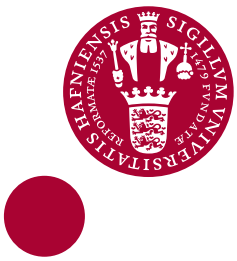

Design, synthesis and molecular pharmacological characterization of novel peptide analogs of human peptide YY (PYY)

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FACULTY OF LIFE SCIENCES
UNIVERSITY OF COPENHAGEN

Design, synthesis and molecular pharmacological characterization of novel peptide analogs of human peptide YY (PYY)

Ph.D. Thesis

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IGM, Section of Bioorganic Chemistry

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Ph.D. Thesis

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Abstract

The prevalence of obesity has illuminated the demand for safer and more efficacious anti-obesity therapeutics. Gut hormones, such as PYY3-36, has been shown to play a role in the control of body energy homeostasis. However, in general gut hormones have poor metabolic stability and some have moderate selectivity, thus novel analogs needed.

The present thesis describes the design, synthesis, and characterization of several novel PYY3-36 analogs. The ligand design was based on a recently presented hypothesis claiming that the PP-fold family of peptides (PYY, NPY and PP) associates with the membrane prior to receptor activation. Four different design strategies were chosen: *i*) Modifications of the *C*-terminal Tyr-36 of the receptor-recognizing pentapeptide of PYY3-36 to increase the selectivity towards the Y2 receptor. *ii*) Glyco-scan using human PYY3-36 as a model peptide - to investigate the impact of glycosylation on affinity, selectivity, and stability. *iii*) Branching the PYY3-36 sequence at the turn motif to create more selective agonists. *iv*) Introduction of an additional amphipathic α-helix either at the end of the *N*-terminus as a branch or instead of the *N*-terminal, to increase the affinity towards the membrane and thereby the Y receptor.

The results demonstrate numerous novel PYY3-36 analogs with increased selectivity. Successfully, several ligands were found to improve or maintain sub-nanomolar affinity towards the Y2 receptor. Interesting analogs were further investigated in acute or chronic mouse models of obesity and showed to reduce body-weight and food intake in comparison to PYY3-36.

The work conducted during this thesis has increased the understanding of the ligand-receptor interaction as well as further confirmed the hypothesis that the PP-fold peptides recognizes their target-receptors via a membrane-bound intermediate.

Resumé (In Danish)

Den globale fedme epidemi understreger behovet for ikke mindst bedre forebyggelse, men også udviklingen af nye sikre og effektive lægemidler til behandlingen af overvægt. Tarmhormoner, såsom PYY3-36, har vist sig at spille en rolle i kontrollen af kropsvægt samt i energibalancen, men flere af tarmhormonerne er dårlige lægemidler pga. ringe metabolisk stabilitet og/eller moderat selektivitet overfor forskellige receptorer. Studier i både gnavere og mennesker har påvist, at PYY3-36 virker appetitnedsættende via Y2 receptoren, og at denne receptor kan være et relevant farmaceutisk target. I den nærværende Ph.D.-afhandling beskrives designet, syntesen og karakteriseringen af 4 nye serier af PYY3-36 analoger. Designet af PYY3-36 analogerne blev inspireret af en nyligt frembragt hypotese, der erklærer, at 'PP-fold' peptiderne (PYY, NPY og PP) binder til membranen, inden de interagerer med deres specifikke receptorer. Der blev fokuseret på fire forskellige designstrategier: *i*) Modificering af den *C*-terminal Tyr-36, som er en del af det receptorgenkendende pentapeptid af PYY3-36, for at øge selektiviteten mod Y2 receptoren. *ii*) 'Glyco-scan' med PYY3-36 som modelpeptid - for at undersøge indvirkningen af glykosylering på affiniteten, selektiviteten og stabiliteten. *iii*) Forgrening af PYY3-36 sekvensen i turn regionen for at skabe en mere selektiv agonist. *iv*) Introduktion af en ekstra amfipatisk α-helix; a) som en forgrening i enden eller b) i stedet for *N*-terminalen for at øge affiniteten til membranen, og derved også til Y receptorerne.

Resultaterne viser adskillige nye PYY3-36 analoger med øget selektivitet. Flere ligander opretholder PYY3-36's sub-nanomolære affinitet mod Y2 receptoren, og nogle udviser endda øget affinitet og aktivitet. De mest interessante af disse analoger blev endvidere undersøgt i musemodeller for fedme, både akut og kronisk, og det viste sig, at stofferne reducerede både kropsvægten og fødeindtaget i samme grad som PYY3-36.

Arbejdet udført i løbet af dette projekt har ført til øget forståelse af interaktionen mellem ligand og receptor, samt understøttet den hypotese, at PP-fold peptiderne genkender deres receptor via et membranbundet intermediat.

Preface

The present thesis is the result of work carried out during my Ph.D. studies, in the period from December 2006 till December 2009, in collaboration with Rheoscience A/S and the Faculty of Life Sciences, University of Copenhagen. The Ph.D.-project was funded by the Ministry of Science, Technology and Innovation, and conducted under the supervision of Prof. Knud J. Jensen, University of Copenhagen and Dr. Niels Vrang, Rheoscience A/S (now at gubra ApS).

First, I would like to thank Prof. Knud J. Jensen for his strong commitment as well as encouragement. I am very grateful for his help and support. Also Dr. Niels Vrang is thanked for his encouragement, loyalty, and valuable feedback – he has been a great mentor both scientifically and businesswise.

Associate Prof. Dr. Birgitte Holst is thanked for conducting the signal transduction assays and for providing Y1 receptor transfected SK-N-MC cells. Dr. Leif Kongskov Larsen, Dr. Jenny Frederiksson, and Anja Daniel Andersen are tanked for their help in setting up the radioligand competition binding assay and for assistance in the cell laboratory. A special thanks to the former management and employees at Rheoscience A/S for their great company and openheartedness.

Moreover, I would like to give a special thanks to Kasper K. Sørensen, Dr. Mikkel Boas Thygesen, and Dr. Kim Larsen for the introduction to the lab, technical assistance and friendship. Additionally, Kasper is thanked for his research contribution to the article describing the semi-automated microwave peptide synthesizer. Furthermore, present and former members of the Knud J. Jensen group are thanked, especially, Dr. Pernille T. Shelton, Leila Malik, Dr. Emiliano Cló, Henrik Munch, and Dr. Masood Hosseini. Moreover, additional members of the Bioorganic Chemistry Section are also thanked.

Finally, I would like to thank my family for being supportive - especially my fiancée Linda.

Søren Ljungberg Pedersen

Copenhagen, December 2009

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Publications

Peer-reviewed articles and manuscripts in preparation

1. Søren L. Pedersen, Birgitte Holst, Niels Vrang and Knud J. Jensen. Modifying the conserved *C*-terminal tyrosine of the peptide hormone PYY3-36 to improve Y2 receptor selectivity, *J. Pept. Sci.*, **2009**, *15*, 11, 753-759.

2. Søren L. Pedersen, Catharina Steentoft, Niels Vrang and Knud J. Jensen. Glyco-scan: Varying glycosylation in the sequence of the peptide hormone PYY3-36 and its effect on receptor selectivity, *ChemBioChem*, **2009**, accepted.

3. Søren L. Pedersen, Pottayil G. Sasikumar, Shekar Chelur, Birgitte Holst, Andreas Artmann, Knud J. Jensen, and Niels Vrang. Re-design of a peptide hormone: *N*-terminally branched PYY3-36 isoforms give improved lipid and fat cell metabolism in diet-induced obese mice, *in manuscript* (Organic and Biomolecular Chemistry).

4. Søren L. Pedersen, Pottayil G. Sasikumar, Niels Vrang, and Knud J. Jensen. Peptide-architecture: Adding an α-helix to PYY Lys side-chain maintains binding affinity and demonstrate body-weight lowering effects, *ChemMedChem*, submitted.

5. Søren L. Pedersen, Birgitte Holst, Maria H. Pedersen, Niels Vrang and Knud J. Jensen. Unique design concept of novel PYY3-36 analogs: Introducing an amphipathic *de novo* designed α-helix leads to increased Y2 receptor potency and selectivity, *in manuscript*.

6. Søren L. Pedersen, Kasper K. Sørensen and Knud J. Jensen. Semi-automated microwave-assisted SPPS: Optimization of protocols and synthesis of difficult sequences, *Biopolymers*, **2009**, accepted.

Oral presentations

Kasper K. Sørensen, Søren L. Pedersen and Knud J. Jensen. Semi-Automated Solid-Phase Microwave Peptide Synthesis, Biotage Usergroup Meeting **2007**, Copenhagen, Denmark.

