 $[M + Na]^+$; Anal. ($C_{18}H_{26}N_4O_3 \cdot 0.1H_2O$) C, H, N: calcd, 16.09; found, 15.57.

Succinic acid azepane L-prolyl-2(*S*)-cyanopyrrolidine amide (12). Boc-2(*S*)-cyanopyrrolidine (1.3 g, 6.8 mmol) was deprotected using 27 ml trifluoroacetic acid in 70 ml DCM according to procedure C. **10a** (2.01 g, 6.78 mmol) and the 2(*S*)-cyanopyrrolidine trifluoroacetic acid salt were coupled according to procedure A. The product was purified by flash chromatography (8-10% MeOH in EtOAc). Yield 320 mg, 13%. ^{1}H NMR δ 1.47-1.59 (4 H, m), 1.62-1.76 (4 H, m), 1.92-2.06 (2 H, m), 2.10-2.33 (6 H, m), 2.45-2.58 (2 H, m), 2.76-2.89 (2 H, m), 3.35-3.87 (8 H, m), 4.58 (1 H, dd, *J* = 3.7 Hz, 8.1 Hz), 4.80-4.83 (1 H, m); ^{13}C NMR δ 24.8, 25.4, 26.9, 27.1, 27.6, 27.9, 28.7, 29.0, 29.5, 29.7, 46.0, 46.3, 46.5, 47.3, 47.7, 57.5, 118.6, 171.2, 171.3, 171.4; MS (ESI, +) *m/z* 375 $[M + H]^+$; Anal. ($C_{20}H_{30}N_4O_3 \cdot 0.2H_2O$) C, H, N.

Glutaric acid azepane L-prolyl-pyrrolidine amide (13). 10c (0.51 g, 1.64 mmol) was coupled with pyrrolidine (0.14 ml, 1.68 mmol) according to procedure A. The product was purified by flash chromatography (15% MeOH in EtOAc) to give the product as colourless oil. Yield 0.34 g, 57%. ^{1}H NMR δ 1.51-1.58 (4 H, m), 1.65-1.74 (4 H, m), 1.78-2.29 (10 H, m), 2.31-2.48 (4 H, m), 3.35-3.72 (9 H, m), 3.80-3.85 (1 H, m), 4.57 (0.1 H, dd, *J* = 2.8 Hz, 8.8 Hz), 4.64 (0.9 H, dd, *J* = 3.8 Hz, 8.4 Hz); ^{13}C NMR δ 20.4, 24.1, 24.9, 26.2, 26.9, 27.2, 27.7, 28.9, 29.2 32.3, 33.8, 45.9, 45.9, 46.3, 47.4, 47.9, 57.7, 170.7, 171.4, 172.3; MS (ESI, +) *m/z* 364 $[M + H]^+$; Anal. ($C_{20}H_{33}N_3O_3 \cdot 0.1H_2O$) C, H, N.

Glutaric acid azepane L-prolyl-L-proline amide. 10c (0.38 g, 1.22 mmol) and L-proline methyl ester HCl salt (0.20 g, 1.21 mmol) were coupled according to procedure A. The product was purified by flash chromatography (10-20% MeOH in EtOAc) to give the product as oil. Yield 0.42 g, 82%. The methyl ester group of the obtained product was hydrolyzed using $LiOH \cdot H_2O$ (63 mg, 1.5 mmol) according to procedure B to give the white solid product. Yield 0.39 g, 95%. ^{1}H NMR δ 1.50-1.61 (4 H, m), 1.65-1.77 (4H, m), 1.82-2.54 (14 H, m), 3.42 (2 H, t, *J* = 6.0 Hz), 3.51 (2 H, t, *J* = 6.0 Hz), 3.54-3.72 (3 H, m), 3.77-3.86 (1 H, m), 4.32 (0.2 H, dd, *J* = 3.4 Hz, 8.1 Hz), 4.44-4.48 (0.2 H, m), 4.60-4.64 (1.6 H, m).

Glutaric acid azepane L-prolyl-L-prolinamide amide. To a solution of glutaric acid azepane L-prolyl-L-proline amide (170 mg, 0.42 mmol) and Et_3N (59 µl, 0.42 mmol) in 8 ml THF was added ethyl chloroformate (40 µl, 0.42 mmol) in 4 ml THF dropwise at -10 °C. After 20 min 25% NH_3 aq. (143 µl, 2 .1 mmol) was added and the reaction mixture was stirred overnight at rt. The reaction mixture was evaporated,

dissolved in DCM and filtered. The filtrate was washed with sat. $NaHCO_3$ aq, dried and evaporated yielding the white solid product (150 mg, 88%). ^{1}H NMR δ 1.50-1.60 (4 H, m), 1.66-1.76 (4 H, m), 1.78-2.23 (9 H, m), 2.29-2.61 (5 H, m), 3.38-3.84 (8 H, m), 4.28-4.30 (0.4 H, m), 4.39 (0.4 H, dd, *J* = 5.9 Hz, 7.4 Hz), 4.61-4.68 (1.2 H, m), 5.24 (0.5 H, br s), 5.53 (0.5 H, br s), 6.91 (0.5 H, br s), 8.26 (0.5 H, br s).

Glutaric acid azepane L-prolyl-2(*S*)-cyanopyrrolidine amide (14). To a solution of glutaric acid azepane L-prolyl-L-prolinamide amide (150 mg, 0.37 mmol) and Et_3N (155 µl, 1.11 mmol) in 8 ml THF was added trifluoroacetic anhydride (80 µl, 0.56 mmol) in 4 ml THF dropwise at 0 °C. The reaction mixture was stirred 2 h at rt, evaporated and dissolved in DCM. The organic phase was washed with 30% citric acid aq, dried and evaporated. The product was purified by flash chromatography (10% MeOH in EtOAc) yielding colorless oil (76 mg, 53%). ^{1}H NMR δ 1.52-1.59 (4 H, m), 1.66-1.73 (4 H, m), 1.90-2.06 (4 H, m), 2.09-2.31 (6 H, m), 2.35-2.47 (4 H, m), 3.39-3.44 (2 H, m), 3.45-3.64 (4 H, m), 3.66-3.71 (1 H, m), 3.85-3.90 (1 H, m), 4.55 (0.9 H, dd, *J* = 4.2 Hz, 8.6 Hz), 4.60-4.62 (0.1 H, m), 4.65-4.68 (0.1 H, m), 4.79-4.81 (0.9 H, m); ^{13}C NMR δ 20.3, 25.0, 25.4, 26.9, 27.2, 27.7, 28.8, 29.1, 29.7, 32.1, 33.7, 45.9, 46.3, 46.5, 47.4, 47.8, 57.4, 118.6, 171.4, 171.7, 172.1; MS (ESI, +) *m/z* 389 $[M + H]^+$; Anal. ($C_{21}H_{32}N_4O_3 \cdot 0.2H_2O$) C, H, N.

3,3-Dimethylglutaric acid azepane L-prolyl-L-proline amide. 10d (0.52 g, 1.54 mmol) and L-proline methyl ester HCl salt (0.26 g, 1.57 mmol) were coupled according to procedure A. The product was purified by flash chromatography (10% MeOH in EtOAc). Yield 0.54 g, 78%. ^{1}H NMR δ 1.18 (3 H, s), 1.19 (3 H, s), 1.50-1.58 (4 H, m), 1.66-1.72 (4 H, m), 1.80-2.28 (8 H, m), 2.37 (1 H, d, *J* = 15.2 Hz), 2.48 (1 H, d, *J* = 14.6 Hz), 2.59 (1 H, d, *J* = 15.2 Hz), 2.63 (1 H, d, *J* = 14.6 Hz), 3.40-3.69 (8 H, m), 3.70 (3 H, s), 3.40-3.56 (4 H, m), 3.58-3.69 (3 H, m), 3.70 (3 H, s), 3.81-3.87 (1 H, m), 4.54-4.59 (1 H, m), 4.67 (0.9 H, dd, *J* = 3.8 Hz, 7.8 Hz), 4.75-4.77 (0.1 H, m). The methyl ester group of the obtained product was hydrolyzed using $LiOH \cdot H_2O$ (76 mg, 1.8 mmol) according to procedure B. Yield 0.54 g, 100%.

3,3-Dimethylglutaric acid azepane L-prolyl-L-prolinamide amide. To a solution of 3,3-dimethylglutaric acid azepane L-prolyl-L-proline amide (0.54 g, 1.24 mmol) and Et_3N (0.17 ml, 1.22 mmol) in THF was added ethyl chloroformate (0.12 ml, 1.26 mmol) in THF dropwise at -10 °C. After 20 min 25% NH_3 aq (84 µl, 1.2 mmol) was added and the reaction mixture was allowed to warm up to rt and stirred overnight. The reaction mixture was evaporated, dissolved in DCM and washed with saturated $NaHCO_3$ aq. The organic phase was dried and evaporated. Yield 450 mg, 84%.

3,3-Dimethylglutaric acid azepane L-prolyl-2(*S*)-cyanopyrrolidine amide (15). To a solution of 3,3-dimethylglutaric acid azepane L-prolyl-L-prolinamide amide (0.45 g, 1.04 mmol) and Et_3N (0.43 ml, 3.09 mmol) in THF was added trifluoroacetic anhydride (0.22 ml, 1.58 mmol) in THF dropwise at 0 °C. The reaction mixture was stirred for 6 h at rt, and the reaction was then quenched with 5 ml of water. The reaction mixture was evaporated, dissolved in DCM and washed with 30% citric acid aq, saturated NaCl aq and saturated $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified by flash chromatography (30-50% acetonitrile in EtOAc), yielding white solid, 250 mg, 58%. ^{1}H NMR δ 1.17 (3 H, s), 1.18 (3 H, s), 1.51-1.58 (4 H, m), 1.67-1.73 (4 H, m), 1.90-1.98 (2 H, m), 2.09-2.28 (6 H, m), 2.35-2.64 (4 H, m), 3.40-3.75 (7 H, m), 3.82-3.87 (1 H, m), 4.56 (0.9 H, dd, *J* = 4.3 Hz, 8.4 Hz), 4.73-4.86 (1.1 H, m); ^{13}C NMR δ 25.0, 25.4, 26.8, 27.0, 27.7, 28.5, 28.6, 28.7, 29.1, 29.7, 34.0, 42.1, 43.8, 45.9, 46.2, 46.5, 48.1, 48.4, 57.4, 118.7, 171.1, 171.4, 171.5; MS (ESI, +) *m/z* 417 $[M + H]^+$; Anal. ($C_{23}H_{36}N_4O_3 \cdot 0.1H_2O$) C, H, N.

Phthalic acid pyrrolidine L-prolyl-pyrrolidine amide (16). 9d (0.90 g, 2.84 mmol) was coupled with pyrrolidine (0.26 ml, 3.11 mmol) according to procedure A. The product was purified by flash chromatography (10% MeOH in EtOAc). Yield 860 mg, 83%. ^{1}H NMR δ 1.49-1.58 (1 H, m), 1.65-1.74 (1 H, m), 1.81-2.06 (8 H, m), 2.14-2.29 (1.5 H, m), 2.48-2.52 (0.5 H, m) 3.00-3.05 (0.5 H, m), 3.14-3.89 (9.5 H, m), 4.69 (0.5 H, dd, *J* = 3.3 Hz, 8.2 Hz), 4.79 (0.5 H, dd, *J* = 6.2 Hz, 8.3 Hz), 7.29-7.42 (3.5 H, m), 7.51-7.55 (0.5 H, m); ^{13}C NMR δ 23.3, 24.0, 24.2, 24.5, 24.6, 25.2, 25.9, 25.9, 26.0, 26.2, 29.2, 30.5, 45.6, 45.6, 45.7, 45.7, 46.0, 46.3, 46.8, 48.8, 48.9, 49.8, 57.6, 59.4, 125.7, 126.4, 127.4, 127.5, 128.5, 128.7, 128.9, 128.9, 135.2, 135.6, 135.7, 135.8, 168.6, 168.7, 168.8, 169.0, 170.3, 171.1; MS (ESI, +) *m/z* 370 $[M + H]^+$; Anal. ($C_{21}H_{27}N_3O_3 \cdot 0.2H_2O$) C, H, N.

Phthalic acid pyrrolidine L-prolyl-2(*S*)-cyanopyrrolidine amide (17). Boc-2(*S*)-cyanopyrrolidine (980 mg, 5.0 mmol) was deprotected using 10 ml trifluoroacetic acid in 50 ml DCM according to procedure C. **9d** (1.58 g, 4.99 mmol) and the 2(*S*)-cyanopyrrolidine trifluoroacetic acid salt were coupled according to procedure A. The product was purified with a silica plate chromatotron (2% MeOH in DCM). Yield 250 mg, 13%. ^{1}H NMR δ 1.65-2.12 (8 H, m), 2.15-2.33 (4 H, m), 3.11-3.37 (2 H, m), 3.42-3.73 (5 H, m), 3.79-3.94 (1 H, m), 4.43-4.45 (0.3 H, m), 4.72 (0.7 H, dd, *J* = 6.3 Hz, 8.3 Hz), 4.77 (0.3 H, dd, *J* = 3.4 Hz, 8.2 Hz), 4.81-4.84 (0.7 H, m), 7.26-7.33 (1 H, m), 7.34-7.44 (2 H, m), 7.46-7.51 (1 H, m); ^{13}C NMR δ 24.6, 25.3, 25.4, 26.0, 29.2, 29.7, 45.7, 46.3, 46.6, 49.0, 49.7, 57.4, 118.6, 126.5, 127.2, 129.1, 129.1, 135.4, 135.7,

168.6, 168.9, 171.0; MS (ESI, +) *m/z* 395 $[M + H]^+$; Anal. ($C_{22}H_{26}N_4O_3 \cdot 0.2H_2O$) C, H, N.

Terephthalic acid pyrrolidine L-prolyl-L-proline amide. 9c (0.50 g, 1.58 mmol) and L-proline methyl ester HCl salt (0.27 g, 1.63 mmol) were coupled according to procedure A. The product was purified by flash chromatography (5-10% MeOH in EtOAc). Yield 0.45 g, 66%. ^{1}H NMR δ 1.82-2.01 (6.5 H, m), 2.06-2.34 (5.5 H, m), 3.17-3.39 (2 H, m), 3.46-3.51 (1 H, m), 3.61-3.71 (4 H, m), 3.73 (3 H, s), 3.77-4.00 (1 H, m), 4.26-4.28 (0.2 H, m), 4.31-4.33 (0.2 H, m), 4.61 (0.8 H, dd, *J* = 4.5 Hz, 8.6 Hz), 4.87 (0.8 H, dd, *J* = 5.0 Hz, 8.3 Hz), 7.40-7.42 (0.4 H, m), 7.46-7.47 (0.4 H, m), 7.52-7.53 (1.6 H, m), 7.58-7.60 (1.6 H, m). The methyl ester group of the product was hydrolyzed using $LiOH \cdot H_2O$ (69 mg, 1.6 mmol) according to procedure B. Yield 0.43 g, 95%.

Terephthalic acid pyrrolidine L-prolyl-L-prolinamide amide. The compound was prepared from terephthalic acid pyrrolidine L-prolyl-L-proline amide (0.43 g, 1.04 mmol) and 25% NH_3 aq (71 µl, 1.0 mmol) according to the method for preparation of 3,3-dimethylglutaric acid azepane L-prolyl-L-prolinamide amide. The product was purified by flash chromatography (10-30% MeOH in EtOAc). Yield 260 mg, 61%.

Terephthalic acid pyrrolidine L-prolyl-2(*S*)-cyanopyrrolidine amide (18). Compound **18** was prepared from terephthalic acid pyrrolidine L-prolyl-L-prolinamide amide (0.26 g, 0.63 mmol) according to the method for the preparation of **15**. The reaction time was 2 h. The product was purified by flash chromatography (30-50% acetonitrile in EtOAc), yielding yellowish solid, 145 mg, 59%. ^{1}H NMR δ 1.86-2.33 (12 H, m), 3.22-3.26 (0.1 H, m), 3.33 (0.2 H, t, *J* = 6.6 Hz), 3.38 (1.8 H, t, *J* = 6.6 Hz), 3.50-3.55 (1 H, m), 3.60-3.91 (4.0 H, m), 3.70-4.02 (0.9 H, m), 4.27-4.29 (0.1 H, m), 4.39-4.41 (0.1 H, m), 4.75 (0.9 H, dd, *J* = 6.1 Hz, 8.0 Hz), 4.85-4.87 (0.9 H, m), 7.37-7.39 (0.2 H, m), 7.43-7.45 (0.2 H, m), 7.54-7.55 (1.8 H, m), 7.58-7.60 (1.8 H, m); ^{13}C NMR δ 24.4, 25.4, 25.6, 26.4, 29.0, 29.8, 46.2, 46.5, 46.6, 49.5, 50.2, 58.0, 118.6, 127.1, 127.3, 137.3, 139.0, 168.8, 168.9, 171.1; MS (ESI, +) *m/z* 395 $[M + H]^+$; Anal. ($C_{22}H_{26}N_4O_3 \cdot 0.5H_2O$) C, H, N.

Isophthalic acid pyrrolidine L-prolyl-2(*S*)-cyanopyrrolidine amide (19). Boc-2(S)-cyanopyrrolidine (980 mg, 5.0 mmol) was deprotected using 20 ml trifluoroacetic acid in 50 ml DCM according to procedure C. **9b** (1.58 g, 4.99 mmol) and the 2(*S*)-cyanopyrrolidine trifluoroacetic acid salt were coupled according to procedure A. The product was purified by flash chromatography (1.5-3% MeOH in DCM). Yield 730 mg, 37%. ^{1}H NMR δ 1.82-2.13 (7 H, m), 2.15-2.32 (5 H, m), 3.21-3.43 (2 H, m), 3.53-3.90

(5 H, m), 3.98-4.02 (1 H, m), 4.25-4.27 (0.2 H, m), 4.55-4.57 (0.2 H, m), 4.75 (0.8 H, dd, *J* = 6.3 Hz, 8.1 Hz), 4.85-4.87 (0.8 H, m), 7.43-7.47 (1 H, m), 7.58-7.62 (2 H, m), 7.68-7.69 (1 H, m); ^{13}C NMR δ 24.5, 25.4, 25.7, 26.4, 29.0, 29.8, 46.2, 46.5, 46.6, 49.6, 50.3, 58.0, 118.6, 125.8, 128.5, 128.6, 129.0, 136.1, 137.5, 168.8, 168.8, 171.1; MS (ESI, +) *m/z* 395 $[M + H]^+$; Anal. ($C_{22}H_{26}N_4O_3 \cdot 0.2H_2O$) C, H, N.