Poster presentations

1. Søren L. Pedersen, Pottayil Sasikumar, Niels Vrang and Knud J. Jensen. Novel *N*-terminally modified PYY3-36 analogs: Design, structural analysis, affinity study and *in vivo* evaluation, American Peptide Symposium **2009**, Bloomington, Indiana, USA, *Biopolymers*, **2009**, *92*, 4, 323.

2. Søren L. Pedersen, Kasper K. Sørensen and Knud J. Jensen. Semi-Automated Microwave Assisted Solid-Phase Peptide Synthesis, European Peptide Symposium **2008**, Helsinki, Finland, *J. Pept. Sci. Suppl.*, **2008**, *14*, 8, 145.

Patents

Vrang, Niels; Jensen, Knud Jørgen; Pedersen, Søren Ljungberg; Chelur, Shekar Siddalingaiah; Sasikumar, Pottayil Govindan; Dhakshinamoorthy, Saravanakumar, Ramachandra, Muralidhara; 'Y2 receptor agonists', International application number: PCT/EP2008/067606; International publication number: WO 2009/080608 A1.

Abbreviations

AgRP	agouti gene related transcript
Ahx	6-aminohexanoic acid
ANOVA	analysis of variance
ARC	arcuate nucleus
BMI	body mass index
Boc	*tert*-butoxycarbonyl
cAMP	cyclic adenosine monophosphate
Cbz	benzyloxycarbonyl
CD	circular dichroism
CNS	central nervous system
COMU	1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium hexafluorophosphate
Cy	Cyclohexyl
DAG	diacylglycerol
DCC	dicyclohexylcarbodiimide
DIC	diisopropylcarbodiimide
DIO	diet induced obese
DIPEA	diisopropylethylamine (Hünig's base)
DMF	dimethylformamide
DPC	dodecylphosphocholine
DPP-IV	dipeptidyl peptidase IV
ER	endoplasmatic reticulum

Fmoc	fluoren-9-ylmethyloxycarbonyl
FRET	fluorescence resonance energy transfer
Gal	galactose
GDP	guanosine-diphosphate
Glc	glucose
GLP-1 or 2	glucagon-like peptide-1 or 2
GPCR	G-protein coupled receptor
GTP	guanosine-triphosphate
HATU	*N*-[(dimethylamino)-1*H*-1,2,3-triazole[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HBTU	*N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HF	hydrofluoric acid
HMBA	4-hydroxymethylbenzoic acid
HMPA	4-hydroxymethyl-phenoxyacetic acid
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
IP_1	inositol monophosphate
IP_3	inositol 1,4,5-triphosphate
*i*Pr	isopropyl
LC-MS	liquid chromatography-mass spectrometry
MC	melanocortin
MSH	melanocyte stimulating hormone
NMP	*N*-methyl-2-pyrrolidone
NMR	nuclear magnetic resonance (spectroscopy)
NMRI	Naval Medical Research Institute
NPY	neuropeptide Y

O-BAL	*ortho*-backbone amide linker
Pbf	2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl
PEG	poly(ethylene glycol)
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC-β	phospholipase Cβ
POMC	proopiomelanocortin
PP	pancreatic polypeptide
PVN	paraventricular nucleus
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
PYY	peptide YY, peptide tyrosine tyrosine
RP-HPLC	reverse phase-high-performance liquid chromatography
SEM	standard error of mean
SPPS	solid phase peptide synthesis
*t*Bu	*tert*-butyl
TFA	triflouroacetic acid
Trt	trityl
UV	ultraviolet (spectroscopy)
θ	molar ellipticity

Proteinogenic amino acids; Three and one-letter codes:

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Project aim and outline

The aim of the present Ph.D. project is to use solid-phase peptide synthesis (SPPS) to make novel peptide analogs of the gut hormone peptide YY (PYY), that act as high affinity agonist towards the Y2 receptor. The project focus is on developing novel high-affinity Y2 receptor agonists with improved selectivity (Y2 over Y1) and stability, when compared to the anorectic gut hormone PYY3-36. Recently published data obtained from NMR studies of PYY have shed new light on the binding mechanism and conformation of the PP-fold family of peptides;[1-5] results that are being exploited in the design of new ligands with enhanced affinity for the Y2 receptor and better plasma stability. Finally, the Ph.D.-project aims at delivering lead peptide candidates for future clinical development.

As a side-project the use of a new custom-made microwave-assisted peptide synthesizer has been conducted. The work has resulted in one peer-reviewed article (Appendix 6). The article describes the utilization of the custom-made semi-automated peptide synthesizer and describes a set of optimized protocols for this unique platform. Moreover, the optimized protocols have been used to synthesize long and 'difficult' sequences.

Outline of thesis

Part 1 'Introduction':

Chapter 1: Herein the control and regulation of food intake is described with special focus on the gut-brain axes. Moreover, the hormonal changes subsequent to a gastric bypass operation are described.

Chapter 2: The PP-fold peptide structures interact with plasma membrane prior to receptor recognition, which are exploited in the design of the novel PYY analogs of this project. Besides the membrane compartment model ligand-receptor interactions are also described in this chapter.

Part II ‘Methods’:

Chapter 3: Here a brief presentation of solid-phase peptide synthesis, circular dichroism spectroscopy, binding and functional assays, and finally animal models of obesity are given.

Part III ‘Results and discussion’:

Chapter 4: The rationale for the different designs and the main results for each of the four projects of the thesis are described and discussed in this chapter. The work is moreover described thoroughly in the papers and manuscripts that are located in the appendices.

Chapter 5: Overall conclusion and perspective.

Part IV ‘References’

Appendix 1-5: PYY papers - reprints of one published paper, one accepted article, as well as three papers in manuscript.

Appendix 6: MW article. The manuscript of one paper is given (accepted for publication).

Part I
‘Introduction’

Chapter 1. Obesity: Controlling of energy homeostasis

Obesity (BMI > 30 kg/m^2) is increasing with an alarming rate worldwide, hence intensive research have been invested in understanding the physiological mechanisms underlying human food intake and body-weight regulation. Although our knowledge of appetite and body-weight regulation has increased tremendously over past decades, still only few pharmacological therapies targeting obesity exist.

The health consequences of obesity are many and serious: Insulin resistance and type II diabetes, cardiovascular disease, osteoarthritis and certain types of cancers are just a few of the many diseases strongly associated with obesity. It is generally agreed that today's obesity epidemic is the result of both environment (i.e. increased consumption of energy-dense food combined with a sedentary life style) and inheritance.[6] Several monogenic diseases as well as Mendelian disorders have been identified and characterized in the search for genes involved in the development of obesity. However, individuals affected by mutations causing these diseases only represent a minor fraction of the population and these genes are clearly not sufficient to explain the obesity prevalence of today.[7]

In normal-weight individuals, the cumulative energy intake corresponds precisely to the energy expenditure. Most healthy humans maintain a steady body-weight, although, an increasing number of individuals worldwide, consume more calories than needed for their level of physical activity, leading to an enhanced storage of energy (fat). For several years it has been anticipated that homeostatic regulators, such as peptide hormones, control the energy balance.[8] Despite an array of experiments distracted from the initial report, it took more than 25 years before energy homeostatic molecules were identified. The understanding of physiological processes that control food intake, has grown rapidly the latest two decades; consequently, we are now familiar with numerous circulating molecules to have considerable influence on the control of food intake and energy expenditure. Most of these compounds act on the hypothalamus, the brainstem, or afferent autonomic nerves and primarily come from fat cells, the endocrine pancreas and the gastrointestinal tract (Figure 1.1).

Along with the growing obesity epidemic the past few years has seen a nearly exponential rise in obesity surgeries.[9] One of the most dramatic and efficient types of obesity surgery is the Roux-en-Y gastric bypass whereby gastric volume is drastically reduced and food is

conveyed directly into the upper jejunum.[10] Aside from marked weight-loss this type of gastric bypass surgery often leads to a rapid (usually within days) resolution of type II diabetes in more than 80% of obese type II diabetics.[11] The mechanisms mediating the dramatic weight-loss and resolution of diabetes are unknown. However, changes in gut-brain communication involving both hormones and neural pathways are thought to be the major candidates for these beneficial effects.[12] Nevertheless, it is well known that Roux-en-Y gastric bypass leads to a rise in plasma levels of a number of gut hormones with either anorectic and/or anti-diabetic effects (e.g. PYY3-36, GLP-1, PP).[13]

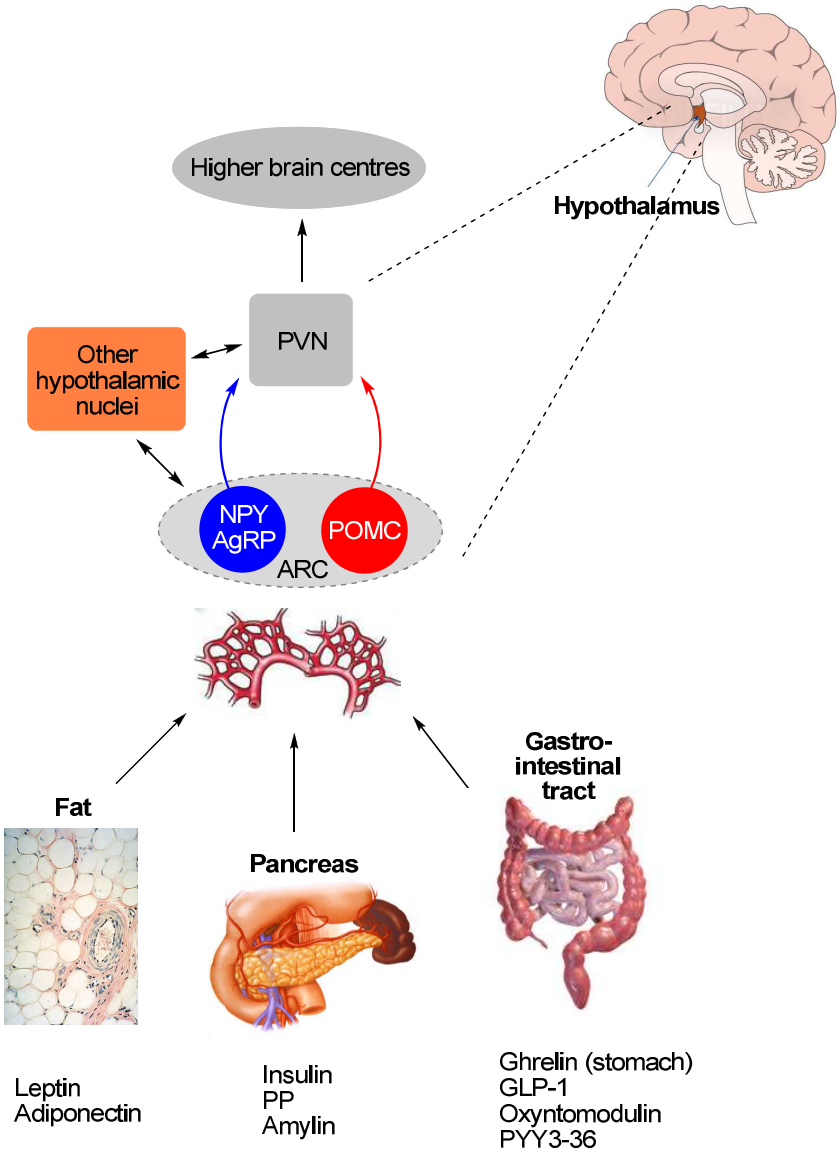


Figure 1.1. *The pathways in which gut hormones regulate energy homeostasis.*

1.1 Gut-brain interactions

The brain regions regulating energy homeostasis receives neural, metabolic and humoral signals from the stores of body-energy, thus determining the levels of intake and expenditure. Pivotal studies on the arcuate nucleus (ARC) of the hypothalamus have led to identification of the actual regions involved in energy homeostasis.[14-18] The neurons of the ARC are anatomically placed in close proximity to capillaries at the base of the hypothalamus, giving them access to humoral signals that otherwise are restricted to the periphery due to the blood brain barrier.[19,20] Two sets of neurons, with opposite effect on feeding, have been identified in the ARC. The neurons that express proopiomelanocortin (POMC) are anorexigenic, and the neuropeptide Y (NPY) and agouti gene related transcript (AgRP) are orexigenic (Figure 1.1 and 1.2).

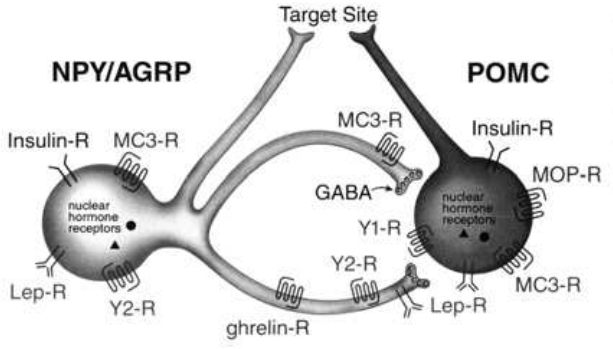


Figure 1.2. *Schematic drawing of the ARC and the sites of various homeostatic receptors.[20] Abbreviations: Lep-R, leptin receptor; insulin-R, insulin receptor; MC3-R, melanocortin-3 receptor; Y1-R, Y2-R, neuropeptide Y1 or Y2 receptors; MOP-R, m-opioid receptor.*

The cleavage products of the POMC precursor, α- and β-melanocyte stimulating hormones (α- and β-MSH), are the main reason for the anorexigenic effect of POMC, and act on the melanocortin receptor subtype 3 and 4 (MC3-R and MC4-R).[21-23] NPY and AgRP potently stimulate food intake:[24] NPY, via the Y1 receptor and AgRP, acts as a natural antagonist of the MC3 and MC4 receptors, thereby blocking for α- and β-MSH (Figure 1.2).[24,25]

1.1.1 Neuropeptide Y

Neuropeptide Y (NPY) is one of the most abundant and potent orexigenic[26] peptides in the hypothalamus.[27] The ARC of the hypothalamus is the major site of expression for NPY, and the most notable effect is the stimulation of feeding (Figure 1.2). NPY synthesis and secretion are up-regulated during energy deficiency and metabolic demand, such as starvation, insulin-dependent diabetes mellitus and exercise.[28] Thus, the primarily physiological role of the ARC NPY neurons probably is to restore normal energy balance and body-fat stores under conditions of energy deficit.

1.1.2 Glucagon-like peptide-1

Glucagon-like peptide-1 (GLP-1) is a product of the preproglucagon precursor molecule and is released postprandially. Besides GLP-1, preproglucagon is processed to glucagon-like peptide-2 (GLP-2) and oxyntomodulin. GLP-1 is metabolized to several forms, but the most abundant circulating form is the *N*-terminal truncated analog which is cleaved between residue 6 and 7.[29] Administration of GLP-1 as well as analogs thereof, stimulates insulin release and inhibits appetite.[30-35]

1.1.3 Pancreatic polypeptide

Pancreatic polypeptide (PP) is a member of the PP-fold family of peptides,[36] and is secreted from F-cells in the pancreas (Figure 1.1).[37] PP release reflects the calorie content of a meal and is a strong and selective agonist towards the Y4 receptor leading to a reduction in appetite. The anorectic effects may partly result from a delayed gastric emptying.[38,39] Repeated peripheral administration of PP in rodents reduced food intake[40,41] and intravenous infusion of PP in normal weight human subjects resulted in a 25% reduction in food intake.[38]

1.1.4 Peptide YY

Peptide YY in full length (PYY1-36) is co-secreted with the preproglucagon derived hormones (GLP-1, GLP-2 and oxyntomodulin) from L-cells lining the gut.[42] PYY1-36 is released postprandially, and subsequently processed by the ubiquitously expressed enzyme, dipeptidyl peptidase IV (DPP-IV) to yield PYY3-36.[43,44] PYY3-36 is the major circulating form of PYY in both the fed and fasted state.[45,46] Following a meal PYY3-36 concentrations start to increase after 15 minutes and peaks after 1-2 hours and stay elevated for several hours thereafter.[42] Peak concentrations reflect the calorific content of the meal.[42,45,47] PYY3-36 is a potent agonist for the Y2 receptor, a member of the so-called Y receptors,[48] which also bind NPY and PP. The Y2 receptor is located throughout the central nervous system (CNS),[49-51] and substantial evidence suggests that PYY3-36 exerts its effects on feeding via the ARC of the hypothalamus (Figure 1.1 and 1.2).[49,52] However, PYY3-36 may also indirectly influence hypothalamic control of feeding via the brainstem and vagal afferents.[51] Radiolabled PYY3-36 has been reported to accumulate in around the brainstem and the hypothalamus.[53] Vagotomy (removing some of the vagus nerve) has been shown to reduced the anorectic effects of PYY3-36.[51,54] Moreover, PYY1-36 and PYY3-36 exert a number of actions on the gastrointestinal function, including inhibition of pancreatic exocrine secretion,[55-58] inhibition of the gastrointestinal motility,[59-62] inhibition of the gastric acid secretion,[63,64] and stimulation of the gallbladder contraction[65].