Isophthalic acid azepane L-prolyl-2(*S*)-cyanopyrrolidine amide (20). Boc-2(*S*)-cyanopyrrolidine (940 mg, 4.8 mmol) was deprotected using 19 ml trifluoroacetic acid in 50 ml DCM according to procedure C. **10b** (1.67 g, 4.85 mmol) and the 2(*S*)-cyanopyrrolidine trifluoroacetic acid salt were coupled according to procedure A. The product was purified by flash chromatography (1.5-2.5% MeOH in DCM). Yield 920 mg, 45%. ^{1}H NMR δ 1.53-1.66 (6 H, m), 1.78-2.32 (10 H, m), 3.21-3.35 (2 H, m), 3.53-3.90 (5 H, m), 3.97-4.02 (1 H, m), 4.24-4.26 (0.2 H, m), 4.53 (0.2 H, dd, *J* = 3.3 Hz, 6.8 Hz), 4.74 (0.8 H, dd, *J* = 6.4 Hz, 7.9 Hz), 4.85-4.87 (0.8 H, m), 7.42-7.45 (2 H, m), 7.56-7.60 (2 H, m); ^{13}C NMR δ 25.4, 25.7, 26.5, 27.2, 27.8, 29.0, 29.4, 29.8, 46.3, 46.5, 46.6, 49.8, 50.3, 58.0, 118.6, 125.3, 127.9, 128.3, 128.6, 136.2, 137.6, 168.8, 170.6, 171.1; MS (ESI, +) *m/z* 423 $[M + H]^+$; Anal. ($C_{24}H_{30}N_4O_3 \cdot 0.1H_2O$) C, H, N.

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6 A CYCLOPENT-2-ENECARBONYL GROUP MIMICS PROLINE AT THE P2 POSITION OF PROLYL OLIGOPEPTIDASE INHIBITORS*

Abstract. With the aim to replace the natural amino acid proline by a proline mimetic structure, a cyclopent-2-enecarbonyl moiety was studied at the P2 position of prolyl oligopeptidase (POP) inhibitors. The cyclopent-2-enecarbonyl moiety proved to be an excellent proline mimetic at the P2 position of POP inhibitors. The replacement is particularly useful when increased lipophilicity is needed.

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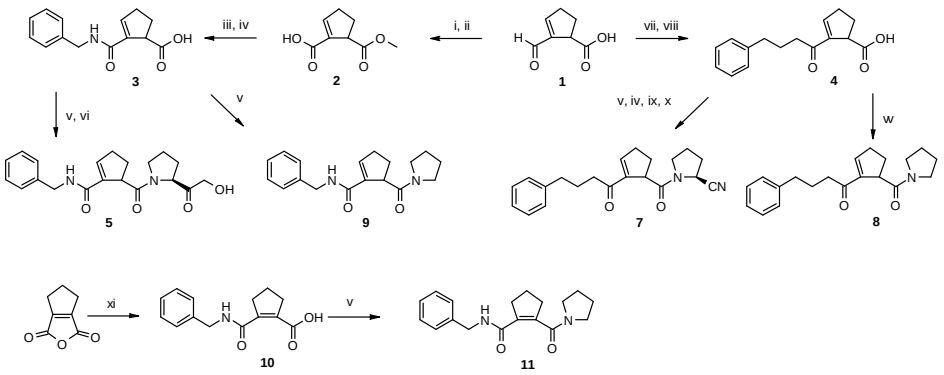
6.1 Introduction

Several protease inhibitors are recently under investigation as potential drugs. Many of them have a peptide-like structure, and they suffer from poor bioavailability and susceptibility to nonspecific degradation. These problems have led to the development of amino acid mimetics that could be used to replace the natural amino acids. The unique structure of proline makes it challenging to replace (De Nanteuil *et al*. 1998, Leung *et al*. 2000).

L-Proline is the preferred amino acid at the P2 position of POP inhibitors. Only a few successful replacements of this proline have been reported (Tsuru *et al*. 1988, De Nanteuil 1996, Barelli *et al*. 1999, Wallén *et al*. 2003). The L-prolyl residue has been replaced by groups that contain a five-membered carbon ring, such as a cyclopentanecarbonyl group and a cyclopent-1-enecarbonyl group, but the resulting compounds had a decreased inhibitory activity (Ohno 1988, Faraci 1991). In the present study, the pyrrolidine ring was successfully replaced by a cyclopent-2-enecarbonyl group. The same replacement has previously been studied at the P2 position of thrombin inhibitors with moderate results (Nöteberg *et al*. 2000).

6.2 Methods

Synthetic chemistry. The synthetic routes are presented in Scheme 1. (±)-2-Formyl-cyclopent-2-enecarboxylic acid (**1**) and (±)-cyclopent-2-ene-1,2-dicarboxylic acid 1-methyl ester (**2**) were synthesized as described in the literature with small modifications (Stevens *et al*. 1971, Nöteberg *et al*. 2000). DCC and HOBt were used to couple benzylamine with **2**, and the methyl ester of the obtained product was hydrolyzed with LiOH to obtain (±)-2-benzylcarbamoylcyclopent-2-enecarboxylic acid (**3**). Reaction of **1** with (3-phenylpropyl)magnesium bromide (prepared *in situ*) produced a secondary alcohol, which was oxidized with oxalyl chloride/DMSO to obtain ((±)-2-(4-phenylbutyryl)cyclopent-2-enecarboxylic acid (**4**). Compounds **3** and **4** were activated with trimethylacetyl chloride and reacted with pyrrolidine to yield end products **9** and **8**, respectively. The enantiomers of **9** and **8** were not separated. Compound **3** was activated with trimethylacetyl chloride and reacted with 2(*S*)-(acetoxyacetyl)-pyrrolidine. The acetyl group was hydrolyzed with K_2CO_3 in water and methanol, yielding end product **5**. The diastereomers of **5** were separated on silica support. The separated compounds were confirmed to be diastereomers by NMR. Compound **4** was activated with



Scheme 1. i) $CH(OCH_3)_3$, *p*-TsOH; ii) resorcinol, $NaH_2PO_4 \cdot H_2O$, $NaClO_2$/*t*-BuOH, H_2O; iii) benzylamine, HOBt, Et_3N, DCC/CH_3CN, 0-20 ºC; iv) LiOH/MeOH, H_2O; v) 1. Et_3N, $(CH_3)_3CCOCl/CH_2Cl_2$, 0 ºC, 2. Et_3N, an amine or a salt of an amine; vi) K_2CO_3/MeOH, H_2O, 0-20 ºC; vii) 1. Mg, I_2, 1-bromo-3-phenylpropane/Et_2O, 2. compound **1**; viii) 1. oxalyl chloride, DMSO/CH_2Cl_2, -78 ºC, 2. Et_3N; ix) 1. Et_3N, C_2H_5OCOCl/THF, -10 ºC, 2. 25% NH_3 aq./THF, room temperature; x) Et_3N, trifluoroacetic anhydride/THF, 0-20 ºC; xi) benzylamine/THF, 0-20 ºC.

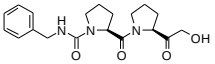
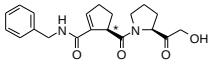
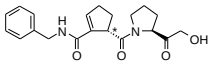
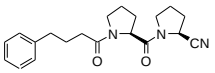
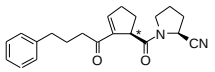
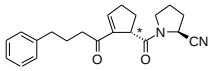
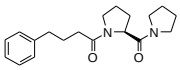
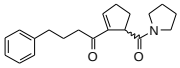
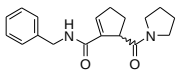
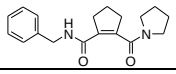
trimethylacetyl chloride and reacted with L-proline methyl ester. The methyl ester group was hydrolyzed with LiOH in water and methanol. Thus, the obtained free carboxylic acid was activated with ethyl chloroformate and treated with 25% aqueous NH_3. The carboxylic acid amide was then dehydrated with trifluoroacetic anhydride to yield end product **7**. The diastereomers of **7** were separated on silica support. The separated compounds were confirmed to be diastereomers by NMR. Compound **10** was prepared from cyclopent-1-ene-1,2-dicarboxylic anhydride and benzylamine. Compound **10** was then activated with trimethylacetyl chloride and reacted with pyrrolidine to yield reference compound **11**. Reference compounds **6**, SUAM-1221 (Houssin *et al*. 1988, Saito *et al*. 1991, Arai *et al*. 1993), and JTP-4819 (Kobayashi 1996) were prepared as described in the literature.

The log P values. The partition coefficients (*P*) were determined in a 1-octanol-phosphate buffer system with the traditional shake-flask method as described in section 4.5.

6.3 Results and discussion

The inhibitory activities of the novel and reference compounds are shown in Table 1. The tight-binding inhibitors are highly potent compounds with the IC_{50} values in the same range as the enzyme concentration in the *in vitro* assay. The K_i values were determined for tight-binding inhibitors to obtain the accurate inhibitory potencies.

Table 1. Structures, inhibitory activities (95% confidence intervals), and log P values of the reference and novel compounds.

Compound	Structure	IC_{50} (nM)		K_i (nM)[a]		Log P
JTP-4819		0.2	(0.16-0.27)	0.06	(0.023-0.087)	0.2
5a[b]		0.3	(0.18-0.56)	0.15	(0.080-0.220)	0.6
5b[c]		52	(39-71)			
6		0.2	(0.17-0.29)	0.02	(0.014-0.033)	1.6
7a[b]		0.2	(0.12-0.36)	0.03	(0.014-0.043)	2.4
7b[c]		5.0	(3.9-6.5)			
SUAM-1221		2.2	(1.9-2.5)			1.8
8		1.3	(0.7-2.7)			
9		9.0	(5.5-15)			
11		230	(160-330)			

[a] The K_i-values were determined against purified porcine POP for those tight-binding inhibitors that had the IC_{50}-value in the subnanomolar range. [b] The chiral center marked with the asterisk was assumed to have *R*-configuration. The absolute configuration was not verified. [c] The chiral center marked with the asterisk was assumed to have *S*-configuration. The absolute configuration was not verified.

The activities of diastereomers **5a** and **5b** differed considerably from each other. The IC_{50} value of **5a** was 0.3 nM, while the IC_{50} value of **5b** was 52 nM. At the P2 position L-amino acids have repeatedly caused better activities than D-amino acids (Yoshimoto *et al*. 1991, Arai *et al*. 1993, Portevin *et al*. 1996). Consequently, the configurations of **5a** and **5b** were assigned assuming that the more active diastereomer **5a** has the L-proline mimetic at the P2 position. The configurations were not verified. Diastereomer **5a** was almost equipotent with reference compound JTP-4819.

Compound **7a** was equipotent with reference compound **6**. It had a K_i value of 0.03 nM and was also assumed to have the L-proline mimetic at the P2 position. The

absolute configuration was not verified. The other diastereomer **7b** was less active with an IC_{50} of 5 nM.

The 2(*S*)-hydroxyacetyl derivative **5a** and 2(*S*)-cyano derivative **7a** are tight-binding inhibitors of prolyl oligopeptidase. Normally, these electrophilic groups increase the inhibitory activity over 10-fold as compared to an unsubstituted pyrrolidine. Compound **8** has the unsubstituted pyrrolidine at the P1 position. It was tested as a mixture of enantiomers, and despite that, the IC_{50} value was as low as 1.30 nM. On the basis of the activity differences between **5a** and **5b** and between **7a** and **7b**, it can be assumed that the more active enantiomer is even more potent than the reference compound SUAM-1221.

POP inhibitors, which contain a cyclopent-1-enecarbonyl group at the P2 position, have been published in the literature (Faraci 1991). One such inhibitor (**11**) and the corresponding cyclopent-2-enecarbonyl analogue (**9**) were synthesized. As expected, **11** was less active, with an IC_{50} of 230 nM, than **9** with an IC_{50} of 9 nM. The structure of the cyclopent-1-enecarbonyl group is not a good proline mimetic and is therefore moderately active. The cyclopent-2-enecarbonyl group mimics a proline residue rather well, as observed by the inhibitory activity.

To study the effect of the cyclopent-2-enecarbonyl moiety on the lipophilicity, the log P values were determined for a selection of compounds (Table 1). The replacement of the prolyl moiety by the cyclopent-2-enecarbonyl moiety changed the urea group of JTP-4819 to the amide group of **5a**. This change increased the log P value by 0.4 units. The change of the amide group of **6** to the ketone group of **7a** increased the log P value by 0.8 units. On the basis of these results, it seems that the replacement of a proline with a cyclopent-2-enecarbonyl group increases the log P value but the magnitude depends on the substituents.

The replacement of the L-prolyl residue by the cyclopent-2-enecarbonyl group introduced an α,β-unsaturated ketone or amide to the novel compounds. The α,β-unsaturated ketones gave valuable information about the structure-activity relationships, but their stability needs further evaluation because of the activated double bond. However, the α,β-unsaturated amides are not as reactive because of the electron-donating effect of the nitrogen atom.

6.4 Conclusions

The L-prolyl moiety was replaced by the cyclopent-2-enecarbonyl structure at the P2 position of prolyl oligopeptidase inhibitors. In all cases, the cyclopent-2-enecarbonyl

analogue proved to be equipotent with the reference compound. Furthermore, this replacement increased the lipophilicity of the compounds. This study shows that a cyclopent-2-enecarbonyl structure can be used to replace a prolyl residue at the P2 position of POP inhibitors.

6.5 Synthetic procedures and analytical data

(±)-2-Formylcyclopent-2-enecarboxylic acid (1). Dichloroacetyl chloride (30.0 ml, 312 mmol) and freshly distilled cyclopentadiene (26.0 ml, 312 mmol) were dissolved in 250 ml of hexane. Et_3N (44.0 ml, 316 mmol) in 200 ml of hexane was added at 0 °C during 2 hours. The reaction was let to proceed overnight at rt. The reaction mixture was filtered, evaporated and dissolved in 1 l of 1 M $NaHCO_3$. The mixture was stirred for 4 hours at 75 °C and, after cooling, filtered, washed with Et_2O and acidified with 3 M HCl. Water was then evaporated and the residue was stirred overnight with DCM and Na_2SO_4. The mixture was filtered and evaporated. The product was first purified by flash chromatography (EtOAc:toluene:CH_3COOH 50:50:1) and then crystallized from cyclohexane, yielding the product as white needles (9.7 g, 22%). ^{1}H NMR δ 2.32-2.46 (m, 2 H), 2.63-2.71 (m, 1 H), 2.77-2.85 (m, 1 H), 3.81-3.85 (m, 1 H), 7.11-7.12 (m, 1 H), 9.77 (s, 1 H).

(±)-2-Formylcyclopent-2-enecarboxylic acid methyl ester. To a solution of **1** (4.5 g, 32 mmol) in 70 ml of trimethyl orthoformate was added *p*-toluenesulfonic acid (3.1 g, 16 mmol) and the mixture was stirred for three days. 20 ml of water was added and the mixture was stirred for 30 min. More water was added and the product was extracted with DCM and the organic phase was dried over Na_2SO_4 and evaporated. The product was purified by flash chromatography (25% EtOAc in PE) Yield 3.6 g, 72%. ^{1}H NMR δ 2.14-2.21 (m, 1 H), 2.33-2.41 (m, 1 H), 2.60-2.68 (m, 1 H), 2.74-2.82 (m, 1 H), 3.70 (s, 3 H), 3.75-3.80 (m, 1 H), 7.02-7.03 (m, 1 H), 9.77 (s, 1 H).

(±)-Cyclopent-2-ene-1,2-dicarboxylic acid 1-methyl ester (2). To a solution of (±)2-formylcyclopent-2-enecarboxylic acid methyl ester (3.6 g, 23 mmol) in *t*-BuOH (70 ml) and H_2O (35 ml) were added resorcinol (4.5 g, 41 mmol), $NaH_2PO_4 \cdot H_2O$ (4.8 g, 35 mmol) and 80% $NaClO_2$ (3.1 g, 34 mmol). The mixture was stirred for 5 hours at rt. 10 ml of sat. Na_2SO_3 aq. was added, the mixture was acidified with 2 M HCl and extracted with DCM. The organic phase was extracted with sat. $NaHCO_3$ aq., which was acidified with 2 M HCl and extracted with DCM. The second organic phase was dried over Na_2SO_4 and evaporated. Crystallization from diethyl ether-hexane gave the product as white crystals. Yield 2.7 g, 68%. ^{1}H NMR δ (DMSO) 1.89-1.95 (m, 1 H),

2.26-2.34 (m, 1 H), 2.45-2.59 (m, 2 H), 3.59 (s, 3 H), 3.62-3.67 (m, 1 H), 6.82-6.83 (m, 1 H).

(±)-2-Benzylcarbamoyl-cyclopent-2-enecarboxylic acid methyl ester. 2 (0.68 g, 4.00 mmol), benzylamine (0.65 ml, 5.95 mmol), HOBt (contained 12% of H_2O, 0.92 g, 6.0 mmol) and Et_3N (0.85 ml, 6.10 mmol) were dissolved in 30 ml of CH_3CN. DCC in 10 ml of CH_3CN was added dropwise at 0 °C and the mixture was stirred overnight at rt. The mixture was filtered and evaporated and the residue was dissolved in DCM. The organic phase was washed with 30% citric acid, sat. NaCl and sat. $NaHCO_3$, dried over Na_2SO_4 and evaporated. The methyl ester was purified by flash chromatography (50% EtOAc in PE). Yield 1 g, 96%. ^{1}H NMR δ 2.20-2.31 (m, 2 H), 2.45-2.53 (m, 1 H), 2.60-2.68 (m, 1 H), 3.65 (s, 3 H), 3.79-3.83 (m, 1 H), 4.46 (dd, *J* = 5.6, 14.8 Hz, 1 H), 4.53 (dd, *J* = 5.9, 14.8 Hz, 1 H), 6.53 (br s, 1 H), 6.65-6.67 (m, 1 H), 7.24-7.34 (m, 5 H).

(±)-2-Benzylcarbamoyl-cyclopent-2-enecarboxylic acid (3). To a solution of (±)-2-benzylcarbamoyl-cyclopent-2-enecarboxylic acid methyl ester (1.0 g, 3.9 mmol) in 6 ml of MeOH was added $LiOH·H_2O$ (0.24 g, 5.72 mmol) in 2 ml of H_2O at rt. The mixture was stirred overnight. Methanol was evaporated and the mixture was diluted with water and washed with Et_2O. The aqueous phase was acidified with 2 M HCl and extracted with Et_2O. The organic phase was dried over Na_2SO_4, filtered and evaporated. Yield 0.86 g, 91%.

2-(1-Hydroxy-4-phenyl-butyl)-cyclopent-2-enecarboxylic acid. Catalytic amount of I_2 and magnesium (0.51 g, 21 mmol) were placed in a flask under nitrogen atmosphere. 1-Bromo-3-phenylpropane (3.2 ml, 21 mmol) in 4 ml of Et_2O was added and the mixture was heated until reaction started. When the reaction had ended, **1** (1.4 g, 10 mmol) in 50 ml of Et_2O was added dropwise and the mixture was stirred for 2 h. The mixture was poured into 100 ml of ice cold, sat. NH_4Cl solution. The aqueous phase was acidified with 2 M HCl and the layers were separated. The aqueous phase was extracted with DCM and the combined organic phases were dried over Na_2SO_4 and evaporated. The product was purified by flash chromatography (PE:EtOAc:CH_3COOH 66:33:1). Yield 1.8 g, 69%. ^{1}H NMR δ 1.53-1.80 (m, 4 H), 2.18-2.25 (m, 2 H), 2.29-2.37 (m, 1 H), 2.46-2.52 (m, 1 H), 2.58-2.68 (m, 2 H), 3.44-3.47 (m, 0.3 H), 3.55-3.58 (m, 0.7 H), 4.29-4.31 (m, 0.3 H), 4.36-4.39 (m, 0.7 H), 5.79-5.80 (m, 0.7 H), 5.83-5.85 (m, 0.3 H), 7.16-7.19 (m, 3 H), 7.25-7.28 (m, 2 H).

(±)-2-(4-Phenylbutyryl)-cyclopent-2-enecarboxylic acid (4). To a solution of oxalyl chloride (0.6 ml, 6.9 mmol) in 30 ml of DCM was added DMSO (1.47 ml, 20.7 mmol) at -78 ºC. The mixture was stirred for 15 min. 2-(1-Hydroxy-4-phenyl-butyl)-

cyclopent-2-enecarboxylic acid (1.8 g, 6.9 mmol) was added dropwise and the mixture was stirred for 2 h. Et_3N (3.8 ml, 27.6 mmol) was added and the mixture was stirred for 5 min and was let to warm up to rt. The product was extracted with 5% NaOH aq. solution. The aqueous phase was acidified with 2 M HCl and extracted with DCM. The organic phase was dried over Na_2SO_4 and evaporated. The product was purified by flash chromatography (0.5% CH_3COOH in DCM). Yield 0.91 g, 51%. ^{1}H NMR δ 1.77-1.83 (m, 2 H), 1.87-1.94 (m, 1 H), 2.20-2.28 (m, 1 H), 2.49-2.64 (m, 4 H), 2.67-2.70 (m, 2 H), 3.54-3.59 (m, 1 H), 6.96-6.97 (m, 1 H), 7.15-7.19 (m, 3 H), 7.25-7.29 (m, 2 H).

TFA salt of 2(*S*)-(acetoxyacetyl)-pyrrolidine. To a solution of Boc-protected 2(*S*)-(acetoxyacetyl)-pyrrolidine (0.95 g, 3.50 mmol) in 18 ml of DCM was added 7 ml of TFA at 0 ºC, the mixture was stirred for 2.5 h and evaporated. The salt was stored under vacuum not longer than 24 h.

Cyclopent-2-ene-1,2-dicarboxylic acid 2-benzylamide 1-[2(*S*)-(acetoxyacetyl)-pyrrolidine] amide. To a solution of **3** (0.86 g, 3.51 mmol) and Et_3N (0.54 ml, 3.87 mmol) in DCM was added trimethylacetyl chloride (0.43 ml, 3.49 mmol) dropwise at 0 ºC and the mixture was stirred for 2.5 h. Et_3N (1.6 ml, 11.6 mmol) and TFA salt of 2(*S*)-(acetoxyacetyl)-pyrrolidine (3.5 mmol) in DCM were added in this order and the icebath was removed. The mixture was stirred for 3 h at rt. The mixture was washed with 30% citric acid, sat. NaCl and sat. $NaHCO_3$, dried over Na_2SO_4 and evaporated. The product was purified by flash chromatography (20% PE in EtOAc) monitoring carefully that the distereomers were not separated. Yield 0.82 g, 60%.

Cyclopent-2-ene-1,2-dicarboxylic acid 2-benzylamide 1-[2(*S*)-(hydroxyacetyl)-pyrrolidine] amide (5). To a solution of cyclopent-2-ene-1,2-dicarboxylic acid 2-benzylamide 1-[2(*S*)-(acetoxyacetyl)-pyrrolidine] amide (0.82 g, 2.06 mmol) in 8.5 ml of MeOH and 8.5 ml of H_2O was slowly added K_2CO_3 (0.32 g, 2.3 mmol) at 0 ºC. The mixture was stirred 10 min at 0 ºC and 30 min at rt. Methanol was evaporated and the product was dissolved in DCM. The organic phase was washed with sat. NaCl, dried over Na_2SO_4 and evaporated. The product was first purified by flash chromatography (EtOAc). Yield 0.74 g, 99%. The diastereomers **5a** and **5b** were then separated by flash chromatography (4% 2-propanol in DCM). Analytical data for **5a**: ^{1}H NMR δ 1.83-1.89 (m, 1 H), 1.97-2.18 (m, 4 H), 2.27-2.34 (m, 1 H), 2.49-2.56 (m, 1 H), 2.62-2.69 (m, 1 H), 3.65-3.69 (m, 1 H), 3.90-3.95 (m, 1 H), 4.07-4.10 (m, 1 H), 4.23-4.41 (m, 3 H), 4.54-4.60 (m, 2 H), 6.40-6.41 (m, 1 H), 6.45-6.51 (m, 1 H), 7.25-7.33 (m, 5 H). ^{13}C

NMR δ 25.3, 27.6, 28.5, 32.9, 43.4*, 43.4, 47.6, 49.0, 60.9, 67.1*, 67.1, 127.4, 127.4*, 127.6, 127.7*, 128.7, 128.7*, 137.6*, 137.6, 138.3*, 138.4, 139.4*, 139.4, 165.2*, 165.2, 173.8*, 173.8, 209.2, 209.2*. Anal. ($C_{20}H_{24}N_2O_4 \cdot 0.1\ H_2O$) C, H, N. ESI-MS *m/z* 357.1 $[M + H]^+$. Analytical data for **5b**: ^{1}H NMR δ 1.96-2.18 (m, 5 H), 2.23-2.30 (m, 1 H), 2.49-2.56 (m, 1 H), 2.65-2.73 (m, 1 H), 3.61-3.66 (m, 1 H), 3.95-3.99 (m, 1 H), 4.04-4.07 (m, 1 H), 4.26 (dd, *J* = 5.0, 19.1 Hz, 1 H), 4.33 (dd, *J* = 5.0, 19.1 Hz, 1 H), 4.45-4.55 (m, 3 H), 6.34-6.38 (m, 1 H), 6.48-6.50 (m, 1 H), 7.24-7.34 (m, 5 H). ^{13}C NMR δ 25.2, 28.0, 28.4, 32.9, 43.6, 47.8, 48.9, 62.9, 67.0, 127.4, 127.9, 128.7, 138.2, 138.7, 139.4, 165.2, 173.8, 209.4. Anal. ($C_{20}H_{24}N_2O_4 \cdot 0.2\ H_2O$) C, H, N. ESI-MS *m/z* 357.1 $[M + H]^+$.

1-[2-(4-Phenylbutyryl)-cyclopent-2-enecarbonyl]-pyrrolidine-2(*S*)-carboxylic acid methyl ester. The product was synthesized from **4** (0.58 g, 2.25 mmol) and HCl salt of L-proline methyl ester (0.72 g, 2.25 mmol) as described for cyclopent-2-ene-1,2-dicarboxylic acid 2-benzylamide 1-[2(*S*)-(acetoxyacetyl)-pyrrolidine] amide. The product was purified by flash chromatography (50% EtOAc in PE). Yield 0.64 g, 77%.

1-[2-(4-Phenylbutyryl)-cyclopent-2-enecarbonyl]-pyrrolidine-2(*S*)-carboxylic acid. To a solution of 1-[2-(4-Phenylbutyryl)-cyclopent-2-enecarbonyl]-pyrrolidine-2(*S*)-carboxylic acid methyl ester (0.64 g, 1.7 mmol) in 10 ml of MeOH was added $LiOH \cdot H_2O$ (0.11 g, 2.6 mmol) in 3.5 ml of H_2O at rt. The mixture was stirred overnight. Methanol was evaporated and the mixture was diluted with water and washed with DCM. The aqueous phase was acidified with 3 M HCl and extracted with DCM. The organic phase was dried over Na_2SO_4, filtered and evaporated. Yield 0.58 g, 94%.

1-[2-(4-Phenylbutyryl)-cyclopent-2-enecarbonyl]-pyrrolidine-2(*S*)-carboxylic amide. To a solution of 1-[2-(4-Phenylbutyryl)-cyclopent-2-enecarbonyl]-pyrrolidine-2(*S*)-carboxylic acid (0.58 g, 1.63 mmol) and Et_3N (0.23 ml, 1.65 mmol) in THF was added ethyl chloroformate (0.16 ml, 1.67 mmol) in THF dropwise at -10 ºC. After 20 min 25% NH_3 aq. (0.55 ml, 8.1 mmol) was added and the mixture was stirred overnight at rt. THF was evaporated and the residue was dissolved in DCM. The organic phase was washed with sat. $NaHCO_3$, dried over Na_2SO_4 and evaporated. The product was purified by flash chromatography (10% MeOH in EtOAc). Yield 0.50 g, 86%. ^{1}H NMR δ 1.88-1.98 (m, 3 H), 1.99-2.15 (m, 3 H), 2.17-2.29 (m, 1 H), 2.35-2.43 (m, 0.8 H), 2.47-2.52 (m, 0.2 H), 2.56-2.67 (m, 3 H), 2.68-2.74 (m, 2 H), 2.75-2.85 (m, 1 H), 3.53-3.77 (m, 1.5 H) 3.84-3.99 (m, 1.5 H), 4.34-4.36 (m, 0.2 H), 4.58-4.61 (m, 0.8 H), 5.89

* Minor rotamer.

(br s, 0.8 H), 5.64 (br s, 0.2 H), 6.81-6.85 (m, 0.5 H), 6.88-6.89 (m, 0.5 H), 7.04 (br s, 0.8 H), 7.15-7.20 (m, 3 H), 7.25-7.29 (m, 2 H), 7.72 (br s, 0.2 H).

1-[2-(4-Phenylbutyryl)-cyclopent-2-enecarbonyl]-pyrrolidine-2(*S*)-nitrile (7). To a solution of 1-[2-(4-Phenylbutyryl)-cyclopent-2-enecarbonyl]-pyrrolidine-2(*S*)-carboxylic amide (0.50 g, 1.41 mmol) and Et_3N (0.59 ml, 4.23 mmol) in dry THF was added trifluoroacetic anhydride (0.30 ml, 2.16 mmol) in dry THF dropwise at 0 ºC. The mixture was stirred for 3 h at rt. 5 ml of water was added and THF was evaporated. The product was dissolved in DCM and the organic phase was washed with 30% citric acid, sat. NaCl and sat. $NaHCO_3$, dried over Na_2SO_4 and evaporated. The product was purified and the diastereomers were separated by flash chromatography (33% EtOAc in PE). Yield 0.46 g, 96%. Analytical data for **7a**: ^{1}H NMR δ 1.89-1.95 (m, 2 H), 2.09-2.15 (m, 1 H), 2.18-2.27 (m, 5 H), 2.55-2.64 (m, 3 H), 2.67-2.70 (m, 2 H), 2.80-2.88 (m, 1 H), 3.66-3.69 (m, 1 H), 3.83-3.87 (m, 1 H), 3.89-3.94 (m, 1 H), 4.75-4.77 (m, 1 H), 6.83-6.85 (m, 1 H), 7.16-7.19 (m, 3 H), 7.26-7.29 (m, 2 H). ^{13}C NMR δ 25.2, 25.7, 27.9, 30.0, 33.7, 35.1, 37.8, 46.5, 46.8, 47.5, 118.8, 125.9, 128.3, 128.5, 141.6, 144.6, 146.4, 173.7, 198.3. Anal. ($C_{21}H_{24}N_2O_2 \cdot 0.1\ H_2O$) C, H, N. ESI-MS *m/z* 337.0 $[M + H]^+$. Analytical data for **7b**[*]: ^{1}H NMR δ 1.88-2.00 (m, 2 H), 2.05-2.44 (m, 6 H), 2.53-2.73 (m, 5 H), 2.81-2.92 (m, 1 H), 3.45-3.54 (m, 1 H), 3.56-3.61 (m, 0.6 H), 3.87-3.93 (m, 1 H), 4.19-4.23 (m, 0.4 H), 4.66-4.68 (dd, *J* = 2.1, 8.3 Hz, 0.4 H), 5.35-5.37 (dd, *J* = 2.9, 7.4 Hz, 0.6 H), 6.80-6.82 (m, 0.4 H), 6.86-6.88 (m, 0.6 H), 7.15-7.20 (m, 3 H), 7.25-7.29 (m, 2 H). ^{13}C NMR δ 23.4, 25.1, 25.8, 25.8, 28.1. 28.5, 30.3, 32.2, 33.7, 33.9, 35.1, 35.1, 37.8, 37.9, 46.3, 46.6, 46.7, 47.6, 47.7, 48.0, 118.0, 119.5, 125.8, 126.0, 128.3, 128.4, 128.5, 128.6, 141.5, 141.8, 144.9, 144.9, 145.7, 147.3, 173.7, 173.9, 197.9, 198.8. Anal. ($C_{21}H_{24}N_2O_2 \cdot 0.2\ H_2O$) C, H, N. ESI-MS *m/z* 337.1 $[M + H]^+$.

(±)-1-[2-(4-Phenylbutyryl)-cyclopent-2-enecarbonyl]-pyrrolidine (8). The product was synthesized from **4** (0.23 g, 0.89 mmol) and pyrrolidine (75 µl, 0.9 mmol) as described for cyclopent-2-ene-1,2-dicarboxylic acid 2-benzylamide 1-[2(*S*)-(acetoxyacetyl)-pyrrolidine] amide, but in the 2nd addition of Et_3N, 1.1 eq instead of 3.3 eq was used. The product was purified by flash chromatography (50% EtOAc in PE). Yield 0.21 g, 77%. ^{1}H NMR δ 1.80-2.08 (m, 7 H), 2.14-2.22 (m, 1 H), 2.50-2.57 (m, 1 H), 2.59-2.74 (m, 4 H), 2.77-2.84 (m, 1 H), 3.38-3.51 (m, 3 H), 3.84-3.93 (m, 2 H), 6.78-6.80 (m, 1 H), 7.15-7.18 (m, 3 H), 7.25-7.28 (m, 2 H). ^{13}C NMR δ 24.4, 25.7, 26.1,

[*] Compound **7b** clearly shows two rotamers for each carbon.

28.1, 33.6, 35.2, 38.0, 45.8, 46.9, 47.8, 125.8, 128.3, 128.5, 141.8, 145.3, 145.4, 172.9, 198.3. Anal. ($C_{20}H_{25}NO_2$) C, H, N. ESI-MS *m/z* 312.2 $[M + H]^+$.

(±)-Cyclopent-2-ene-1,2-dicarboxylic acid 2-benzylamide 1-pyrrolidine amide (9). The product was synthesized from **3** (0.46 g, 1.88 mmol) and pyrrolidine (0.16 ml, 1.92 mmol) as described for cyclopent-2-ene-1,2-dicarboxylic acid 2-benzylamide 1-[2(*S*)-(acetoxyacetyl)-pyrrolidine] amide, but in the 2nd addition of Et_3N, 1.1 eq instead of 3.3 eq was used. The product was purified by flash chromatography (EtOAc). Yield 0.39 g, 69%. ^{1}H NMR δ 1.80-1.86 (m, 2 H), 1.89-2.07 (m, 3 H), 2.23-2.30 (m, 1 H), 2.46-2.53 (m, 1 H), 2.62-2.69 (m, 1 H), 3.37-3.50 (m, 3 H), 3.77-3.81 (m, 1 H), 4.03-4.07 (m, 1 H), 4.32 (dd, *J* = 5.3, 15.0 Hz, 1 H), 4.57 (dd, *J* = 6.4, 15.0 Hz, 1 H), 6.45-6.46 (m, 1 H), 6.69 (br t, 1 H), 7.22-7.26 (m, 1 H), 7.27-7.32 (m, 4 H). ^{13}C NMR δ 24.4, 26.1, 28.1, 32.8, 43.4, 45.9, 46.9, 49.5, 127.2, 127.6, 128.6, 137.6, 138.6, 140.0, 165.6, 173.2. Anal. ($C_{18}H_{22}N_2O_2 \cdot 0.2\ H_2O$) C, H, N. ESI-MS *m/z* 299.2 $[M + H]^+$.

2-Benzylcarbamoyl-cyclopent-1-enecarboxylic acid (10). To a solution of benzylamine (1.2 ml, 10.9 mmol) in 5 ml of THF was added cyclopent-1-ene-1,2-dicarboxylic anhydride (0.5 g, 3.6 mmol) in 8 ml of THF dropwise at 0 ºC. The mixture was stirred overnight at rt. THF was evaporated and the residue was dissolved in 2 M NaOH aq. The aqueous phase was washed with DCM, acidified with 3 M HCl and extracted with DCM. The organic phase was dried over Na_2SO_4 and evaporated. Yield 0.74 g, 83%.

1-[2-Benzylcarbamoyl-cyclopent-1-enecarbonyl]-pyrrolidine (11). The product was synthesized from **10** (0.25 g, 1.02 mmol) and pyrrolidine (85 µL, 1.0 mmol) as described for cyclopent-2-ene-1,2-dicarboxylic acid 2-benzylamide 1-[2(*S*)-(acetoxyacetyl)-pyrrolidine] amide, but in the 2nd addition of Et_3N, 1.1 eq instead of 3.3 eq was used. The product was purified by flash chromatography (EtOAc). Yield 0.20 g, 66%. ^{1}H NMR δ 1.79-1.83 (m, 4 H), 1.97 (qui, *J* = 7.6 Hz, 2 H), 2.71-2.78 (m, 4 H), 3.28-3.30 (m, 2 H), 3.34-3.37 (m, 2 H), 4.45 (d, *J* = 5.9 Hz, 2 H), 7.22-7.31 (m, 5 H). ^{13}C NMR δ 22.2, 24.1, 25.8, 33.8, 35.7, 43.2, 45.4, 47.3, 127.3, 127.7, 128.6, 136.9, 138.6, 142.0, 165.0, 168.2. Anal. ($C_{18}H_{22}N_2O_2$) C, H, N. ESI-MS *m/z* 299.1 $[M + H]^+$.

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7 AN INTRODUCTION OF A PYRIDINE GROUP INTO THE STRUCTURE OF PROLYL OLIGOPEPTIDASE INHIBITORS*

Abstract. A series of ionizable prolyl oligopeptidase inhibitors were developed through the introduction of a pyridyl group to the P3 position of the prolyl oligopeptidase inhibitor structure. The study was performed on previously developed prolyl oligopeptidase inhibitors with proline mimetics at the P2 position. The 3-pyridyl group resulted in equipotent compounds as compared to the parent compounds. It was shown that the pyridyl group improves water solubility, and in combination with a 5(*R*)-*tert*-butyl-L-prolyl group at the P2 position, good lipophilicity can be achieved.