Batterham *et al*. initially showed that peripheral administration of PYY3-36 reduced food intake in rodents.[49] Although Tschop *et al*. questioned the initial findings of Batterham and

co-workers,[66] subsequent reports from numerous independent groups have reproduced the initial results found by Batterham and co-workers and further confirmed the anorectic properties of PYY3-36,[44,52,54,67-75] e.g. in male DIO C57BL/6J mice and DIO male Sprague-Dawley rats (Figure 1.3)[75].

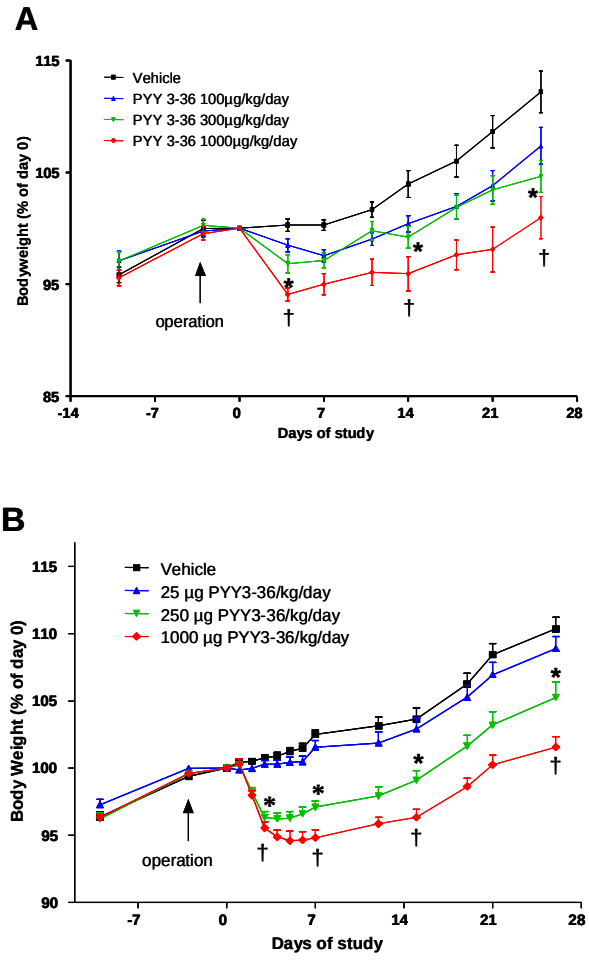


Figure 1.3. *Body-weight in male DIO C57BL/6J mice (A) and male DIO Sprague-Dawley rats (B) administrated subcutaneously by minipump.*[75]

PYY3-36 has also been reported to reduce feeding in obese and normal-weight humans at physiological concentrations.[76-79] Intravenous administration of PYY3-36 for 90 minutes to healthy and obese humans has been shown to reduce appetite and caloric intake,[76] further indicating a possible therapeutic role of PYY3-36 in appetite and weight control (Figure 1.4).[76,77] Given that obese retain their PYY3-36 sensitivity; PYY3-36 has potential as a therapeutic for the treatment of obesity.

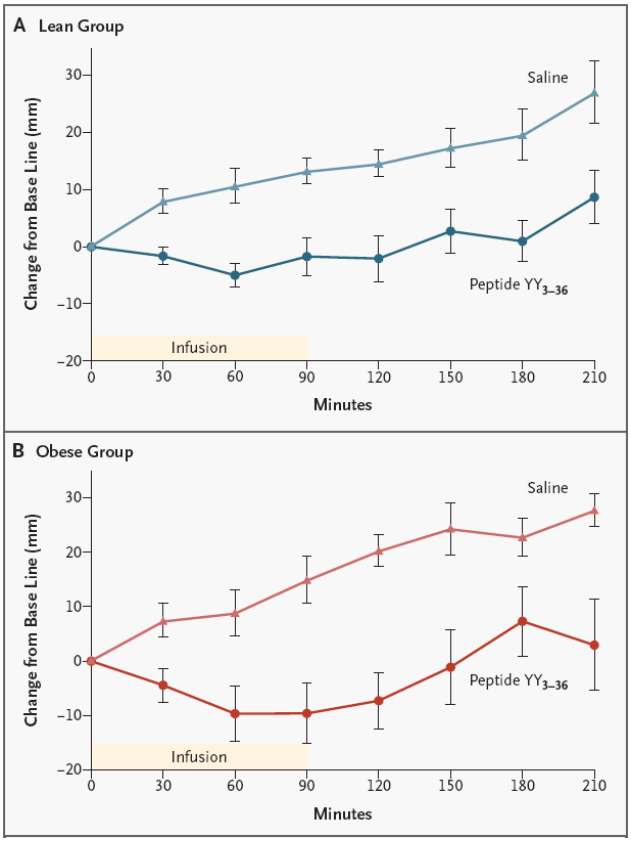


Figure 1.4. *Appetite scores for lean and obese humans.*[76]

Despite the anorectic properties of peripherally administrated PYY3-36, it suffers from low metabolic stability as well as being a non-selective Y receptor agonist engaging both the Y1 and Y2 receptor subtypes.[80] The half-life of intravenous administration of PYY3-36 in mice[81] and rabbits[82] has been reported to 13 and 19 minutes, respectively. Thus, longer-acting and metabolic stable as well as highly selective Y2 agonists are therefore on demand.

1.1.5 The Y receptors

The PP-fold family of peptides (PYY, NPY and PP)[36] binds to Y receptors, a G-protein-coupled receptor (GPCR) family that belong to the rhodopsin-like superfamily (class A) of receptors.[48] Four Y receptor subtypes have been cloned from human tissue, the Y1, Y2, Y4 and Y5 receptors,[48] furthermore, an additional subtype, the y6 receptor, has been cloned, but is not expressed in mammal tissue.[83] The receptors all couple to inhibitory G-proteins (G_i) thus mediating the inhibition of cyclic adenosine monophosphate synthesis.[48] The Y receptor subtypes all differ in their potency from the PP-fold peptides (NPY, PP and PYY1-36 as well as PYY3-36) (Table 1.1).

The *N*-terminal domain of the Y receptors (*N*-Y) is located in the extracellular space and is potential interaction sites for the ligands. It has recently been suggested that PP interacts

with the *N*-Y4 receptor[84] and later studies has indicated that all the peptides of the PP-fold family interact with the *N*-terminal region the Y receptors, however, much weaker than PP to the *N*-Y4.[85] These *N*-terminal interactions may help to guide and engage the ligands onto the rigid Y receptor prior to activation.[84,85]

The Y1 receptor is mainly expressed in the hypothalamus,[86] but also in adipose tissue[87] and vascular smooth muscle cells[88] leading to effects on food intake and vasoconstriction. Full length of NPY or PYY are essential for Y1 receptor activity, which has been shown by *N*-terminal truncated analogs of NPY or PYY that leads to 1000-fold decreases in affinity.[80]

The Y2 receptor is predominantly expressed in the hypothalamus,[49] but also in adipose tissue,[89] and the digestive tract[63] leading to effects primarily on food intake. The Y2 receptor has high affinity for both NPY as well as PYY1-36 (Table 1.1), and, in contrast to the Y1 receptor, also to the *N*-terminal truncated analogs, such as NPY3-36, PYY3-36, NPY13-36, PYY13-36, and even NPY18-36 and NPY22-36 have resulted in nanomolar affinities towards the Y2 receptor.[80]

Table 1.1. The PP-fold peptides affinity (K_i) towards the Y receptor subtypes.

	Y1 [nM]	Y2 [nM]	Y4 [nM]	Y5 [nM]
PYY1-36	1.16 ± 0.26	0.012 ± 0.001	1.057 ± 0.068	0.62 ± 0.04
NPY	0.81 ± 0.12	0.016 ± 0.001	1.900 ± 0.003	0.19 ± 0.05
PP	48.9 ± 27.5	>1000	0.008 ± 0.001	7.25 ± 1.98

Determined by radioligand competition binding assay.[90] Tracers; Y1R and Y5R: ^{125}I-NPY, Y2R: ^{125}I-PYY, and Y4R: ^{125}I-PP.

The Y4 receptor is primarily expressed in the gastrointestinal tract, but is also found in the heart and in the hypothalamus.[91] The Y4 receptor has a high sequence homology with the Y1 receptor, 46%, and less with the Y2 and Y5 receptors, 33% and 23% respectively. The Y4 receptor is also referred to as the PP-preferring receptor due to high selectivity of PP over NPY and PYY (Table 1.1). The Y4 receptor is primarily involved in the regulation of food intake and motility of the gastrointestinal tract.