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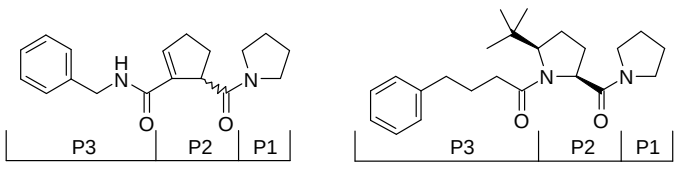
7.1 Introduction

Unlike many small-molecule drugs, typical POP inhibitors are unionizable compounds. An ionizable group may improve water solubility and it also allows salt formation. In later stages of drug development, the proper choice of salt can be used to modify stability, solubility, and pharmaceutical processing properties.

Two unionizable POP inhibitors with different proline mimetics at the P2 positions (Figure 1) were earlier presented by our group (Wallén *et al*. 2003, Chapter 6). These compounds were further investigated as to whether an ionizable pyridyl group could be introduced into the structure. The pK_a value of pyridine is 5.2 and thus, it is mostly in the unionized form at the physiological pH 7.4. However, a significant fraction is in the ionized form in the slightly acidic environment of the duodenum (Dressman *et al*. 1990), which allows dissolution that is a prerequisite for the absorption.

A literature search revealed that a pyridyl moiety has occasionally been included in some series of POP inhibitors, but no systematic study regarding the position and the substitution of the pyridine group has been performed (Tsuru *et al*. 1988, Saito *et al*. 1991, Nakajima *et al*. 1992, Tsutsumi *et al*. 1994, Tsuda *et al*. 1996a, Tsuda *et al*. 1996b, Vendeville *et al*. 2002). One potent peptide-like POP inhibitor, which possessed a pyridine-2-carboxyl amide moiety at the P3 position, was reported by Tsuda *et al*. (1996a). In the present study, the phenyl group at the P3 site was successfully replaced by a pyridyl group in two series of POP inhibitors. The chain length and the substitution pattern were optimized. The effect of the pyridyl moiety on lipophilicity and water solubility was also studied.

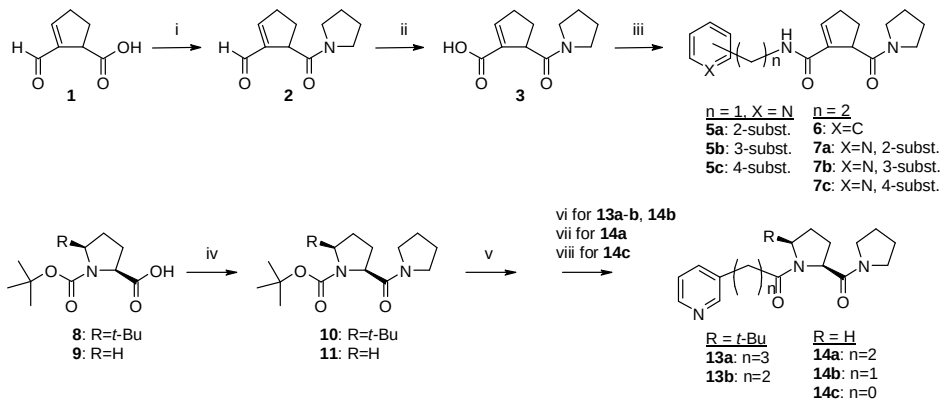


IC_{50} = 9.0 nM (compound **4**) IC_{50} = 1.2 nM (compound **12**)

Figure 1. Previously published POP inhibitors, which possess proline mimetics at the P2 site.

7.2 Methods

The synthetic routes for the novel compounds are presented in Scheme 1. Compounds **1**, **4** (Chapter 6), and **12** (Wallén *et al*. 2003) were synthesized as described earlier. Compound **1** was activated with trimethylacetyl chloride and reacted with pyrrolidine at 0 °C to obtain (±)-5-(pyrrolidine-1-carbonyl)-cyclopent-1-enecarbaldehyde (**2**). The aldehyde group of compound **2** was oxidized with $NaClO_2$ to obtain (±)-5-(pyrrolidine-1-carbonyl)-cyclopent-1- enecarboxylic acid (**3**). Compound **3** was reacted with DCC, HOBt, and an appropriate amine to yield compounds **5a**–**c**, **6**, and **7a**–**c**. Boc-5(*R*)-*tert*-butyl-L-proline (**8**) was prepared according to published procedures (Beausoleil *et al*. 1996, Sim and Rapoport 1999)**,** with slight modifications as described earlier (Wallén *et al*. 2003). Boc-5(*R*)-*tert*-butyl-L-proline (**8**) and Boc-L-proline (**9**) were activated with trimethylacetyl chloride and reacted with pyrrolidine to yield compounds **10** and **11**, respectively. Compounds **10** and **11** were deprotected in HCl-saturated ethyl acetate to yield 5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine and L-prolyl-pyrrolidine. 4-(Pyridin-3-yl) butyric acid and 3-(pyridin-3-yl)propionic acid were reacted with EDC, HOBt, and 5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine to yield compounds **13a** and **13b**, respectively. 4-(Pyridin-3-yl)butyric acid was prepared according to a published procedure (Menghin *et al*. 2003) with small modifications. L-Prolyl-pyrrolidine was reacted with 3-(pyridin-3-



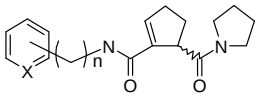
Scheme 1. (i) 1. Et_3N, $(CH_3)_3CCOCl/CH_2Cl_2$, 0 °C, 2. Et_3N, pyrrolidine/CH_2Cl_2, 0 °C; (ii) resorcinol, $NaH_2PO_4{\cdot}H_2O$, $NaClO_2$/*t*-BuOH, H_2O; (iii) an appropriate amine, Et_3N, HOBt, DCC/CH_3CN, 0–20 °C; (iv) 1. Et_3N, $(CH_3)_3CCOCl/CH_2Cl_2$, 0 °C, 2. Et_3N, pyrrolidine/CH_2Cl_2, room temperature; (v) HCl/EtOAc; (vi) an appropriate carboxylic acid, Et_3N, HOBt, EDC·HCl/CH_2Cl_2, 0–20 °C; (vii) 3-pyridin-3-yl-propionyl chloride HCl, Et_3N/CH_2Cl_2, 0–20 °C; (viii) DMAP, nicotinoyl chloride HCl/pyridine.

yl)propionyl chloride and nicotinoyl chloride to yield compounds **14a** and **14c**, respectively. EDC and HOBt were used to couple L-prolyl-pyrrolidine with (pyridin-3-yl)acetic acid to obtain compound **14b**.

7.3 Results and discussion

Compound **4** (Chapter 6) possesses a cyclopentenecarbonyl moiety at the P2 position. The IC_{50} value of compound **4** is 9 nM for the racemic mixture. It was chosen as the parent compound for the first series of compounds presented in Table 1. The replacement of the phenyl group of compound **4** with a 2- or 4-pyridyl group gave compounds **5a** and **5c**, respectively. These substitutions resulted in over 2 times lower potency. However, the replacement with a 3-pyridyl group gave compound **5b**, which was equipotent with the parent compound **4**. In order to further optimize the position of the pyridyl group, the chain length of compounds **4** and **5a**–**5c** was extended by one methylene group, which resulted in compounds **6** and **7a**–**c**, respectively. The extension of the chain of compound **4** caused a 6-fold increase in potency; the IC_{50} value of

Table 1. Inhibitory activities with 95% confidence intervals and log P values of the (±)-5-(pyrrolidine-1-carbonyl)-cyclopent-1-enecarboxylic acid amides.



Compound	X	Subst	n	IC_{50} (nM)[a]	log P[b]
4	C	-	1	9.0 (5.5-15)	1.6[c]
5a	N	2	1	24 (22-26)	0.3[d]
5b	N	3	1	9.7 (8.5-11)	0.4[d]
5c	N	4	1	19 (15-26)	0.4[d]
6	C	-	2	1.5 (1.3-1.9)	1.9[c]
7a	N	2	2	5.2 (4.4-6.2)	0.6[d]
7b	N	3	2	2.2 (1.9-2.6)	0.6[d]
7c	N	4	2	4.8 (3.8-6.1)	0.5[d]

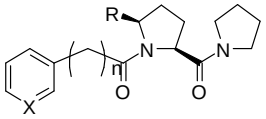
[a] The IC_{50} values were determined against POP from porcine brain (Venäläinen *et al*. 2002).
[b] The reported values are for the unionized species.
[c] Determined with the shake-flask method.
[d] Determined with pH metric titration using a Sirius PCA200 automatic titrator.

compound **6** was 1.5 nM. Again, the replacement of the P3 phenyl group by the 3-pyridyl moiety gave the most potent compound **7b** (2.2 nM), while the 2- and 4-pyridyl analogs, **7a** and **7c**, were less active. Compound **7b** was almost equipotent with compound **6** and over 4 times more potent than its shorter counterpart **5b**. These results confirmed that the 3-pyridyl group can be used to replace the phenyl group at the P3 position of POP inhibitors and the optimal chain length is the same as for the phenyl group, despite the difference in their polarities.

High lipid solubility is a prerequisite for the passive brain penetration of small-molecule drugs (Pardridge 2005) and the theoretical optimum for the log P value is near 2 (Tute 1996). Although lipophilicity is not the only factor that determines brain penetration and the optimal lipophilicity can vary between compound classes, it should be taken into account when novel CNS targeted compounds are designed. The log P values were determined for compounds **4**, **5a**–**c**, **6**, and **7a**–**c** (Table 1). The presented log P values for compounds **5a**–**c** and **7a**–**c** are values for the unionized species, which are mainly found at the physiological pH 7.4 (the pK_a values range between 4.4 and 5.5). The log P value of compound **4** was 1.6. The replacement of the phenyl moiety by the more polar pyridyl moiety dropped the log P values to 0.3–0.4 for compounds **5a**–**5c**. The extension of the chain increased the log P value to 1.9 for compound **6**, but again the replacement of the phenyl moiety by the pyridyl moiety dropped the log P values to 0.5–0.6 for compounds **7a**–**7c**.

Compound **12** (Wallén *et al*. 2003) possesses a lipophilic 5(*R*)-*tert*-butyl-L-prolyl moiety at the P2 position. The IC_{50} value of compound **12** is 1.2 nM and the log P value is 3.3. It was chosen as the parent compound for the second series of compounds presented in Table 2. In this series, only the 3-pyridyl group was studied because it had given the most potent compounds in the first series. The replacement of the P3 phenyl group of compound **12** by the 3-pyridyl group resulted in compound **13a** having an IC_{50} value of 2.1 nM. It was only slightly less potent than compound **12** and equipotent with compound **7b**, which was the most potent pyridyl derivative in the first series. However, it has to be kept in mind that **13a** possesses the more active configuration of L-proline, while **7b** was tested as a racemic mixture. To study whether the change of the skeleton affects the optimal position of the pyridyl group, the P3 chain of compound **13a** was shortened by one methylene group resulting in compound **13b**. The results were consistent with the first series; a decrease in the chain length decreased the potency and the IC_{50} value of compound **13b** was 13 nM. The effect of even shorter chain lengths was studied with compounds **14a**–**c**, which have an L-prolyl residue at the P2 site. The

Table 2. Inhibitory activities with 95% confidence intervals and log P values of the *N*-alkanoyl 5(*R*)-*tert*-butyl-L-prolyl-pyrrolidines and *N*-alkanoyl L-prolyl-pyrrolidines.



Compound	X	n	R	IC_{50} (nM)[a]	log P[b]
12	C	3	*t*-Bu	1.2 (1.0-1.4)	3.3[c]
13a	N	3	*t*-Bu	2.1 (1.9-2.4)	2.0[d]
13b	N	2	*t*-Bu	13 (8.4-20)	1.8[d]
14a	N	2	H	30 (24-39)	0.3[d]
14b	N	1	H	56 (46-70)	-0.2[d]
14c	N	0	H	44 (29-67)	-0.3[d]
15	C	3	H	2.2 (1.9-2.5)	1.8[c]

[a] The IC_{50} values were determined against POP from porcine brain (Venäläinen et al. 2002).
[b] The reported values are for the unionized species.
[c] Determined with the shake-flask method.
[d] Determined with pH metric titration using a Sirius PCA200 automatic titrator.

change to an L-prolyl group at the P2 site was made to avoid steric hindrance that the bulky 5(*R*)-*tert*-butyl group causes when the P3 ring is brought closer to the P2 ring. The removal of the 5(*R*)-*tert*-butyl group of compound **13b** resulted in compound **14a** and in over 2 times lower potency; the IC_{50} value of **14a** was 30 nM. The P3 chain length of compound **14a** was shortened by one and two methylene groups to obtain compounds **14b** and **14c**, respectively. Both compounds were less potent than compound **14a**, confirming that the optimal position for the pyridyl moiety is three carbon atoms away from the carbonyl group.

The log P values of the second series of compounds are presented in Table 2. Again, the reported log P values are for the unionized species. The replacement of the phenyl moiety of compound **12** by the polar pyridyl moiety dropped the log P value from 3.3 to the theoretical optimum 2.0 for compound **13a**. The drop was of the same order of magnitude as in the first series of the compounds; 20-fold drop in partition coefficient P. Compound **13b** with shorter chain length had a log P value 1.8. A comparison between the log P values of compounds **13b** and **14a** (log P = 0.3) shows that the *tert*-butyl group greatly increases lipophilicity and consequently, the log P values of compounds **14a**–**c** were low. The IC_{50} and log P values of compound **13a** are comparable to those of

the unionizable reference compound **15**, SUAM-1221 (Table 2), which is a potent POP inhibitor that can penetrate into the CNS (Atack *et al*. 1991).

The water solubility was determined for the parent compounds of the two series, compounds **4** and **12**, their 3-pyridyl analogs **5b** and **13a**, and for the reference compound **15**, SUAM-1221. The solubilities were determined after 3-day-shaking in 50 mM phosphate buffer (pH 7.4, ionic strength 0.15 M) up to 6 mg/ml, which can be considered adequate even for high-dose compounds with poor permeability (Lipinski 2000). While the solubility of compound **15** was at least 6 mg/ml, compounds **4** and **12** with proline mimetics at the P2 sites had decreased values; 3.0 and 0.9 mg/ml, respectively. However, the change of the phenyl moiety to the 3-pyridyl moiety overcame this decrease and the solubilities of compounds **5b** and **13a** were at least 6 mg/ml.

7.4 Conclusions

The present study proved that a pyridyl group can be introduced to the P3 position of the POP inhibitor structure. However, the inhibitory activity was dependent on the substitution of the pyridyl group and the P3 chain length. The 3-pyridyl group gave the most potent compounds and its optimal position was the same as for the phenyl group; three atoms between the P3 carbonyl group and the aromatic ring. The studied parent compounds had decreased water solubility. The introduction of an ionizable pyridyl group gave excellent water solubility to the novel compounds. In conclusion, the introduction of the 3-pyridyl group at the P3 position in combination with a 5(*R*)-*tert*-butyl-L-prolyl moiety at P2 position was used to optimize the physico-chemical properties while maintaining an excellent inhibitory activity.

7.5 Synthetic procedures and analytical data

General procedure A. A solution of trimethylacetyl chloride (1 eq.) in DCM is added dropwise to a solution of carboxylic acid (1.0 eq.) and triethylamine (1.1 eq.) in DCM at 0 °C. The activation of carboxylic acid is let to proceed 1 h at 0 °C. Triethylamine (1.1 eq.) and amine (1.0-1.1 eq.) are added in this order at 0 °C (if the amine is in the form of a trifluoroacetic acid salt or HCl salt, then 3.3 eq. of triethylamine is used). The reaction mixture is stirred appropriate time at 0 °C or at room temperature (rt). The DCM solution is washed with 30% citric acid aq, saturated NaCl aq. and saturated $NaHCO_3$ aq., dried over anhydrous Na_2SO_4 and evaporated, yielding the crude product.

General procedure B. Carboxylic acid (1 eq.), amine (1.5 eq.), HOBt (1.5 eq.) and Et_3N (1.5 eq.) were dissolved in CH_3CN. DCC (1.5 eq.) in CH_3CN was added dropwise at 0 °C. The reaction mixture was stirred 30 min. at 0 °C and overnight at rt. The mixture was filtered and evaporated and the residue was dissolved in DCM. The organic phase was washed with sat. NaCl aq. and sat. $NaHCO_3$ aq., dried over Na_2SO_4 and evaporated.

General procedure C. Compound **10** or compound **11** (1 eq.) was dissolved in 2-3 M HCl in EtOAc (1 mmol/2-3 ml). The mixture was stirred for 45-60 min. and evaporated. The yielded 5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine hydrochloride or L-prolyl-pyrrolidine hydrochloride, carboxylic acid (1 eq.), HOBt·H_2O (1.5 eq.) and Et_3N (4 eq.) were dissolved in DCM. EDC hydrochloride (1.5 eq.) in DCM was added dropwise at 0 °C. The reaction mixture was stirred 30 min. at 0 °C and overnight at rt. The mixture was diluted with DCM and the organic phase was washed with sat. NaCl aq. and sat. $NaHCO_3$ aq., dried over Na_2SO_4 and evaporated.

(±)-2-Formylcyclopent-2-enecarboxylic acid (1). Dichloroacetyl chloride (30.0 ml, 312 mmol) and freshly distilled cyclopentadiene (26.0 ml, 312 mmol) were dissolved in 250 ml of hexane. Et_3N (44.0 ml, 316 mmol) in 200 ml of hexane was added at 0 °C during 2 hours. The reaction was let to proceed overnight at rt. The reaction mixture was filtered, evaporated and dissolved in 1 l of 1 M $NaHCO_3$ aq. The mixture was stirred for 4 hours at 75 °C and, after cooling, filtered, washed with Et_2O and acidified with 3 M HCl. Water was then evaporated and the residue was stirred overnight with DCM and Na_2SO_4. The mixture was filtered and evaporated. The product was first purified by flash chromatography (EtOAc:toluene:CH_3COOH 50:50:1) and then crystallized from cyclohexane, yielding the product as white needles (9.7 g, 22%). ^{1}H NMR δ 2.32-2.46 (m, 2 H), 2.63-2.71 (m, 1 H), 2.77-2.85 (m, 1 H), 3.81-3.85 (m, 1 H), 7.11-7.12 (m, 1 H), 9.77 (s, 1 H).