The Y5 receptor is predominantly expressed in the CNS and rarely in the peripheral tissues.[92,93] The Y5 receptor has a high affinity to NPY and PYY1-36, but not PP (Table 1.1). In contrast to the Y1 receptor, the *N*-terminal truncated analogs, NPY2-36 and NPY3-36, maintained their high potency towards the Y5 receptor.[91]

Chapter 2. PP-fold family of peptides

2.1 Sequences and secondary structures of PYY, NPY and PP

There is a high degree of homology between the sequences of the PP-fold peptides.[36] They all contain 36 amino acid residues, are *C*-terminally amidated, and have a high number of tyrosine, arginine and proline residues. The sequence identity between NPY and PYY is 69% and between PP and PYY it is 50% (Table 2.1).[36]

Table 2.1. Sequences of human PYY1-36, PYY3-36, NPY and PP.

PYY1-36	H-YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2
PYY3-36	H-IKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2
NPY	H-YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-NH_2
PP	H-APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-NH_2

Conserved residues are underlined.[36]

In 1981, Blundell reported the crystal structure of avian pancreatic polypeptide (aPP),[94] and showed that PP consists of an amphipathic α-helical segment between residues 14-31, a *N*-terminal type II polyproline helix comprising residues 1-8, connected by a spacer and a β-turn, and finally, a flexible *C*-terminal pentapeptide. The amphipathic α-helix was shown to be back-folded onto the polyproline *N*-terminus to generate a stable well-packed hydrophobic core. It is this back-folded structure of PP that has led to the general term, the PP-fold. The X-ray structure also revealed that PP is able to form antiparallel dimers.[94] Both the PP-fold structure and the dimer-formation have subsequently been confirmed by NMR studies of PP in solution.[95-98] The X-ray structure and mutational scanning experiments of PP have revealed three stabilizing interactions at the PP-PP dimer interface (Figure 2.1.A): *i*) An intermolecular π-stacking between each Tyr-7 side-chain (Figure 2.1.B), *ii*) an intramolecular edge-to-face interaction with Phe-20 (Figure 2.1.B), and *iii*) an intermolecular π-stacking between each Tyr-21 (Figure 2.1.C).[98]

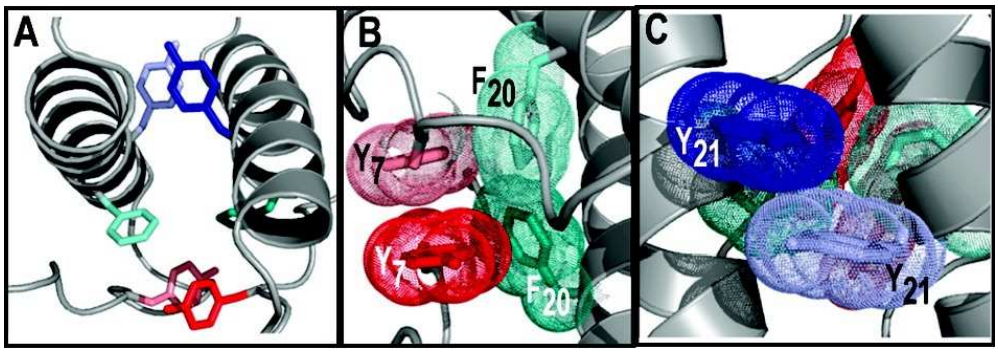


Figure 2.1. *Ribbon diagram of the PP dimer.[94] (A) Highlighting: Tyr-7 side chain pair: red and pink, Phe-20 side chain pair: teal and light teal, and Tyr-21 side chain pair: blue and light blue. (B) Close-up of the inter- and intramolecular interactions of Tyr-7 and Phe-20. (C) Close-up on the intermolecular interaction of Tyr-21.[98]*

Unlike PP, PYY1-36 does not self-associate into dimers, however, NMR analysis has revealed that both PP and PYY1-36 have a hydrophobic core due to back-folding.[4,99,100] Initial reports also suggested that the PP-fold was found in human NPY.[101] Today, however, we know that this is not correct, as NMR and FRET studies has shown that NPY is very flexible in the *N*-terminus.[1,102] The solution structure predicted by Keire *et al*. indicate that the α-helical part of PYY1-36 is interrupted at residue 23-25, which was not observed by the other members of the PP-fold family of peptides.[99] Nevertheless, Lerch *et al.* have later repeated the initial experiments and found the secondary structure of residue 14-31 primarily to be of α-helical nature.[4]

2.2 The membrane compartment model

Despite the solution structures of PP, NPY and PYY1-36 shows major differences, Zerbe and co-workers reported that the PP-fold family of peptides most likely associate with the membrane prior to receptor recognition.[1-5]

Several years ago Kaiser and Kezdy proposed the hypothesis that amphipathic ligands targeting membrane bound receptors recognize their targets from the membrane bound state.[103,104] Moreover, they suggested that the active site of the receptor only occupies a few amino acid residues, and that the activation of the receptor is usually mediated by a few stereospecific interactions between ligand and receptor. The remainder of the amphipathic α-helix of the peptide hormone act as an anchor onto the membrane as well as a conformational guide for the receptor recognizing part of the peptide.[103,104] Subsequently, Schwyzer *et al*. formulated the membrane compartment model: Prior to binding to the receptor, the ligand associates to the membrane surface and accumulates in the vicinity of the receptor. The residues of an amphipathic α-helix interact with the membrane in which the hydrophobic face of the α-helix penetrates into the space between the phospholipid

head groups allowing interaction with the long lipophilic chains of the phospholipids. The hydrophilic face of the helix is orientated towards the aqueous environment. Residues such as Tyr are generally located in the water-membrane interface. The interaction with the membrane leads to conformational changes and reduced entropic loss upon receptor binding and activation (exemplified by PYY1-36; Figure 2.2).[105,106]

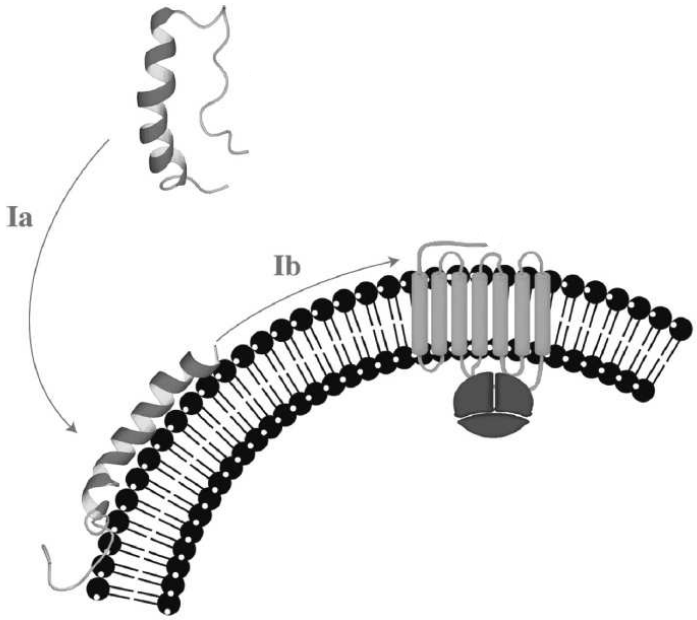


Figure 2.2. *Schematic presentation of the membrane compartment model. Membrane association (Ia) followed by lateral diffusion to the receptor (Ib).*[2]

Zerbe and co-workers recently predicted by NMR that the PP-fold peptides recognize the receptor via the membrane compartment model.[1,3,4] Besides, the high sequence homology of NPY and PYY1-36, the affinities towards the Y receptors are also very similar - in contrast, the solution structures are markedly different (Figure 2.3).[2] The solution structure of the PP-fold peptides shows that PP and PYY1-36 are back-folded, and NPY is not.[2] Nevertheless, NPY and PYY1-36 bound to dodecylphosphocholine (DPC) micelles show high structural similarities (Figure 2.3). PP, on the other hand, has a different structure in the micelle bound state (Figure 2.3) and also a very different receptor-binding profile compared to PYY1-36 and NPY (Table 1.1).[1,2,4] Moreover, high sequence identity and stability of the *C*-terminal pentapeptide of NPY and PYY1-36, and similar membrane anchoring residues of the amphipathic α-helix has been identified.[2] Finally, the membrane-bound state of PP differs significantly from NPY and PYY - especially in the *C*-terminal receptor recognizing pentapeptide and in *N*-terminus.[2] The almost identical binding profiles between PYY1-36 and NPY and the very similar secondary structures as revealed by NMR studies conducted in the presence of micelles, indeed suggest that these peptides interact with their specific receptors from their membrane-bound state.

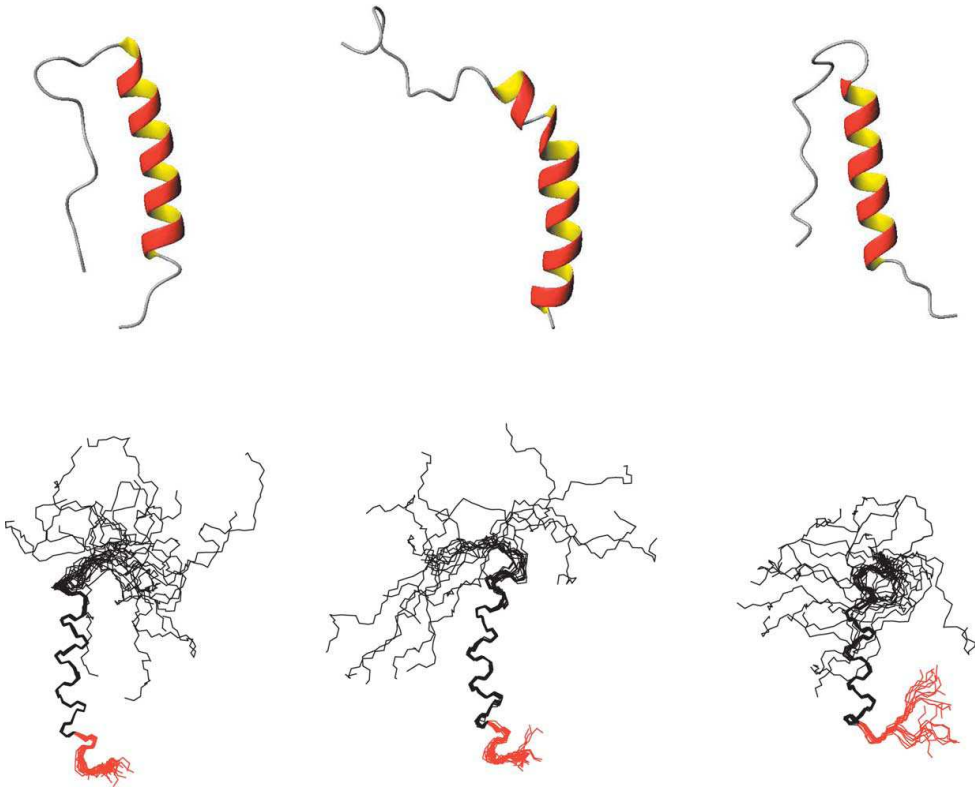


Figure 2.3 *PYY (left), NPY (middle), and PP (right) structures. Top: Solution structures (single conformer). Bottom: Micelle-bound structure (superposition of NMR ensemble).*[4]

To summarize; the PP-fold family of peptides are hypothesized to bind to the Y receptors via a multistage scenario. First, electrostatic interactions attract the peptide towards the membrane. Secondly, the peptide changes conformation such that the hydrophobic side-chains of the amphipathic α-helix penetrates into the hydrophobic interior of the phospholipids of the membrane, and subsequently diffuses laterally along the membrane until receptor binding.[2] As Kaiser and Kezdy originally proposed,[103,104] it is generally believed that the membrane-bound state of the peptide is close to the state from which the peptide is recognized by the receptor. The rigid scaffolds of the GPCRs do not allow large rearrangements of the extracellular loops. It is not likely that the initial preorganized state undergoes further conformational changes by an induced-fit mechanism.

2.3 Ligand-receptor interactions

Ala-mutagenesis and structure affinity/activity relationship studies can be used to indentify the important interactions between ligand and receptor. As a gold standard to evaluate residues within the ligand that are involved in ligand-receptor interactions, Ala-scan studies can be conducted. In an Ala-scan all amino acids are substituted with the smallest

chiral amino acid, Alanine (Ala). Moreover, to evaluate the importance of the *N*- or *C*-terminal amino acids the native ligands can be truncated in the *N*- or *C*-terminus. Ala-scan of an entire GPCR is however not feasible thus; single amino acid replacement by Ala or other amino acids within the extracellular loops are the method of choice. Additionally, to identify the direct interaction partners between the ligand and the receptor complementary mutagenesis could be applied.

2.3.1 Ligand side

Ala-scans has been conducted both on NPY[107] and PYY3-36[108] and showed residues 25, 27, and 32 to 36 to be the most critical residues in the recognition of NPY and PYY3-36 by the Y receptors. The importance of Arg-25 is most profound in the interaction with the Y2 and Y5 receptor subtypes, in contrast to Arg-33 that is most critical for the Y1 and Y4 receptor subtypes.[108] The PYY3-36 *C*-terminal Arg-35 is a highly important residue for all Y receptor subtypes.[108] Moreover, the Tyr residues of the PYY3-36 *C*-terminal is highly critical for receptor binding, except Tyr-27 towards the Y2 receptor subtype.[108] The *N*-terminal segment of NPY and PYY is essential for Y1 receptor activation, in contrast to the Y2 receptor and to some extent also the Y4 and Y5 receptors.[80,109-115] The α-helix is however essential for activation of all the Y receptor subtype,[80] which is in alignment with the membrane compartment model. Analogs of NPY containing the conformational restricted Pro residue at position 34 has resulted in selective Y1/Y5 agonism.[116,117]

2.3.2 Receptor side

Selected residues of the Y receptors have been point mutated.[91,118-120] Walker *et al*. have showed that several aspartic acid residues of the Y1 receptor are highly important for NPY binding,[119] which were partly confirmed by Beck-Sickinger and co-workers:[118] Asp-2.68 and Asp-6.59 are essential and Asp-5.27 is moderately important for Y1/Y4 receptor activation. Additionally, Asp-6.59 is important for Y2/Y5 receptor activation and in contrast to the Y1/Y4 receptor residue Glu-5.24 is also a critical residue in ligand binding towards the Y2/Y5 receptor.[118] Several groups have additionally reported Tyr-2.64, Phe-6.58 and His-7.31 of the Y1 receptor to be important for ligand binding.[121-124] Even though a rhodopsin-derived Y1 receptor structure showed these residues to be to far apart,[125] the initial hypothesis that the residues form a hydrophobic pocket that directly interacts with the *C*-terminal amide and Tyr-36 of the ligand is still feasible.[123]

2.3.3 Complementary mutagenesis

Asp-6.59 of the Y2/Y5 receptors has been shown to interact with Arg-33 of NPY and Asp-6.59 of the Y1/Y4 receptors interact with Arg-35 of NPY or PP trough ionic interactions.[118] No contact point has been established between ligand and Glu/Asp-5.27,

however it has been speculated that Glu-5.27 is partially blocking the active site of the Y2/Y5 receptors such that Arg-35 of the ligand can not interact with Asp-6.59.[118]

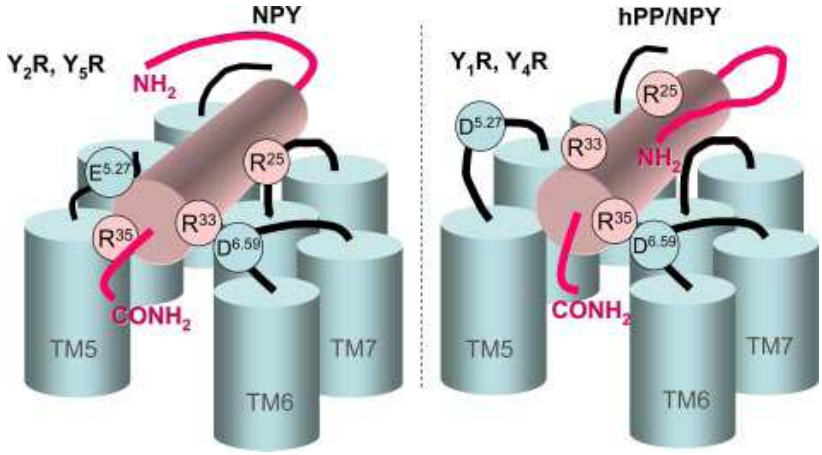


Figure 2.4. *NPY and PP interactions sites between the Y1, Y2, Y4 and Y5 receptor subtypes. Two conserved Arg residues, Arg-33 and Arg-35, at the C-terminal of the NPY and PP participate in the formation of high affinity interactions to Y receptors.*[118]

Part II
‘Methods’