(±)-5-(Pyrrolidine-1-carbonyl)-cyclopent-1-enecarbaldehyde (2). Compound **2** was synthesized from compound **1** (2.0 g, 14.3 mmol) and pyrrolidine (1.2 ml, 14.3 mmol) according to the general procedure A. The reaction mixture was stirred 1 h at 0 °C. The product was purified by flash chromatography (2-10% MeOH in EtOAc) yielding white solid, 2.6 g, 94%. ^{1}H NMR δ 1.81-2.07 (m, 4 H), 2.16-2.30 (m, 2 H), 2.58-2.65 (m, 1 H), 2.85-2.93 (m, 1 H), 3.40-3.53 (m, 3 H), 3.86-3.91 (m, 2 H), 7.04-7.05 (m, 1 H), 9.75 (s, 1 H).

(±)-5-(Pyrrolidine-1-carbonyl)-cyclopent-1-enecarboxylic acid (3). To a solution of compound **2** (900 mg, 4.7 mmol) in *t*-BuOH (15 ml) and H_2O (7 ml) were added

resorcinol (900 mg, 8.2 mmol), NaH_2PO_4 (840 mg, 7.0 mmol) and 80% $NaClO_2$ (630 mg, 5.6 mmol). The mixture was stirred overnight at rt. Saturated Na_2SO_3 aq. (10 ml) was added and the mixture was washed with Et_2O. The mixture was acidified with 2 M HCl and extracted with $CHCl_3$:EtOH 1:1. The organic phase was dried over Na_2SO_4 and evaporated. Yield 900 mg, 91%. ^{1}H NMR δ 1.82-1.87 (m, 2 H), 1.92-2.00 (m, 2 H), 2.04-2.12 (m, 1 H), 2.27-2.34 (m, 1 H), 2.49-2.56 (m, 1 H), 2.70-2.77 (m, 1 H), 3.42-3.56 (m, 3 H), 3.71-3.76 (m, 1 H), 3.91-3.94 (m, 1 H), 7.01-7.02 (m, 1 H).

(±)-5-(Pyrrolidine-1-carbonyl)-cyclopent-1-enecarboxylic acid (pyridin-2-ylmethyl)-amide (5a). Compound **5a** was synthesized from compound **3** (120 mg, 0.57 mmol) and 2-(aminomethyl)pyridine (89 µl, 0.86 mmol) according to general procedure B in 7 ml of CH_3CN. The product was purified by flash chromatography (25% MeOH in EtOAc). Yield 100 mg, 59%. ^{1}H NMR δ 1.81-2.09 (m, 5 H), 2.24-2.31 (m, 1 H), 2.49-2.55 (m, 1 H), 2.68-2.75 (m, 1 H), 3.40-3.53 (m, 3 H), 3.81-3.85 (m, 1 H), 4.04-4.07 (m, 1 H) 4.56 (dd, *J* = 5.1, 16.4 Hz, 1 H), 4.62 (dd, *J* = 5.3, 16.4 Hz, 1 H), 6.54-6.55 (m, 1 H), 7.16-7.18 (m, 1 H), 7.23 (br, 1 H), 7.26-7.28 (m, 1 H), 7.62-7.66 (m, 1 H), 8.51-8.52 (m, 1 H). ^{13}C NMR δ 24.4, 26.2, 28.2, 32.8, 44.5, 45.9, 46.9, 49.3, 121.9, 122.2, 136.7, 137.9, 139.9, 148.9, 156.8, 165.5, 173.1. Anal. ($C_{17}H_{21}N_3O_2$·0.2 H_2O) C, H, N: calcd, 13.87; found, 14.28. ESI-MS *m/z* 300.2 $[M + H]^+$.

(±)-5-(Pyrrolidine-1-carbonyl)-cyclopent-1-enecarboxylic acid (pyridin-3-ylmethyl)-amide (5b). Compound **5b** was synthesized from compound **3** (190 mg, 0.91 mmol) and 3-(aminomethyl)pyridine (139 µl, 1.36 mmol) according to general procedure B. The product was purified by flash chromatography (25% MeOH in EtOAc). Yield 140 mg, 51%. ^{1}H NMR δ 1.80-1.89 (m, 2 H), 1.90-2.03 (m, 3 H), 2.25-2.33 (m, 1 H), 2.48-2.54 (m, 1 H), 2.59-2.65 (m, 1 H), 3.39 (t, *J* = 6.9 Hz, 2 H), 3.46-3.50 (m, 1 H), 3.75-3.80 (m, 1 H), 4.04-4.07 (m, 1 H), 4.25 (dd, *J* = 5.5, 15.2 Hz, 1 H), 4.60 (dd, *J* = 6.5, 15.2 Hz, 1 H), 6.52-6.53 (m, 1 H), 7.22 (dd, *J* = 4.8, 7.7 Hz, 1 H), 7.33 (br, 1 H), 7.65 (d, *J* = 7.7 Hz, 1 H), 8.48 (dd, *J* = 1.3, 4.8 Hz, 1 H), 8.54 (d, *J* = 1.5 Hz, 1 H). ^{13}C NMR δ 24.4, 26.1, 28.1, 32.8, 40.7, 46.0, 46.9, 49.5, 123.5, 134.6, 135.4, 138.2, 139.7, 148.5, 149.2, 165.8, 173.4. Anal.($C_{17}H_{21}N_3O_2$·0.2 H_2O) C, H, N. ESI-MS *m/z* 300.2 $[M + H]^+$.

(±)-5-(Pyrrolidine-1-carbonyl)-cyclopent-1-enecarboxylic acid (pyridin-4-ylmethyl)-amide (5c). Compound **5c** was synthesized from compound **3** (150 mg, 0.72 mmol) and 4-(aminomethyl)pyridine (110 µl, 1.08 mmol) according to general procedure B. The product was purified by flash chromatography (MeOH:EtOAc 1:1). Yield 170 mg, 79%. ^{1}H NMR δ 1.81-1.86 (m, 2 H), 1.90-2.06 (m, 3 H), 2.28-2.35 (m, 1

H), 2.50- 2.57 (m, 1 H), 2.62-2.69 (m, 1 H), 3.39 (t, *J* = 6.9 Hz, 2 H), 3.46-3.50 (m, 1 H), 3.73-3.78 (m, 1 H), 4.05-4.08 (m, 1 H), 4.30 (dd, *J* = 5.8, 16.1 Hz, 1 H), 4.57 (dd, *J* = 6.5, 16.1 Hz, 1 H), 6.55-6.56 (m, 1 H), 7.15 (t, 1 H), 7.21 (d, *J* = 5.6 Hz, 2 H), 8.52 (d, 2 H). ^{13}C NMR δ 24.3, 26.0, 28.0, 32.8, 42.0, 45.9, 46.9, 49.5, 122.1, 138.4, 139.6, 148.1, 149.7, 165.8, 173.5. Anal.($C_{17}H_{21}N_3O_2 \cdot 0.2\ H_2O$) C, H, N. ESI-MS *m/z* 300.2 $[M + H]^+$.

(±)-5-(Pyrrolidine-1-carbonyl)-cyclopent-1-enecarboxylic acid phenethyl-amide (6). Compound **6** was synthesized from compound **3** (167 mg, 0.80 mmol) and phenethylamine (150 µl, 1.20 mmol) according to general procedure B in 11 ml of CH_3CN. The residue was dissolved in EtOAc and the organic phase was washed with 30% citric acid, sat. NaCl aq. and sat. $NaHCO_3$ aq. The product was purified by flash chromatography (5% MeOH in EtOAc). Yield 130 mg, 52%. ^{1}H NMR δ 1.80-2.04 (m, 5 H), 2.21-2.28 (m, 1 H), 2.44-2.50 (m, 1 H), 2.59-2.65 (m, 1 H), 2.81 (t, *J* = 7.0 Hz, 2 H), 3.35-3.49 (m, 4 H), 3.56-3.63 (m, 1 H), 3.75-3.80 (m, 1 H), 3.99-4.02 (m, 1 H), 6.33-6.34 (m, 1 H), 6.48 (t, 1 H), 7.18-7.21 (m, 3 H), 7.27-7.30 (m, 2 H). ^{13}C NMR δ 24.4, 26.1, 28.1, 32.7, 35.7, 40.6, 45.9, 46.9, 49.4, 126.3, 128.5, 128.8, 137.3, 139.2, 140.1, 165.6, 173.3. Anal.($C_{19}H_{24}N_2O_2 \cdot 0.2\ H_2O$) C, H, N. ESI-MS *m/z* 313.1 $[M + H]^+$.

(±)-5-(Pyrrolidine-1-carbonyl)-cyclopent-1-enecarboxylic acid (2-pyridin-2-yl-ethyl)-amide (7a). Compound **7a** was synthesized from compound **3** (150 mg, 0.72 mmol) and 2-(2-aminoethyl)pyridine (130 µl, 1.09 mmol) according to general procedure B in 14 ml of CH_3CN. The product was purified by flash chromatography (25% MeOH in EtOAc). Yield 183 mg, 81%. ^{1}H NMR δ 1.79-2.06 (m, 5 H), 2.19-2.27 (m, 1 H), 2.44-2.51 (m, 1 H), 2.63-2.71 (m, 1 H), 3.00 (t, *J* = 6.4 Hz, 2 H), 3.39-3.52 (m, 3 H), 3.57-3.63 (m, 1 H), 3.69-3.75 (m, 1 H), 3.80-3.84 (m, 1 H), 3.99-4.02 (m, 1 H), 6.35-6.37 (m, 1 H), 7.03 (t, 1 H), 7.13-7.15 (m, 1 H), 7.17 (d, *J* = 7.7 Hz, 1 H), 7.61 (dt, *J* = 1.8, 7.7 Hz, 1 H), 8.52 (d, *J* = 4.9 Hz, 1 H). ^{13}C NMR (solvent as an internal standard, 77.16 ppm) δ 24.3, 26.1, 28.1, 32.7, 36.9, 38.7, 45.8, 46.8, 49.1, 121.5, 123.5, 136.6, 137.1, 140.2, 149.1, 159.7, 165.3, 173.1. Anal.($C_{18}H_{23}N_3O_2 \cdot 0.3\ H_2O$) C, H, N: calcd, 13.18; found, 13.73. ESI-MS *m/z* 314.2 $[M + H]^+$.

(±)-5-(Pyrrolidine-1-carbonyl)-cyclopent-1-enecarboxylic acid (2-pyridin-3-yl-ethyl)-amide (7b). Compound **7b** was synthesized from compound **3** (600 mg, 2.9 mmol) and 3-(2-aminoethyl)pyridine (530 mg, 4.3 mmol) according to general procedure B in 14 ml of CH_3CN. The product was purified by flash chromatography (20% MeOH in EtOAc). Yield 225 mg, 25%. ^{1}H NMR δ 1.82-2.05 (m, 5 H), 2.24-2.31 (m, 1 H), 2.45-2.51 (m, 1 H), 2.57-2.63 (m, 1 H), 2.83 (t, *J* = 7.1 Hz, 2 H), 3.34-3.49

(m, 4 H), 3.56-3.62 (m, 1 H), 3.73-3.78 (m, 1 H), 3.99-4.01 (m, 1 H), 6.37-6.38 (m, 1 H), 6.73 (t, 1 H), 7.21-7.24 (m, 1 H), 7.55 (d, *J* = 7.8 Hz, 1 H), 8.44-8.46 (m, 2 H). ^{13}C NMR δ 24.4, 26.1, 28.1, 32.6, 32.9, 40.3, 46.0, 46.9, 49.5, 123.5, 134.8, 136.4, 137.7, 139.9, 147.7, 150.1, 165.9, 173.3. Anal.($C_{18}H_{23}N_3O_2 \cdot 0.8\ H_2O$) C, H, N. ESI-MS *m/z* 314.2 $[M + H]^+$.

(±)-5-(Pyrrolidine-1-carbonyl)-cyclopent-1-enecarboxylic acid (2-pyridin-4-yl-ethyl)-amide (7c). Compound **7c** was synthesized from compound **3** (470 mg, 2.2 mmol) and 4-(2-aminoethyl)pyridine (410 mg, 3.4 mmol) according to general procedure B in 14 ml of CH_3CN. The product was purified by flash chromatography (20% MeOH in EtOAc). Yield 460 mg, 65%. ^{1}H NMR δ 1.82-2.03 (m, 5 H), 2.24-2.32 (m, 1 H), 2.44-2.52 (m, 1 H), 2.57-2.64 (m, 1 H), 2.83 (t, *J* = 7.1 Hz, 2 H), 3.38-3.49 (m, 4 H), 3.56-3.63 (m, 1 H), 3.71-3.75 (m, 1 H), 3.97-4.01 (m, 1 H), 6.35-6.36 (m, 1 H), 6.62 (t, 1 H), 7.14 (d, *J* = 5.9 Hz, 2 H), 8.50 (d, *J* = 5.9 Hz, 2 H). ^{13}C NMR δ 24.4, 26.1, 28.1, 32.6, 35.1, 39.6, 46.0, 46.9, 49.6, 124.3, 137.6, 139.9, 148.4, 149.7, 165.9, 173.2. Anal.($C_{18}H_{23}N_3O_2 \cdot 0.6\ H_2O$) C, H, N. ESI-MS *m/z* 314.1 $[M + H]^+$.

Boc-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine (10). Compound **10** was synthesized from Boc-5(*R*)-*tert*-butyl-L-proline (**8**) (540 mg, 2.0 mmol) and pyrrolidine (170 µl, 2.0 mmol) according to general procedure A. The reaction mixture was stirred 2.5 h at rt. The product was purified by flash chromatography (PE:EtOAc 2:1). Yield 650 mg, 100%. ^{1}H NMR δ 1.01 (s, 9 H), 1.42 (s, 9 H), 1.80–2.11 (m, 8 H), 3.36–3.70 (m, 4 H), 3.79 (d, *J* = 7.9 Hz, 1 H), 4.39 (br s, 1 H).

Boc-L-prolyl-pyrrolidine (11). Compound **11** was synthesized from Boc-L-proline (**9**) (1.72 g, 7.99 mmol) and pyrrolidine (670 µl, 8.0 mmol) according to general procedure A. The reaction mixture was stirred 5 h at rt. The product was purified by flash chromatography (5% MeOH in DCM). Yield 2.1g, 98%.

4-(Pyridin-3-yl)butyric acid. STEP 1. To a solution of 3-(pyridin-3-yl)propan-1-ol (1.3 ml, 10 mmol) in DCM (28 ml) was added $SOCl_2$ (3.6 ml, 50 mmol) in DCM (7 ml) dropwise at 0 °C. The mixture was stirred overnight at rt and evaporated to yield 3-(3-chloropropyl)pyridine hydrochloride (100%). ^{13}C NMR δ 29.8, 32.7, 43.3, 126.9, 138.9, 140.8. 141.5, 145.8. The product was stored as the HCl salt. It was dissolved in DCM, washed with sat. $NaHCO_3$ aq., dried over Na_2SO_4 and evaporated just before further use. STEP 2. To a suspension of NaCN (280 mg, 5.8 mmol) and KI (catalytic amount) in dry DMSO (5 ml) was added 3-(3-chloropropyl)pyridine (690 mg, 4.4 mmol) in dry DMSO (15 ml) dropwise at 80 °C. The mixture was refluxed for 9 h, let to cool down to rt and evaporated to yield 4-(pyridin-3-yl)butyronitrile. The product was used without

purification. ^{13}C NMR (DMSO, solvent as an internal standard, 39.52 ppm) δ 15.8, 26.1, 31.0, 120.1, 123.4, 135.7, 135.7, 147.4, 149.5.

STEP 3. All product from STEP 2 was dissolved in EtOH (10 ml) and KOH (1.0 g, 17.8 mmol) in H_2O (10 ml) was added. The mixture was refluxed overnight. EtOH was evaporated and the aqueous phase washed with DCM. The pH of the aqueous phase was adjusted to 5 with HCl aq. and the product was extracted with DCM (continuous extraction). Yield (steps 2 and 3) 400 mg, 55%. ^{13}C NMR (solvent as an internal standard, 77.16 ppm) δ 25.7, 31.6, 32.8, 122.9, 135.6, 136.5, 146.7, 149.1, 174.6.

4-(Pyridin-3-yl)butanoyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine (13a). Compound **13a** was prepared from compound **10** (280 mg, 0.9 mmol) and 4-(pyridin-3-yl)butyric acid (140 mg, 0.9 mmol) according to general procedure C. The product was purified by flash chromatography (3-5% MeOH in EtOAc). Yield 210 mg, 66%. ^{1}H NMR δ 1.00 (s, 5.3 H), 1.02 (s, 3.7 H), 1.73-2.07 (m, 9.5 H), 2.09-2.18 (m, 1 H), 2.29-2.40 (m, 1 H), 2.47-2.53 (m, 0.5 H), 2.58-2.71 (m, 2 H), 3.30-3.51 (m, 3.2 H), 3.58-3.66 (m, 0.8 H), 3.74-3.79 (m, 0.4 H), 4.28-4.31 (m, 1.2 H), 4.57-4.61 (m, 0.4), 7.19-7.22 (m, 1 H), 7.51-7.53 (m, 1 H), 8.40-8.43 (m, 2 H). ^{13}C NMR δ 23.9, 24.1, 25.9, 26.2, 26.3, 26.4, 26.5, 27.3, 27.7, 28.0, 28.1, 29.6, 32.3, 32.4, 33.0, 33.8, 36.5, 36.6, 45.9, 46.0, 46.0, 46.3, 60.9, 61.1, 65.8, 67.2, 123.4, 123.4, 136.0, 136.2, 137.1, 137.4, 147.3. 147.4, 149.9, 149.9, 170.2, 170.4, 173.5, 173.7. Anal.($C_{22}H_{33}N_3O_2$·0.3 H_2O) C, H, N. ESI-MS *m/z* 372.2 $[M + H]^+$.

3-(Pyridin-3-yl)propionyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine (13b). Compound **13b** was prepared from compound **10** (350 mg, 1.1 mmol) and 3-(pyridin-3-yl)propionic acid (150 mg, 1.0 mmol) according to general procedure C. The product was purified by flash chromatography (10% MeOH in EtOAc). Yield 260 mg, 73%. ^{1}H NMR δ 1.00 (s, 5 H), 1.04 (s, 4 H), 1.66-2.07 (m, 7 H), 2.11-2.17 (m, 0.5 H), 2.29-2.35 (m, 1 H), 2.45-2.51 (m, 0.5 H), 2.64-2.71 (m, 0.5 H), 2.75-2.81 (m, 0.5 H), 2.93-3.06 (m, 2 H), 3.33-3.56 (m, 3 H), 3.60-3.65 (m, 1 H), 3.75-3.80 (m, 0.5 H), 4.30-4.33 (m, 1 H), 4.56-4.60 (m, 0.5 H), 7.18-7.23 (m, 1 H), 7.52-7.56 (m, 1 H), 8.42-8.46 (m, 2 H). ^{13}C NMR (solvent as an internal standard, 77.16 ppm) δ 23.8, 24.0, 26.1, 26.2, 26.3, 27.2, 27.6, 27.9, 28.0, 28.4, 29.0, 29.6, 35.6, 36.2, 36.5, 36.6, 45.9, 45.9, 45.9, 46.3, 60.9, 61.1, 65.8, 67.2, 123.3, 123.4, 136.0, 136.2, 136.8, 137.2, 147.3, 147.5, 149.6, 149.7, 170.1, 170.2, 172.8, 172.8. Anal.($C_{21}H_{31}N_3O_2$) C, H, N. ESI-MS *m/z* 358.2 $[M + H]^+$.

3-(Pyridin-3-yl)propionyl chloride hydrochloride. 3-(Pyridin-3-yl)propionic acid (180 mg, 1.2 mmol) was dissolved in thionyl chloride (875 µl, 12.0 mmol). The mixture was stirred overnight and evaporated.

3-(Pyridin-3-yl)propionyl-L-prolyl-pyrrolidine (14a). Compound **11** (320 mg, 1.2 mmol) was dissolved in HCl treated EtOAc (4 ml, 2.7 M). The mixture was stirred for 45 min. and evaporated to yield L-prolyl-pyrrolidine hydrochloride. To a solution of 3-(pyridin-3-yl)propionyl chloride hydrochloride (1.2 mmol) and Et_3N (840 µl, 6.0 mmol) in 2 ml of DCM was added L-prolyl-pyrrolidine hydrochloride in 5 ml of DCM dropwise at 0 °C. The mixture was stirred overnight at rt. The reaction mixture was diluted with DCM and washed with saturated $NaHCO_3$ aq. and saturated NaCl aq., dried over anhydrous Na_2SO_4 and evaporated. The product was purified by flash chromatography (10-30% MeOH in EtOAc). Yield 160 mg, 44%. ^{1}H NMR δ 1.80-2.28 (m, 8 H), 2.55-2.62 (m, 1 H), 2.65-2.71 (m, 1 H), 2.97-3.00 (m, 2 H), 3.35-3.46 (m, 3 H), 3.56-3.61 (m, 1 H), 3.63-3.68 (m, 1 H), 3.81-3.85 (m, 1 H), 4.65 (dd, *J* = 3.5, 8.5 Hz, 1 H), 7.21 (dd, *J* = 4.9, 7.7 Hz, 1 H), 7.55 (d, *J* = 7.7 Hz, 1 H), 8.44-8.45 (m, 1 H), 8.48 (br s, 1 H). ^{13}C NMR (solvent as an internal standard, 77.16 ppm) δ 24.1, 24.7, 26.2, 27.8, 28.8, 35.8, 45.9, 46.3, 47.2, 57.8, 123.3, 136.0, 136.8, 147.5, 149.8, 170.1, 170.5. Anal.($C_{17}H_{23}N_3O_2$·0.3 H_2O) C, H, N. ESI-MS *m/z* 302.1 $[M + H]^+$.

(Pyridin-3-yl)acetyl-L-prolyl-pyrrolidine (14b). Compound **14b** was prepared from compound **11** (400 mg, 1.5 mmol) and (pyridin-3-yl)acetic acid hydrochloride (260 mg, 1.5 mmol) according to general procedure C, using 5 eq. of Et_3N. The product was purified by flash chromatography (10-20% MeOH in EtOAc). Yield 300 mg, 70%. ^{1}H NMR δ 1.78-2.17 (m, 7 H), 2.24-2.33 (m, 1 H), 3.36-3.61 (m, 4 H), 3.68 (d, *J* = 15.9 Hz, 1 H), 3.71 (d, *J* = 15.9 Hz, 1 H), 3.75-3.81 (m, 2 H), 4.67 (dd, *J* = 3.6, 8.3 Hz, 1 H), 7.26 (dd, *J* = 4.8, 7.8 Hz, 1 H), 7.70 (dt, *J* = 7.8 Hz, 1 H), 8.47-8.49 (m, 2 H). ^{13}C NMR (solvent as an internal standard, 77.16 ppm) δ 24.0, 24.8, 26.1, 28.8, 38.5, 45.9, 46.2, 47.6, 58.0, 123.4, 130.5, 137.0, 148.0, 150.3, 168.4, 170.2. Anal.($C_{16}H_{21}N_3O_2$·0.3 H_2O) C, H, N. ESI-MS *m/z* 288.1 $[M + H]^+$.

Nicotinoyl-L-prolyl-pyrrolidine (14c). Compound **11** (1.1 g, 4.1 mmol) was dissolved in HCl treated EtOAc (12 ml, 1.9 M) and the mixture was stirred for 40 min. The mixture was evaporated and dissolved in pyridine (70 ml), DMAP (150 mg, 1.2 mmol) was added. Nicotinoyl chloride hydrochloride (3.6 g, 20 mmol) was added as five portions and the mixture was stirred overnight. Pyridine was evaporated and the residue was dissolved in DCM. The organic phase was with sat. $NaHCO_3$ aq., dried over Na_2SO_4 and evaporated. The product was purified by flash chromatography (MeOH:EtOAc 1:1). Yield 610 mg, 56%. ^{1}H NMR (DMSO) δ 1.39-1.47 (m, 0.4 H), 1.54-2.00 (m, 6.6 H), 2.22-2.30 (m, 1 H), 2.69-2.74 (m, 0.4 H), 2.86-2.91 (m, 0.4 H), 3.01-3.06 (m, 0.4), 3.12-3.16 (m, 0.4), 3.26-3.39 (m, 1.2 H), 3.45-3.70 (m, 3.2 H), 4.54

(dd, *J* = 4.6, 8.1 Hz, 0.4 H), 4.71 (dd, *J* = 5.4, 8.4 Hz, 0.6 H), 7.40 (ddd, *J* = 0.7, 4.8, 7.8 Hz, 0.4 H), 7.48 (ddd, *J* = 0.7, 4.8, 7.8 Hz, 0.6 H), 7.71 (dt, *J* = 1.9, 7.8 Hz, 0.4 H), 7.92 (dt, *J* = 1.9, 7.8 Hz, 0.6 H), 8.51 (dd, *J* = 0.7, 2.1 Hz, 0.4 H), 8.58 (dd, *J* = 1.7, 4.8 Hz, 0.4 H), 8.66 (dd, *J* = 1.7, 4.8 Hz, 0.6 H), 8.70 (dd, *J* = 0.7, 2.1 Hz, 0.6 H). ^{13}C NMR (DMSO, solvent as an internal standard, 39.52 ppm) δ 22.5, 23.2, 23.5, 24.8, 25.3, 25.6, 28.3, 30.3, 45.1, 45.2, 45.5, 45.6, 46.9, 49.5, 58.1, 59.5, 122.8, 123.2, 132.2, 133.2, 133.9, 134.6, 147.0, 147.6, 149.9, 150.6, 165.5, 166.8, 169.1, 169.4. Anal.($C_{15}H_{19}N_3O_2 \cdot 0.5\ H_2O$) C, H, N. ESI-MS *m/z* 274.1 $[M + H]^+$.

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8 2(*S*)-(CYCLOALK-1-ENECARBONYL)-1-(4-PHENYL-BUTANOYL)PYRROLIDINES AND 2(*S*)-(AROYL)-1-(4-PHENYLBUTANOYL)PYRROLIDINES AS PROLYL OLIGOPEPTIDASE INHIBITORS*

Abstract. In order to replace the P2-P1 amide group, different 1-cycloalkenyls and 2-aryls were studied in the place of the P1 pyrrolidine group of a 4-phenylbutanoyl-L-Pro-pyrrolidine structure, which is a well-known prolyl oligopeptidase inhibitor SUAM-1221. The 1-cyclopentenyl and the 2-thienyl groups gave novel compounds, which were equipotent with the corresponding pyrrolidine-analog SUAM-1221. It was shown that the P2-P1 amide group of POP inhibitors can be replaced by an α,β-unsaturated carbonyl group or the aryl conjugated carbonyl group.

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8.1 Introduction

POP is a proline specific peptidase and the S1 binding site of POP has evolved to fit the pyrrolidine ring of an L-prolyl residue (Fülöp *et al*. 1998). The pyrrolidine group is unquestionably the most utilized structure at the P1 position of POP inhibitors and only few successful replacements of the P1 pyrrolidine ring have been described in the literature (Nakajima *et al*. 1992, Portevin *et al*. 1996). An electrophilic substituent, like a 2(*S*)-formyl or a 2(*S*)-cyano group, at the 2-position of the pyrrolidine ring increases significantly the potency (Wilk and Orlowski 1983, Tanaka *et al*. 1994). However, these groups are chemically reactive, which may limit their usability in drugs.

In a peptide chain, a prolyl residue lacks the main chain amide hydrogen. Consequently, the P1 pyrrolidine group of POP inhibitors (or the substrates of POP) is not stabilized by hydrogen bonding (Fülöp *et al*. 1998, Fülöp *et al*. 2001). Wallen *et al*. have previously replaced the P1 pyrrolidine group of the well-known reference compound SUAM-1221 **1a** (Yoshimoto *et al*. 1991) by a series of alkyl groups and a phenyl group (Wallén *et al*. 2002). The cyclopentyl and the phenyl groups gave equipotent compounds **1b** and **1c** (Figure 1). These compounds were moderately potent but still one order of magnitude less potent than the reference compound **1a**. In the present study, the field between these two replacements was further studied, focusing on mimicking the amide bond geometry.

8.2 Methods

The synthetic routes are presented in Schemes 1, 2 and 3. Cyclopentylidene-hydrazine (**2a**) and cyclohexylidene-hydrazine (**2b**) were prepared by the reaction of hydrazine monohydrate with cyclopentanone and cyclohexanone, respectively. While the reaction with cyclopentanone gave *N,N'*-dicyclopentylidene-hydrazine as a minor side-product (about 5 mol%) the reaction with cyclohexanone produced a significant amount of *N,N'*-

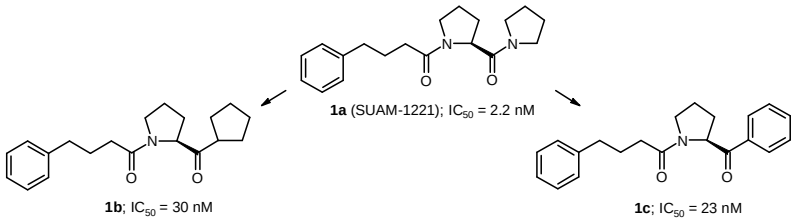
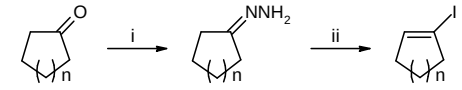
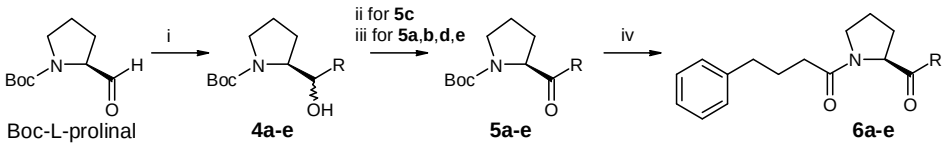


Figure 1. The reference compound **1a** and the previously reported compounds **1b** and **1c** (Wallén *et al*. 2002).



n = 1 or 2 **2a**; n = 1 **2b**; n = 2 **3a**; n = 1 **3b**; n = 2

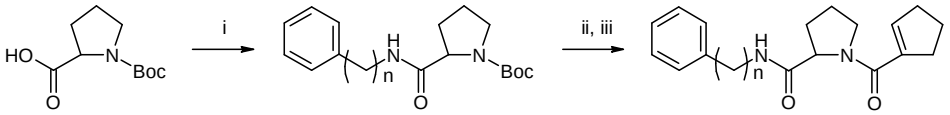
Scheme 1. (i) hydrazine monohydrate/reflux; (ii) 1. I_2, tetramethylguanidine/Et_2O, 2. Compound **2a** or **2b**/Et_2O.



Boc-L-prolinal **4a-e** **5a-e** **6a-e**

4a-6a; R = 1-cyclopentenyl
4b-6b; R = 1-cyclohexenyl
4c-6c; R = 2-pyridyl
4d-6d; R = 2-thienyl
4e-6e; R = 2-furyl

Scheme 2. (i) organolithium reagent (prepared *in situ* or commercial reagent)/THF, -80 °C; (ii) Et_3N, SO_3·pyridine/DMSO; (iii) DMP/CH_2Cl_2; (iv) 1. trifluoroacetic acid/CH_2Cl_2, 2. 4-phenylbutyric acid, HOBt·H_2O, EDC·HCl, Et_3N/CH_2Cl_2, 0-20 °C.



Boc-L-proline or Boc-D-proline **7a**; *S*, n = 1 **7b**; *R*, n = 1 **7c**; *S*, n = 2 **8a**; *S*, n = 1 **8b**; *R*, n = 1 **8c**; *S*, n = 2

Scheme 3. (i) 1. Et_3N, ethyl chloroformate/THF, -15 °C, 2. benzylamine/THF, -15-+20 °C; (ii) 1. trifluoroacetic acid/CH_2Cl_2, 0 °C; (iii) 1. cyclopent-1-enecarboxylic acid, Et_3N, trimethylacetyl chloride/CH_2Cl_2, 0 °C, 2. Et_3N, trifluoroacetic acid salt of the deprotected **6** from reaction (ii).

dicyclohexylidene-hydrazine (about 20 mol%). All attempts to distill **2b** led to the decomposition of the product. The unpurified compounds **2a** and **2b** were reacted with iodine in the presence of tetramethylguanidine to yield 1-iodocyclopentene (**3a**) and 1-iodocyclohexene (**3b**), respectively, which were distilled before further use (Barton *et al*. 1983). Compounds **3a** and **3b** were treated with *tert*-BuLi, and 2-bromopyridine and furan were treated with *n*-BuLi to obtain the corresponding organolithium reagents, which were reacted with Boc-L-prolinal to give compounds **4a**, **4b**, **4c** and **4e**, respectively. Compound **4d** was obtained from the reaction of the commercially available 2-thienyllithium and Boc-L-prolinal. Compounds **4a-e** were oxidized with Dess-Martin periodinane or SO_3·pyridine/DMSO and deprotected. The resulting amines

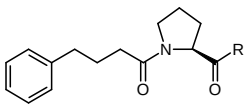
were coupled with 4-phenylbutyric acid using EDC and HOBt to yield compounds **6a-e**. Boc-L-proline and Boc-D-proline were activated with ethyl chloroformate and reacted with benzylamine or phenethylamine to yield compounds **7a-c**. Cyclopent-1-enecarboxylic acid was activated with trimethylacetyl chloride and reacted with **7a-c** after the removal of the Boc-protection group to yield compounds **8a-c**.

8.3 Results and discussion

Compounds **6a-e** are presented in Table 1. The P1 pyrrolidine ring of the reference compound **1a** (Figure 1) has been replaced by a 1-cyclopentene ring in compound **6a**. A similar replacement at the P2 position has previously been shown to give potent POP inhibitors (Chapter 6). Indeed, compound **6a** was equipotent with the reference compound **1a**. Compound **6a** was one order of magnitude more potent than the previously reported compound **1b** with the cyclopentyl ring at the P1 site. The difference in their potencies can be explained by differences in geometry and π-stacking. The P1 amide nitrogen is sp^2 hybridized and, although the replacement by an sp^3 carbon was tolerated (IC_{50} of compound **1b** was 30 nM), an sp^2 carbon provides a better mimetic for the amide nitrogen. Furthermore, the double bond may have beneficial π-stacking with the indole ring of Trp595 (Fülöp *et al*. 1998).

The enlargement of the 1-cyclopentenyl group of compound **6a** resulted in compound **6b** with a 1-cyclohexenyl group at the P1 position. Compound **6b** was 20 times less potent than compound **6a**, which reflects the limited space in the S1 pocket (Fülöp *et al*. 1998). However, Wallén *et al*. have previously reported that a compound

Table 1. Inhibitory activities with 95% confidence intervals of the compounds with different 1-cycloalkenyl and 2-aryl groups at the P1 position.



Compound	R	IC_{50} (nM)[a]
6a	1-cyclopentenyl	3.0 (2.7-3.4)
6b	1-cyclohexenyl	59 (52-69)
6c	2-pyridyl	510 (450-570)
6d	2-thienyl	3.0 (2.7-3.4)
6e	2-furyl	5.20 (4.3-6.1)

[a] The IC_{50} values were determined against POP from porcine brain (Venäläinen *et al*. 2002).

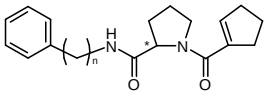
with the same skeleton as **6b** but with a fully saturated cyclohexyl group at the P1 site gives only weak POP inhibitory activity (IC_{50} = 1.1 μM). Compound **6b** was 18 times more potent than this compound, while it was only 2.6 times less potent than the reference compound **1c**, which has the phenyl group at the P1 site. This small decrease in potency in relation to compound **1c** may be explained by the smaller size of the phenyl ring or loss in π-stacking with Trp595.

In order to study different aryls at the P1 position, the 2-pyridyl, 2-thienyl and 2-furyl groups were selected. The pyridyl group has previously been successfully employed at the P1' and P3 positions of POP inhibitors (Tsutsumi *et al*. 1994, Chapter 7). As compared to compound **1c** with the phenyl group at the P1 site, compound **6c** with the 2-pyridyl group had strongly decreased inhibitory activity. In contrast, compound **6d**, with the 2-thienyl group at the P1 site, was one order of magnitude more potent than compound **1c** and equipotent with compound **6a**. The difference between compounds **6d** and **1c** can be explained by the smaller size of the 2-thienyl group. The 1-cyclopentenyl and the 2-thienyl groups instead, are almost equal in size and compound **6d** does not seem to have any beneficial π-stacking over **6a**. The potency of compound **6e** with the 2-furyl group at the P1 site was slightly decreased but the same order of magnitude than the potency of compound **6d**.

The 2-thienyl group has previously been studied at the P1 position of POP inhibitors but with opposite results. It gave a poor POP inhibitor against *Flavobacterium meningosepticum* POP (Portevin *et al*. 1996). On the other hand, an unusual POP inhibitor Y-29794 possesses a 2-thienyl group although it is not known where it binds. Y-29794 was reported to have a K_i value of 0.95 nM against rat POP (Nakajima *et al*. 1992). The excellent POP inhibitory activity of compound **6d** indicates that the 2-thienyl group of Y-29794 may bind to the S1 pocket of POP. It also seems that the S1 subsites of the mammalian and the bacterial POP have differences.