Chapter 3. Methods

Proteins almost exclusively carry out the control of biological processes in living cells and posses an enormous potential for diversity, which makes them well suited for such complicated task. Moreover, numerous peptide hormones act as messengers between cells by high affinity and selectivity engagement to specific receptors. Several of the advantages of utilizing peptides and proteins as therapeutic molecules, compared to small molecules include; high activity and specificity, unique three-dimensional characteristics, low toxicity and relatively low immunogenicity.[126] Peptides have potential as unique therapeutic molecules because they can be designed to engage onto a larger portion of the target protein surface, compared to small molecules, thus providing greater specificity. The protein-protein and protein-peptide interactions surfaces are often relative flat consequently, the likeliness of small molecules to engage into a high-affinity and specific binding is often limited.[127] Moreover, for many protein-protein and protein-peptide interactions the apparent complementarities between the two surfaces involves a significant degree of protein flexibility and adaptivity.[128] The hot-spots of a protein interface may be far apart and are therefore particular adept at binding to proteins and peptides.[127] Another challenge with discovering drug-like small molecules to protein-protein targets is that small molecule ligands may act promiscuously by forming large aggregates, micelles or liposomes consequently leading to an unspecific binding.[129-131] Additionally, some compounds act as protein denaturants or covalent inhibitors consequently inhibiting the function of a number of proteins without binding to a discrete site.[132-134] There is, however, also challenges involved in using peptides as therapeutics, which include; low metabolic stability in bodily fluids, bioavailability, and often higher manufacturing costs.[126]

Insulin was the first protein to be isolated and administrated therapeutically,[135] and is still the most commonly prescribed biopharmaceutical. Approximately 65 peptides has since been approved as pharmaceuticals,[136] and roughly 500 peptides are currently being developed for therapeutic applications in a variety of diseases and infections e.g. cancer, diabetes, cardiovascular diseases, inflammation, CNS diseases and obesity.[126,136]

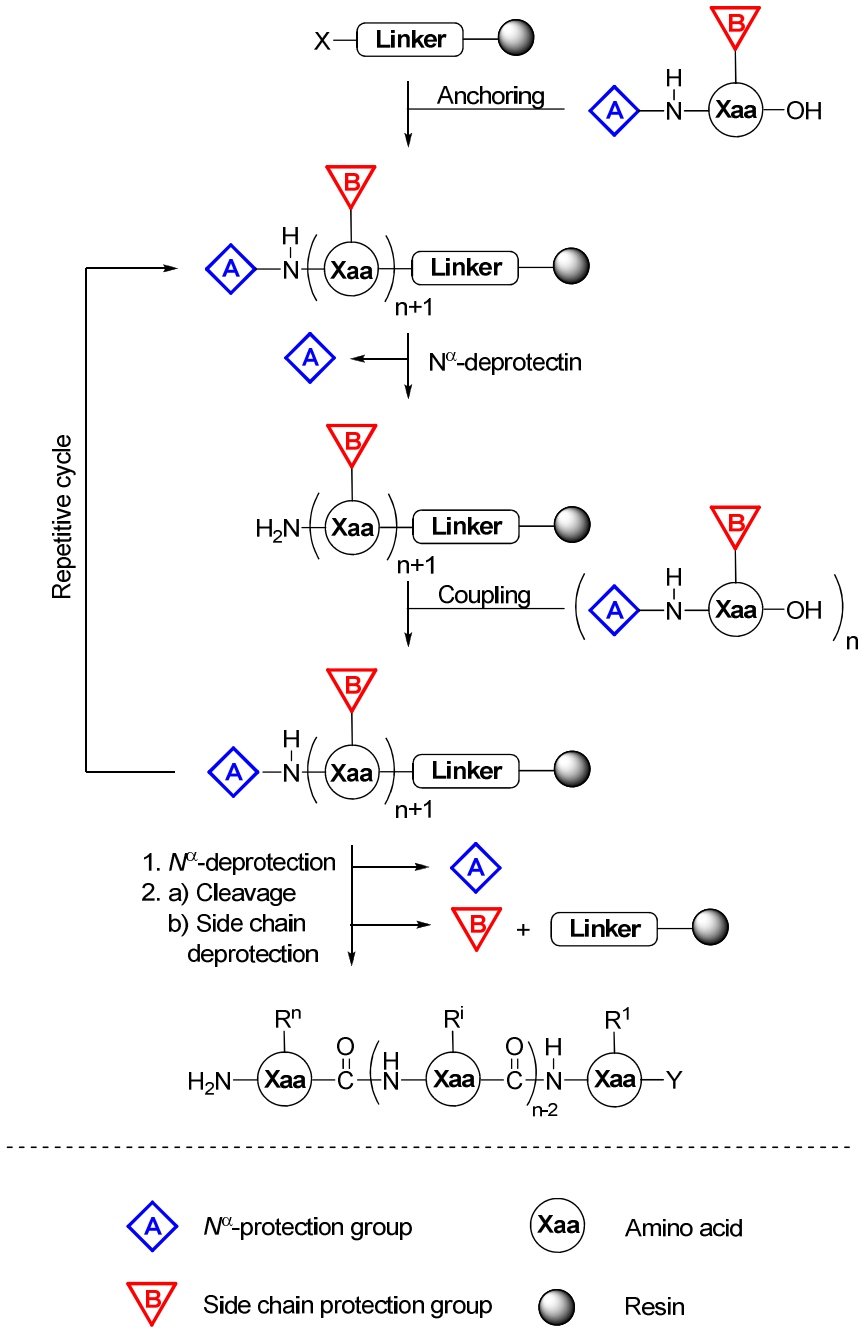
Peptides can be made synthetically, by recombinant methods, or by chemical modification of an isolated natural product. The last 20-30 years of development within the field of synthetic peptide synthesis has increased the possibilities of making successful peptide-based drug discovery programs, even when pursuing long peptide ligands (> 40 residues). Peptide structure evaluation may provide significant information revealing

important characteristics of the secondary structure of the compound of interest. A fast and reliable method for evaluating secondary structures of long peptides and proteins is circular dichroism (CD). In the field of obesity pharmaceuticals different types of assays and animal models has emerged, where the innovative animal model of obesity, especially the dietary induce obesity (DIO) models, has led to a detailed, although not complete, understanding of the regulation of food intake and energy homeostasis.

3.1 Peptide synthesis

The present chapter gives a brief introduction to solid-phase peptide synthesis (SPPS). Since the introduction of chemical synthesis of peptides by Emil Fischer in the beginning of the 20th century,[137] the evolution in the development, analysis, and recently the use of synthetic peptides as therapeutics has been dramatic. Fischer was limited to simple unprotected amino acids and had trouble with the solubility of longer polypeptides. The discovery of the easily removable protecting group, the benzyloxycarbonyl group (Cbz),[138] resulted in the synthesis of a number of native peptides in solution.[139-144] The development of solid-phase peptide synthesis (SPPS) by Bruce Merrifield in 1963 was a quantum leap in peptide science.[145] Consequently, SPPS has led to a widespread use of synthetic peptides in chemical and biomedical investigations. The basis of SPPS, the functionalized solid support that allows for anchoring of an amino acid, revolutionized the field of peptide science and the SPPS methodology has become the method of choice for peptide synthesis.

The strategy of SPPS is to sequentially couple suitably N^{α}- and side chain-protected amino acids to a growing peptide on an insoluble support (resin) in the $C \rightarrow N$ direction. The SPPS reactions are driven to completion by the use of soluble reagents in excess, which can be removed by filtration and washing. Subsequent to anchoring the amino acid at the carboxy terminus to the solid support via a cleavable handle, the N^{α}-protecting group can be removed without affecting the side chain-protecting groups, thus the polypeptide chain is prepared for the next coupling cycle. Following the completion of the desired sequence of amino acids, the peptide is released from the solid support, and simultaneously the permanent side chain-protecting groups are removed. The principle is illustrated in scheme 3.1.