The use of the 1-cyclopentenyl group at the P1 position allowed further modifications on the backbone. Compounds **8a-c** (Table 2) were prepared in order to study whether it is possible to reverse the P2 prolyl residue; the P1-P2 carbonyl group was kept in the original position but the direction of the amide bond was changed. Consequently, compounds **8a-c** possess the α,β-unsaturated amide group instead of the α,β-unsaturated ketone group. α,β-Unsaturated ketones are usually avoided in drug design because of their ability to undergo Michael additions to the double bond. α,β-Unsaturated amides may be less reactive due to the electron-donating effect of the amide nitrogen. Compound **8a** with a reversed L-prolyl residue at the P2 position had an

Table 2. Inhibitory activities with 95% confidence intervals of the compounds with the reversed prolyl residue at the P2 position.



Compound	n	Stereocenter	IC_{50} (nM)[a]
8a	1	*S*	210 (180-240)
8b	1	*R*	44 000 (28000-67000)
8b	2	*S*	110 (91-130)

[a] The IC_{50} values were determined against POP from porcine brain (Venäläinen *et al*. 2002).

IC_{50} value of 210 nM. The potency was significantly decreased as compared to compound **6a**. A D-prolyl residue was also studied but the resulting compound **8b** was over 200 times less active than compound **8a** with the L-prolyl residue. The P3 chain of compound **8a** was elongated with one methylene group resulting in compound **8c**, which had improved inhibitory activity. This reflects the known structure-activity relationships of the P3 site suggesting that the 1-cyclopentenyl group of compounds **8a-c** also binds to the S1 site of POP.

8.4 Conclusions

The 1-cycloalkenyl groups at the P1 position gave better POP inhibitory activities than the corresponding fully saturated cycloalkyl groups. The 1-cyclopentenyl group gave the most potent compound, which was equipotent with the reference compound **1a**. In the series of the aromatic groups, the 2-thienyl group gave the most potent compound. This confirms again that the 5-membered ring is preferred. The reversed P2 L-prolyl residue decreased potency significantly but the effect of the reversed D-prolyl residue was much more pronounced. In conclusion, the P2-P1 amide group of POP inhibitors can be replaced by the α,β-unsaturated carbonyl group or the aryl conjugated carbonyl group.

8.5 Synthetic procedures and analytical data

Method A. 1-Iodo-cyclopentene (3a). STEP 1 (**2a**). Cyclopentanone (8.8 ml, 100 mmol) was added dropwise to hydrazine monohydrate (30 ml, 620 mmol) under vigorous stirring. The mixture was refluxed for 2 h and cooled down to room temperature (rt). DCM was cautiously added at cold water bath and the phases were separated. The organic phase was washed with saturated (sat.) NaCl aq., dried over

Na_2SO_4, filtered and evaporated. The product was used without further purification. Crude product 7.1 g. **STEP 2** (**3a**). Tetramethylguanidine (75 ml, 600 mmol) in Et_2O (110 ml) was added dropwise to iodine (28 g, 110 mmol) in Et_2O (160 ml) during 1.5 h and the mixture was stirred for 2.5 h. **2a** (4.9 g, about 50 mmol) in Et_2O (50 ml) was added dropwise during 2 h and the mixture was stirred overnight. The mixture was refluxed for 2 h, cooled down to rt and filtered. The filtrate was concentrated from Et_2O, heated up to 90-95 °C for 3 h, cooled to rt and diluted with Et_2O. The organic phase was washed with 1 M HCl aq., 20% $Na_2S_2O_3$, sat. $NaHCO_3$ aq. and sat. NaCl aq., dried over Na_2SO_4, filtered and evaporated (with a rotary evaporator at 40 °C and 100 mbar as the lowest pressure point). The product was stored at -18 °C under argon and was distilled before further use at 39 °C and 6 mbar. Yield 5.6 g, 42% for 2 steps. ^{1}H NMR δ 1.90-1.96 (m, 2 H), 2.30-2.34 (m, 2 H), 2.58-2.62 (m, 2 H), 6.09-6.11 (m, 1 H).

1-Iodo-cyclohexene (3b). Prepared according to method A from cyclohexanone (10.4 ml, 100.0 mmol). **STEP 1 (3a)**. Crude product 10.8 g. **STEP 2** (**3b**). Tetramethylguanidine (1.16 mol), I_2 (212 mmol). Compound **3b** was distilled at 50 °C and 1 mbar. Yield 9.9 g, 47% for 2 steps. ^{1}H NMR δ 1.63-1.72 (m, 4 H), 2.07-2.11 (m, 2 H), 2.48-2.52 (m, 2 H), 6.33-6.35 (m, 1 H).

Method B. ((*S*)-1-Boc-pyrrolidin-2-yl)-cyclopent-1-enyl-methanol (4a). 1.7 M *tert*-BuLi (17 ml, 29 mmol) was added dropwise to **3a** (5.6 g, 29 mmol) in THF (30 ml) and the mixture was stirred for 1 h at -80 °C. Boc-L-prolinal (1.9 g, 9.5 mmol) in THF (10 ml) was added dropwise and the mixture was stirred for 3 h at -80 °C. The reaction was quenched with sat. NH_4Cl aq. at -80 °C. The mixture was let to warm up to rt, diluted with H_2O and extracted with DCM. The organic phase was washed with sat. NaCl aq., dried over Na_2SO_4, filtered and evaporated. The product was purified by flash chromatography (15-50% EtOAc in hexane). Yield 1.9 g, 75%.

((*S*)-1-Boc-pyrrolidin-2-yl)-cyclohex-1-enyl-methanol (4b). Prepared according to method B from **3b** (3.1 g, 15 mmol) and Boc-L-prolinal (1.0 g, 5 mmol). The product was purified twice by flash chromatography. One diastereomer was purified with 15% EtOAc in PE but the other diastereomer co-eluted with the unreacted Boc-prolinal. The other diastereomer was purified with 5% acetonitrile in DCM. The diasteromers were combined giving the total yield of 0.54 g, 38%.

((*S*)-1-Boc-pyrrolidin-2-yl)-pyridin-2-yl-methanol (4c). To a solution of 2-bromopyridine (3.2 g, 20 mmol) in 40 ml of THF was added dropwise 1.6 M n-BuLi (12.5 ml, 20.0 mmol) at -70 °C and the mixture was stirred for 1 h. Boc-L-prolinal (3.6 g, 18 mmol) in THF (40 ml) was added and the mixture was stirred for 2 h at -70 °C.

The mixture was poured in water and THF was evaporated. The aqueous phase was extracted with DCM and the organic phase was extracted with 0.1 M HCl aq. The aqueous phase was made basic with 2 M NaOH aq., extracted with DCM, dried over Na_2SO_4, filtered and evaporated. Yield 2.8 g, 56%.

((*S*)-1-Boc-pyrrolidin-2-yl)-thiophen-2-yl-methanol (4d). To a solution of Boc-L-prolinal (0.31 g, 1.56 mmol) in THF was added 1 M 2-thienyllithium solution in THF (1.75 ml, 1.75 mmol) at -70 °C. The mixture was stirred for 3 h and quenched with sat. NH_4Cl aq. at -70 °C. The mixture was let to warm up to rt, diluted with H_2O and extracted with DCM. The organic phase was washed with sat. NaCl aq., dried over Na_2SO_4, filtered and evaporated. The product was purified by the flash chromatography (1% MeOH in DCM). Yield 0.32 g, 71%.

Method C. 1-Boc-2(*S*)-(cyclopent-1-enecarbonyl)pyrrolidine (5a). To a solution of Dess-Martin periodinane (DMP) (1.4 g, 3.3 mmol) in DCM (16 ml) was added **4a** (0.80 g, 2.99 mmol) in DCM (10 ml) and the mixture was stirred for 1 h at rt. 30 % $Na_2S_2O_3$ aq. was added and the mixture was stirred for 5 min. The phases were separated and the organic phase was washed with 30% $Na_2S_2O_3$ aq. and sat. $NaHCO_3$ aq., dried over Na_2SO_4, filtered and evaporated. The product was purified by flash chromatography (20% EtOAc in PE). Yield 0.58 g, 73%. ^{1}H NMR δ 1.34 (s, 5 H), 1.45 (s, 4 H), 1.80-1.96 (m, 5 H), 2.13-2.25 (m, 1 H), 2.50-2.69 (m, 4 H), 3.38-3.61 (m, 2 H), 4.77-4.80 (m, 0.5 H), 4.95-4.97 (m, 0.5 H), 6.76-6.78 (m, 0.5 H), 6.82-6.85 (m, 0.5 H).

1-Boc-2(*S*)-(cyclohex-1-enecarbonyl)pyrrolidine (5b). Prepared according to method C from **4b** (0.54 g, 1.92 mmol) using 1.3 eq. of DMP. No chromatographic purification was performed. Yield 0.54 g, 100%. ^{1}H NMR δ 1.34 (s, 4.7 H), 1.45 (s, 4.3 H), 1.57-1.69 (m, 4 H), 1.75-1.94 (m, 3 H), 2.12-2.39 (m, 5 H), 3.38-3.43 (m, 0.5 H), 3.46-3.62 (m, 1.5 H), 4.90 (dd, *J* = 4.1 Hz, 8.8 Hz, 0.5 H), 5.05 (dd, *J* = 3.4 Hz, 9.1 Hz, 0.5 H), 6.89-6.91 (m, 0.5 H), 6.95-6.97 (m, 0.5 H).

1-Boc-2(*S*)-(pyridine-2-carbonyl)pyrrolidine (5c). To a solution of **4c** (0.60 g, 2.16 mmol) and Et_3N (0.9 ml, 6.5 mmol) in 2.2 ml of DMSO was added SO_3·pyridine (1.03 g, 6.5 mmol) in DMSO (2.2 ml). The mixture was stirred for 1 h and then poured in ice cold water (25 ml). The aqueous phase was extracted with $CHCl_3$ and the organic phase was washed with water and sat. $NaHCO_3$ aq., dried over Na_2SO_4, filtered and evaporated. The product was purified with a chromatotron (1% EtOH in $CHCl_3$). Yield 0.37 g, 61%. ^{1}H NMR δ 1.24 (s, 5.4 H), 1.46 (s, 3.6 H), 1.88-1.98 (m, 3 H), 2.38-2.45 (m, 1 H), 3.48-3.70 (m, 2 H), 5.68-5.70 (m, 0.6 H), 5.77-5.79 (m, 0.4 H), 7.44 (ddd, *J* = 1.0 Hz, 4.8 Hz, 7.6 Hz, 0.4 H), 7.48 (ddd, *J* = 1.1 Hz, 4.8 Hz, 7.7 Hz, 0.6 H), 7.82 (td, *J*

= 1.6 Hz, 7.7 Hz, 0.4 H), 7.86 (td, *J* = 1.7 Hz, 7.8 Hz, 0.6 H), 8.08 (dt, J = 1.0 Hz, 7.8 Hz, 1 H), 8.66 (d, 0.4 H), 8.68 (d, *J* = 4.8 Hz, 0.6 H).

1-Boc-2(*S*)-(thiophene-2-carbonyl)pyrrolidine (5d). Prepared according to method C from **4d** (0.32 g, 1.13 mmol) using 1.3 eq. of DMP. The product was purified by flash chromatography (0.5% MeOH in DCM). Yield 0.27 g, 87%. ^{1}H NMR δ 1.25 (s, 5.4 H), 1.46 (s, 3.6 H), 1.87-2.05 (m, 3 H), 2.25-2.38 (m, 1 H), 3.41-3.50 (m, 0.4 H), 3.56-3.69 (m, 1.6 H), 4.88 (dd, *J* = 5.0 Hz, 8.6 Hz, 0.6 H), 5.10 (dd, *J* = 3.2 Hz, 8.9 Hz, 0.4 H), 7.12-7.16 (m, 1 H), 7.63-7.66 (m, 1 H), 7.78 (d, *J* = 3.8 Hz, 0.6 H), 7.81 (d, *J* = 3.5 Hz, 0.4 H).

1-Boc-2(*S*)-(furan-2-carbonyl)pyrrolidine (5e). 1.6 M n-BuLi (4.7 ml, 7.5 mmol) was added dropwise to furan (10 ml) at 0 °C and the mixture was stirred for 1 h. Boc-L-prolinal (0.30 g, 1.51 mmol) in THF (10 ml) was added dropwise at -80 °C and the mixture was stirred for 3 h. Sat. NH_4Cl aq. was added and the mixture was let to warm up to rt and diluted with DCM. The organic phase was washed with sat. NaCl aq., dried over Na_2SO_4 and evaporated to yield **4e**. Without further purification, the residue was dissolved in DCM, added to a solution of DMP (0.95 g, 2.24 mmol) in DCM (4 ml) and then continued according to method C. The product was purified by flash chromatography (10% EtOAc in PE). Yield 110 mg, 28% (two steps). ^{1}H NMR δ 1.26 (s, 5.4 H), 1.46 (s, 3.6 H), 1.87-2.01 (m, 3 H), 2.23-2.36 (m, 1 H), 3.43-3.67 (m, 2 H), 4.90 (dd, *J* = 4.6 Hz, 8.6 Hz, 0.6 H), 5.08-5.10 (m, 0.4 H), 6.53 (dd, *J* = 1.5 Hz, 3.3 Hz, 0.4 H), 6.56 (dd, *J* = 1.6 Hz, 3.5 Hz, 0.6 H), 7.23 (d, *J* = 3.5 Hz, 0.6 H), 7.26 (m, 0.4 H), 7.58 (d, 0.4 H), 7.61 (d, 0.6).

Method D. 2(*S*)-(Cyclopent-1-enecarbonyl)-1-(4-phenylbutanoyl)pyrrolidine (6a). To a solution of **5a** (110 mg, 0.35 mmol) in DCM (2.5 ml) was added trifluoroacetic acid (TFA) (0.7 ml, 9.4 mmol) at 0 °C. The mixture was stirred for 2.5 h at 0 °C and evaporated. TFA salt of the amine, 4-phenylbutyric acid (58 mg, 0.35 mmol), HOBt·H_2O (81 mg, 0.53 mmol) and EDC·HCl (102 mg, 0.53 mmol) were dissolved/suspended in DCM. Et_3N (240 µl, 1.75 mmol) was added at 0 °C and the mixture was stirred for 0.5 h at 0 °C and for 5 h at rt. The reaction mixture was washed with 30% citric acid, sat. NaCl aq. and sat. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated. The product was purified with a chromatotron (40-50% EtOAc in PE). Yield 50 mg, 46%. ^{1}H NMR δ 1.81-2.06 (m, 7 H), 2.10-2.18 (m, 1 H), 2.23-2.38 (m, 2 H), 2.50-2.69 (m, 6 H), 3.40-3.45 (m, 0.9 H), 3.56-3.62 (m, 1 H), 3.65-3.70 (m, 0.1 H), 4.78 (dd, *J* = 3.0 Hz, 9.2 Hz, 0.1 H), 5.16 (dd, *J* = 3.8 Hz, 8.8 Hz, 0.9 H), 6.64-6.66 (m, 0.1 H), 6.86-6.88 (m, 0.9 H), 7.12-7.29 (m, 5 H). ^{13}C NMR δ 22.5, 24.6, 26.0, 29.4,

30.9, 33.3, 34.2, 35.1, 47.2, 61.2, 125.8, 128.3, 128.6, 141.9, 143.4, 144.2, 171.1, 196.4. Anal. ($C_{20}H_{25}NO_2 \cdot 0.1\ H_2O$) CHN. ESI-MS *m/z* 312.1 $[M + H]^+$.

2(*S*)-(Cyclohex-1-enecarbonyl)-1-(4-phenylbutanoyl)pyrrolidine (6b). Prepared according to method D from **5b** (325 mg, 1.16 mmol) having 20 h reaction time. The product was purified by flash chromatography (50% EtOAc in PE). Yield 160 mg, 49%. ^{1}H NMR δ 1.56-1.67 (m, 4 H), 1.77-1.83 (m, 1 H), 1.87-2.05 (m, 4 H), 2.09-2.38 (m, 7 H), 2.59-2.69 (m, 2 H), 3.40-3.45 (m, 0.9 H), 3.56-3.62 (m, 1 H), 3.64-3.69 (m, 0.1 H), 4.88 (dd, *J* = 2.9 Hz, 9.2 Hz, 0.1 H), 5.24 (dd, *J* = 3.7 Hz, 8.9 Hz, 0.9 H), 6.75-6.77 (m, 0.1 H), 6.99-7.01 (m, 0.9 H), 7.13-7.28 (m, 5 H). ^{13}C NMR δ 21.5, 21.8, 23.2, 24.6, 26.0, 26.1, 29.5, 33.4, 35.1, 47.1, 59.6, 125.8, 128.3, 128.6, 137.4, 140.5, 141.9, 171.0, 198.6. Anal. ($C_{21}H_{27}NO_2 \cdot 0.1\ H_2O$) CHN. ESI-MS *m/z* 326.1 $[M + H]^+$.

1-(4-Phenylbutanoyl)-2(*S*)-(pyridine-2-carbonyl)pyrrolidine (6c). Prepared according to method D from **5c** (280 mg, 1.0 mmol) having a 20 h reaction time. The reaction mixture was not washed with 30% citric acid. The product was purified by flash chromatography (50% EtOAc in PE). Yield 130 mg, 40%. ^{1}H NMR δ 1.79-2.17 (m, 5.5 H), 2.28-2.49 (m, 2.5 H), 2.54-2.59 (m, 0.4 H) 2.68 (t, *J* = 7.5 Hz, 1.6 H), 3.50-3.55 (m, 0.8 H), 3.64-3.71 (m, 1 H), 3.73-3.78 (m, 0.2 H), 5.85 (dd, *J* = 3.0 Hz, 9.4 Hz, 0.2 H), 5.91-5.94 (m, 0.8 H), 7.05-7.08 (m, 0.5 H), 7.13-7.21 (m, 2.5 H), 7.26-7.29 (m, 2 H), 7.46 (ddd, *J* = 1.2 Hz, 4.8 Hz, 7.6 Hz, 0.8 H), 7.53 (ddd, *J* = 1.2 Hz, 4.7 Hz, 7.6 Hz, 0.2 H), 7.83 (td, *J* = 1.7 Hz, 7.7 Hz, 0.8 H), 7.89 (td, *J* = 1.7 Hz, 7.7 Hz, 0.2 H), 8.04-8.08 (m, 1 H), 8.66-8.69 (m, 1 H). ^{13}C NMR δ 25.0, 26.1, 29.3, 33.4, 35.1, 47.5, 60.4, 122.7, 125.8, 127.1, 128.3, 128.6, 136.9, 141.9, 148.8, 152.3, 171.0, 198.6. Anal. ($C_{20}H_{22}N_2O_2$) CHN. ESI-MS *m/z* 323.2 $[M + H]^+$.

1-(4-Phenylbutanoyl)-2(*S*)-(thiophene-2-carbonyl)pyrrolidine (6d). Prepared according to method D from **5d** (270 mg, 1.0 mmol) having a 20 h reaction time and using 2.5 eq. of EDC and HOBt. The product was purified by flash chromatography (15-50% EtOAc in PE). Yield 155 mg, 47%. ^{1}H NMR δ 1.88-2.15 (m, 5.3 H), 2.22-2.44 (m, 2.7 H), 2.52-2.63 (m, 0.3 H), 2.69 (t, *J* = 7.5 Hz, 1.7 H), 3.46-3.51 (m, 0.85 H), 3.63-3.69 (m, 1 H), 3.73-3.77 (m, 0.15 H), 4.93 (dd, *J* = 3.2 Hz, 9.1 Hz, 0.15 H), 5.32 (dd, *J* = 3.6 Hz, 9.0 Hz, 0.85 H), 7.07-7.21 (m, 4 H), 7.26-7.29 (m, 2 H), 7.64 (dd, *J* = 0.9 Hz, 5.0 Hz, 0.85 H), 7.65-7.66 (m, 0.15 H), 7.72 (dd, *J* = 0.9 Hz, 5.0 Hz, 0.15 H), 7.83 (dd, *J* = 0.9 Hz, 3.8 Hz, 0.85 H). ^{13}C NMR δ 24.8, 26.0, 29.6, 33.3, 35.1, 47.2, 61.8, 125.8, 128.1, 128.3, 128.6, 132.3, 133.8, 141.7, 141.8, 171.4, 191.1. Anal. ($C_{19}H_{21}NO_2S \cdot 0.8\ H_2O$) CHN. ESI-MS *m/z* 328.1 $[M + H]^+$.

2(*S*)-(Furan-2-carbonyl)-1-(4-phenylbutanoyl)pyrrolidine (6e). Prepared according to method D from **5e** (100 mg, 0.37 mmol) having a 20 h reaction time. The product was purified by flash chromatography (27% EtOAc in PE). Yield 42 mg, 36%. ^{1}H NMR δ 1.86-2.12 (m, 5.4 H), 2.17-2.40 (m, 2.6 H), 2.53-2.64 (m, 0.3 H), 2.69 (t, *J* = 7.5 Hz, 1.7 H), 3.45-3.50 (m, 0.85 H), 3.61-3.67 (m, 1 H), 3.71-3.76 (m, 0.15 H), 4.97 (dd, *J* = 3.0 Hz, 9.2 Hz, 0.15 H), 5.30 (dd, *J* = 3.9 Hz, 8.8 Hz, 0.85 H), 6.54 (dd, *J* = 1.6 Hz, 3.6 Hz, 0.85 H), 6.59 (dd, *J* = 1.6 Hz, 3.5 Hz, 0.15 H), 7.09-7.22 (m, 3.7 H), 7.26-7.29 (m, 2.3 H), 7.59 (d, *J* = 1.6 Hz, 0.85 H), 7.63 (d, *J* = 1.6 Hz, 0.15 H). ^{13}C NMR δ 24.8, 26.0, 29.1, 33.3, 35.1, 47.2, 60.9, 112.3, 117.9, 125.7, 128.2, 128.6, 141.9, 146.5, 151.4, 171.3, 187.1. Anal. ($C_{19}H_{21}NO_3 \cdot 0.1\ H_2O$) CHN. ESI-MS *m/z* 312.1 $[M + H]^+$.

Method E. 1-Boc-L-proline benzylamide (7a). To a solution of Boc-L-proline (430 mg, 2.0 mmol) and Et_3N (310 µl, 2.2 mmol) in THF (4 ml) was added ethyl chloroformate (190 µl, 2.0 mmol) in THF (3 ml) dropwise at -15 °C, and the mixture was stirred for 30 min. Benzylamine (440 µl, 4.0 mmol) in THF (1 ml) was added at -15 °C and the mixture was stirred overnight at rt. The mixture was diluted with DCM, washed with 30% citric acid, sat. NaCl aq. and sat. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated. Yield 0.55 g, 90%.

1-Boc-D-proline benzylamide (7b). Prepared according to method E from Boc-D-proline (900 mg, 4.2 mmol). At the end of the reaction, the mixture was diluted with EtOAc. Yield 1.08 g, 84%.

1-Boc-L-proline phenethyl amide (7c). Prepared according to method E from Boc-L-proline (250 mg, 2.5 mmol) and phenethylamine (0.63 ml, 5.0 mmol). At the end of the reaction, the mixture was diluted with EtOAc. Yield 0.80 g, 100%.

Method F. 1-(Cyclopent-1-enecarbonyl)-L-proline benzylamide (8a). STEP 1. To a solution of **7a** (550 mg, 1.8 mmol) in DCM (4 ml) was added TFA (3.6 ml, 48 mmol) in DCM (4 ml) dropwise at 0 °C. The mixture was stirred for 1 h at 0 °C and evaporated. **STEP 2.** To a solution of cyclopent-1-enecarboxylic acid (200 mg, 1.8 mmol) and Et_3N (0.28 ml, 2.0 mmol) in DCM (4 ml) was added trimethylacetyl chloride (0.22 ml, 1.8 mmol) in DCM (4 ml) dropwise at 0 °C. The mixture was stirred for 1 h at 0 °C and the ice bath was removed. Et_3N (0.82 ml, 5.9 mmol) and the product of STEP 1 in DCM (4 ml) were added in this order and the mixture was stirred for 2 h. The mixture was washed with 30% citric acid, sat. NaCl aq. and sat. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated. The product was purified by flash chromatography (40-80% EtOAc in PE). Yield 380 mg, 71%. ^{1}H NMR δ 1.85-1.98 (m, 4 H), 2.07-2.15 (m, 1 H), 2.45-2.61 (m, 4 H), 2.66-2.74 (m, 1 H), 3.56-3.65 (m, 2 H), 4.39 (dd, *J* = 5.0

Hz, 15.1 Hz, 1 H), 4.48 (dd, *J* = 5.7 Hz, 15.1 Hz, 1 H), 4.70-4.74 (m, 1 H), 6.15-6.18 (m, 1 H), 7.24-7.32 (m, 5 H), 7.39-7.45 (m, 1 H). ^{13}C NMR δ 22.7, 25.4, 26.8, 33.7, 33.9, 43.4, 49.2, 59.9, 127.2, 127.4, 128.6, 136.3, 138.5, 139.1, 168.7, 171.2. Anal. ($C_{18}H_{22}N_2O_2$) CHN. ESI-MS *m/z* 299.0 $[M + H]^+$.

1-(Cyclopent-1-enecarbonyl)-D-proline benzylamide (8b). Prepared according to method F from **7b** (300 mg, 1.0 mmol). The product was purified by flash chromatography (50-60% EtOAc in hexane). Yield 180 mg, 60%. ^{1}H NMR δ 1.85-1.98 (m, 4 H), 2.07-2.15 (m, 1 H), 2.45-2.61 (m, 4 H), 2.66-2.74 (m, 1 H), 3.56-3.65 (m, 2 H), 4.39 (dd, *J* = 5.2 Hz, 15.1 Hz, 1 H), 4.48 (dd, *J* = 5.9 Hz, 15.1 Hz, 1 H), 4.70-4.74 (m, 1 H), 6.15-6.18 (m, 1 H), 7.24-7.32 (m, 5 H), 7.39-7.45 (m, 1 H). ^{13}C NMR δ 22.7, 25.4, 26.8, 33.7, 33.9, 43.4, 49.2, 59.9, 127.2, 127.4, 128.6, 136.3, 138.5, 139.1, 168.7, 171.2. Anal. ($C_{18}H_{22}N_2O_2$) CHN. ESI-MS *m/z* 299.0 $[M + H]^+$.

1-(Cyclopent-1-enecarbonyl)-L-proline phenethyl amide (8c). Prepared according to method F from **7c** (0.40 g, 1.25 mmol). The product was purified by flash chromatography (50% EtOAc in PE). Yield 330 mg, 85%. ^{1}H NMR δ 1.82-2.05 (m, 5 H), 2.33-2.73 (m, 5 H), 2.75-2.85 (m, 2 H), 3.44-3.66 (m, 4 H), 4.38-4.45 (m, 0.2 H), 4.60-4.65 (m, 0.8 H), 5.87-5.96 (m, 0.2 H), 6.11-6.15 (m, 0.8 H), 6.19-6.29 (m, 0.2 H), 7.00-7.07 (m, 0.8 H), 7.17-7.21 (m, 3 H), 7.25-7.28 (m, 2 H). ^{13}C NMR δ 22.7, 25.3, 27.1, 33.7, 33.8, 35.7, 40.6, 49.2, 60.0, 126.3, 128.4, 128.8, 136.4, 139.1, 139.1, 168.3, 171.3. Anal. ($C_{19}H_{24}N_2O_2$) CHN. ESI-MS *m/z* 313.0 $[M + H]^+$.

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9 GENERAL DISCUSSION

9.1 Modifications at the P3 position and beyond it

A set of POP inhibitors were developed from the previously published larger POP inhibitors (Figure 1). The potencies of the novel compounds did not significantly differ from the potencies of the larger compounds. The common feature of these inhibitors was that they did not have the typical aryl-alkanoyl moiety at the P3 site and the CoMSIA model was created in order to provide information about the binding mode of these inhibitors. Two binding modes, which differed from each other beyond the P3 position, were found. Binding mode A favored lipophilic structures beyond the P3 position and the studied reference compounds occupied this binding mode. On the other hand, binding mode B favored hydrophilic structures beyond the P3 position and was occupied by 40% of the studied inhibitors. Binding mode B has not been described in the literature.

Many POP inhibitors possess a phenyl group at the P3 position. In order to introduce an ionizable group into the POP inhibitor structure, the phenyl group was replaced by a pyridyl group. It was found that the pyridyl moiety gives compounds that are equipotent with their phenyl analogs. Both groups are six-membered aromatic structures but the pyridyl group is more polar and it caused a 20-fold decrease in partition coefficient. The introduction of a polar group is not necessarily a straightforward procedure. At the P1 position, the replacement of the phenyl group by the 2-pyridyl group caused an over 20-fold decrease in potency. Furthermore, the replacement of the azepane or the pyrrolidine moiety by the morpholine moiety decreased the inhibitory activity significantly (Figure 2). The optimal chain length for the pyridyl group reflected the optimal chain length for the phenyl group. This indicates that both groups may bind in a similar manner.

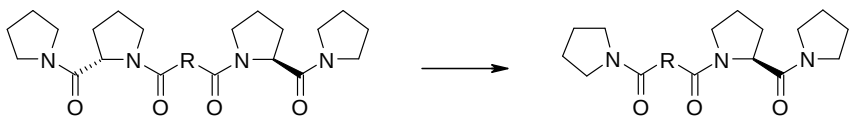
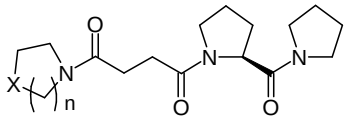


Figure 1. Design of the POP inhibitors with an atypical P3 and beyond structures.



1; X=C, n=1, IC_{50} = 110 nM
2; X=C, n=3, IC_{50} = 41 nM
3; X=O, n=2, IC_{50} = 1200 nM

Figure 2. The decreased potency caused by the polar morpholine group.

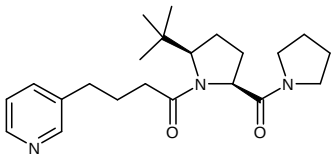


Figure 3. The combination of the 5-*tert*-butyl-L-proline at the P2 position and the 3-pyridyl group at the P3 position gave a potent POP inhibitor with excellent water-solubility and theoretically optimal lipophilicity.

However, according to the CoMSIA model, the P3 site favors the alkyl groups. When the chain of either the pyridyl- or the phenyl-containing compound is shortened, the aromatic ring is brought to this area. Thus, it is also possible that the pyridyl group is positioned into a different area than the phenyl group.

The P3 pyridyl group was studied with two different proline mimetics, the 5-*tert*-butyl-L-prolyl and the cyclopent-1-enecarbonyl groups, at the P2 position. When the pyridyl group was combined with the 5-*tert*-butyl-L-prolyl moiety (Figure 3), the theoretical optimum for lipophilicity was reached. It was also shown that both proline mimetics decreased water-solubility but the introduction of the pyridyl group overcame this decrease. The reference compound SUAM-1221 had also an excellent water-solubility but it is an oily compound, which makes it difficult to handle. The introduced pyridyl group enables salt formation and a solid form can be obtained.

9.2 The amide bond replacements

In the typical POP inhibitor structure, there are pyrrolidine rings at the P2 and P1 positions and the pyrrolidine nitrogens are both involved in the amide bonds. The carbonyl oxygens of these amide bonds form hydrogen bonds with the enzyme and are important for the POP inhibitory activity. Instead, the amide nitrogens do not form hydrogen bonds with POP and the replacements of the P2 and P1 pyrrolidine rings by different carbon rings have been described in the literature. However, these replacements have led to decreased POP inhibitory activities.

The relative potencies of different P1 and P2 pyrrolidine replacements are presented in Figure 4. Amide nitrogens are sp^2 hybridized and amide bonds are planar. Instead, an sp^3 hybridized carbon is tetrahedral and is not able to well mimic the amide nitrogen. This change in geometry can explain the decreased potency of POP inhibitors possessing a cyclopentane ring at the P2 or P1 positions (the data for the P2 position is not from our assay). When the cyclopentane ring with the sp^3 hybridized carbon was

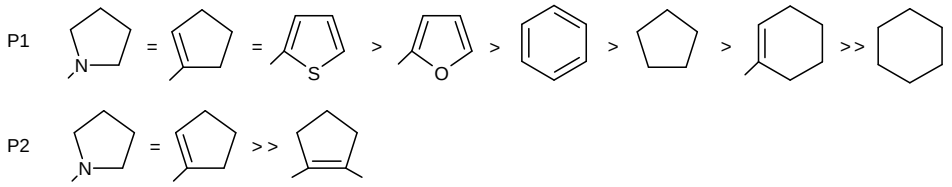


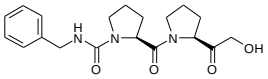
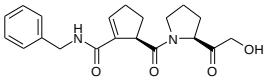
Figure 4. The relative potencies of different P1 and P2 pyrrolidine replacements of the POP inhibitor structure.

replaced by a cyclopentene ring with an sp^2 hybridized carbon, the potencies were increased back to the level of the reference compounds. However, the poor POP inhibitory activity caused by the 1-cyclopentene ring at the P2 position cannot be explained by the wrong geometry at that carbon, which mimics the amide nitrogen. The more probable explanation is the changed hybridization of the neighbouring carbon because it has lost the L-proline mimicking configuration. The relatively poor inhibitory activity of the phenyl group at the P1 position reflects only the fact that the S1 pocket of POP is specific for the 5-membered ring and indeed, the smaller aromatic rings turned out to be one order of magnitude more potent. However, the phenyl group was slightly more potent than the cyclopentyl group and the 1-cyclohexenyl group was more potent than the cyclohexyl group confirming the importance of the sp^2 hybridization.

The beneficial π-stacking with Trp595 was suggested to play some role in the relative potencies of the pyrrolidine replacements at the P1 position. In the literature, there are examples where the replacement of the P1 pyrrolidine ring by a 2,5-dihydropyrrole ring slightly increases inhibitory activity. However, in the present study the aromatic 2-thienyl and 2-furyl groups did not have any beneficial π-stacking effect over the 1-cyclopentene group. It is possible that the increased amount of π-orbitals improves potency but the effect is much smaller than the effect of the hybridization.

The studied amide bond replacements can be used to modify different properties of the compounds. They make the compounds less peptide-like and the replacement of the amide nitrogen by a carbon atom also increases lipophilicity, as was shown with the cyclopent-2-enecarbonyl group at the P2 position. Furthermore, when the P2 L-prolyl residue was changed to the cyclopent-2-enecarbonyl group, it was found that the dissociation half-life of the enzyme-inhibitor complex was decreased (Table 1, unpublished results by Dr. Jarkko Venäläinen). The 5-*tert*-butyl-L-prolyl group has previously been shown to increase the dissociation half-life of the enzyme-inhibitor complex.

Table 1. The effect of the replacement of the P2 L-prolyl group of JTP-4819 by the cyclopent-2-enecarbonyl group on the enzyme-inhibitor binding kinetics.[a]

Structure	K_i (nM)	K_{on} ($M^{-1}s^{-1}$)	K_{off} (s^{-1})	EI $t_{1/2}$ (h)
	0.043	5.59×10^5	2.41×10^{-5}	7.99
	0.074	7.11×10^5	5.27×10^{-5}	3.65

[a] Published with the permission of Dr. Jarkko Venäläinen.

The α,β-unsaturated carbonyl groups are able to undergo Michael addition and are normally avoided in the drug design. The α,β-unsaturated amides may be less reactive than the α,β-unsaturated ketones because of the electron-donating effect of the amide nitrogen. However, this remains to be clarified and it is possible that the further protection of the double bond is needed. An obvious way to provide the further protection would be the substitution of the double bond. An alkyl substituent would most probably be allowed at the P2 position, where the 5-alkyl-L-prolines have been shown to give good inhibitory activities. At the P1 position, the substitution may not be allowed because of the limited space in the S1 pocket. However, the aryl-conjugated carbonyl group should be more stable than the α,β-unsaturated ketone group and provides a useful alternative for the P1 position.

9.3 Conclusions

Novel groups that were able to mimic their parent structures were found for all studied positions. It is concluded that:

1. The P3 position and the positions beyond it give more freedom to drug design than the P1 and P2 positions, and this freedom should be exploited when lead structures are optimized.
2. The amide nitrogens of the P3-P2 and P2-P1 amide bonds of the POP inhibitor structure can be replaced by the sp^2 hybridized carbons and still maintain an excellent POP inhibitory activity.
3. The physico-chemical properties were determined for the selected compounds and it was shown that the studied groups can be used to modify these properties.

9.4 Future perspectives

The research and the development of prolyl oligopeptidase inhibitors have produced a wide variety of extremely potent compounds. Many of these compounds are based on the *N*-acyl-L-Pro-pyrrolidine structure, which have been shown to be highly specific for prolyl oligopeptidase. Furthermore, many compounds based on this structure have undergone pharmacological or clinical studies and have been well-tolerated. However, the physiological role of POP has remained unclear although POP inhibitors have repeatedly been shown to enhance memory in different animal models.

The research should now be focused on the clarification of the physiological role of POP and to get the proof-of-concept of the memory-enhancing (or other) effects of POP inhibitors, possibly in long-term studies. The already developed inhibitors provide good pharmacological tools to perform these studies. The *N*-acyl-L-Pro-pyrrolidine structure and its mimetics have proven to give so potent and specific POP inhibitors that the search for a different kind of lead structures does not seem necessary. The next step in the development of POP inhibitors should be the optimization of the ADMET properties, at the same time or after the proof-of-concept.

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