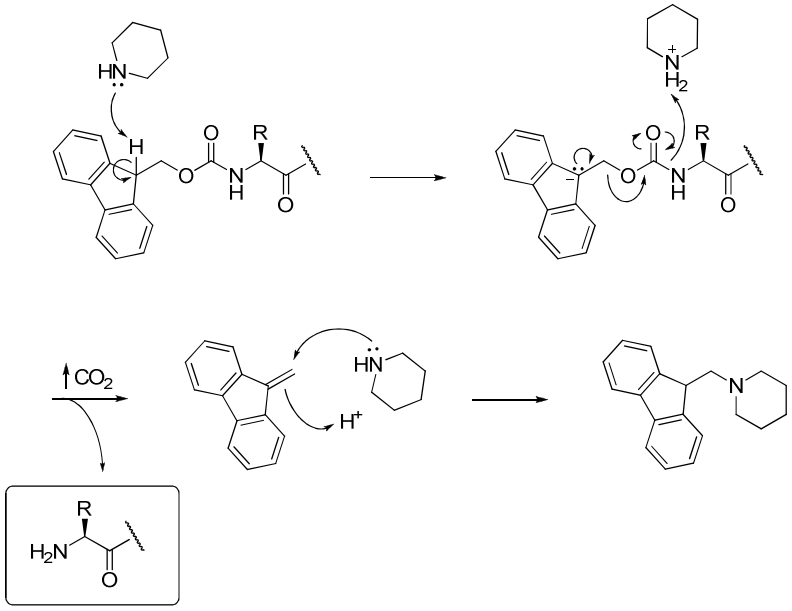


Scheme 3.1. *Overview of the sequential coupling of amino acids used in solid-phase peptide synthesis. X is a nucleophilic functionality of the linker. Y represents the C-terminal functionality of the peptide that varies with the handle of choice.*

3.1.1 Protecting group strategy

The two general protecting group strategies for SPPS are the fluoren-9-ylmethyloxycarbonyl (Fmoc)[146-148] and the *tert*-butoxycarbonyl (Boc)[145,149] named for their application as the N^{α}-protecting group. The Boc strategy, initially introduced by Merrifield, exploits the differences in acid lability of the N^{α}- and side chain-protecting groups, that are

removed by trifluoroacetic acid (TFA) and hydrofluoric acid (HF), respectively.[145,149] Due to the use of the corrosive and toxic nature of HF and the necessity of a HF apparatus, the Fmoc strategy is superior to the Boc strategy for routine synthesis. The Fmoc group can be removed under very mild alkaline conditions, typically 1:4 piperidine-DMF (Scheme 3.2).[150,151] The piperidine induces a β-elimination, resulting in the release of the unprotected N^{α}-amino acid, carbon dioxide and dibenzofulvene. An excess of base will subsequently scavenge the latter (Scheme 3.2).



Scheme 3.2. *N^{α}-deprotecting by piperidine as base and scavenger.*

Semi-permanent protecting groups (side chain-protecting groups) for the Fmoc strategy have been developed extensively during the past decades. For certain amino acid residues, e.g. Cys (cysteine), Asp (aspartic acid), Glu (glutamic acid), Lys (lysine), side chain-protecting is essential for successful peptide synthesis, however, generally all tri-functional amino acids are semi-permanently side chain-protected. The generally used protecting groups are: *tert*-butyl for Glu, Asp, Ser (serine), Thr (threonine), and Tyr (tyrosine); 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for Arg (arginine); and Trt (trityl) for Cys, Asn (asparagine), Gln, and His (histidine).

3.1.2 Coupling conditions

Activation of the carboxylic acid moiety of the amino acid is required to be able to react with the N^{α}-amino group of the growing peptide chain. Initially, carbodiimide-based coupling reagents, such as DCC (*N*,*N'*-dicyclohexylcarbodiimide)[152-154] or DIC (*N*,*N'*-diisopropylcarbodiimide)[155], were used. The carbodiimide-based reagents were adapted from solution-phase chemistry, but has the disadvantage that the *O*-acylisourea

intermediate tends to provoke side-reactions and epimerization, if additives are not added.[156]

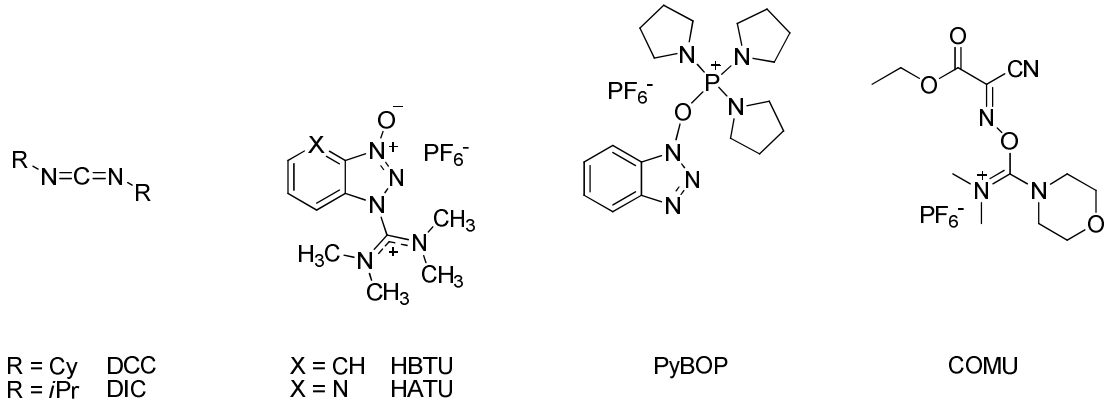
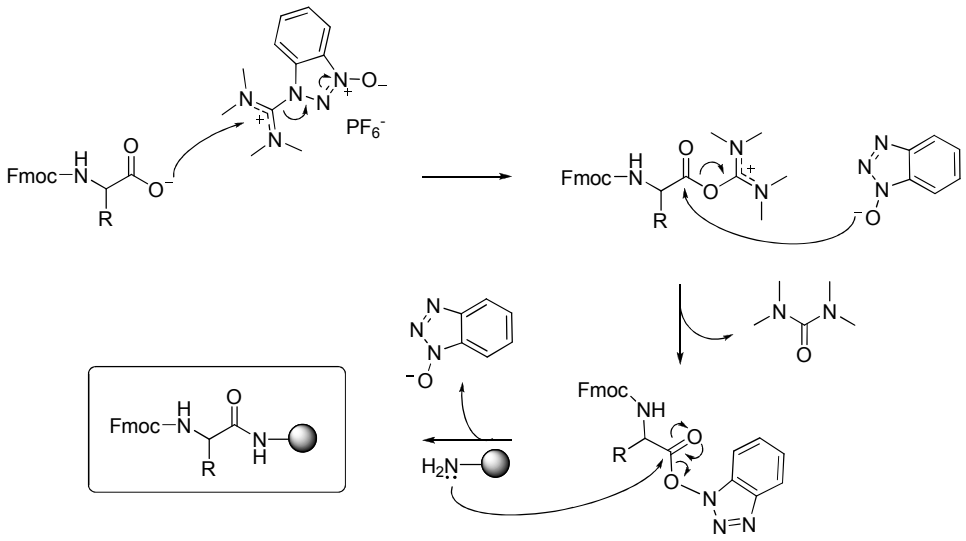


Figure 3.1. *Commonly used coupling reagents.*

Additives, such as HOBt (1-hydroxybenzotriazole),[154,157] ensure that the optical integrity of the stereogenic center at the *C*-terminal of the activated amino acid residue is maintained throughout the coupling step.[154] An array of different coupling reagents has been developed to reduce coupling time and minimize epimerization, since the carbodiimide-based coupling reagents were introduced - the most important are HBTU (*N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexa-fluorophosphate *N*-oxide), HATU (*N*-[(dimethylamino)-1*H*-1,2,3-triazole[4,5-b]pyridine-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide), PyBOP (1-benzotriazolyloxy-tris-pyrrolidinophosphonium hexafluorophosphate) and the novel COMU (1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholino-methylene)] methanaminium hexafluorophosphate)[158] reagents (Figure 3.1).

Probably the most used coupling reagents, due to a combination of price and performance, is HBTU.[159] The coupling with HBTU initiates by deprotonation of the N^{α}-protected amino acid by a non-nucleophilic base, such as *N*,*N*-diisopropylethylamine (DIPEA). The deprotonated amino acid reacts with the coupling reagent by a nucleophilic attack at the carboxylate of the positively charged aminium carbon. Consequently, HOBt is displaced from the highly reactive *O*-acylisourea cation and subsequently HOBt attacks the carboxyl carbon of the amino acid to form the activated ester (Scheme 3.3).

The peptides investigated in the present Ph.D. thesis were generally synthesized using either (i) HBTU as coupling reagent, HOBt-HOAt (9:1) as additive and DIPEA as base, or (ii) DIC and HOBt as coupling reagent and additive.



Scheme 3.3. *Coupling mechanism for HBTU and HOBt.*

3.1.3 Handles

Generally the first amino acid of the growing peptide chain is attached at the *C*-terminus to the linker. The linkers are bifunctional handles that at one end can be permanently attached to the solid support and at the other end work as the *C*-terminal protecting group. The handles for Fmoc-based SPPS are designed to be easily cleavable by strong acid, such as TFA. The handle also determines the final *C*-terminal functionality of the peptide. The peptides investigated in this Ph.D. rapport were made using the Rink amide linker[160] (Figure 3.2) to obtain *C*-terminal amides.

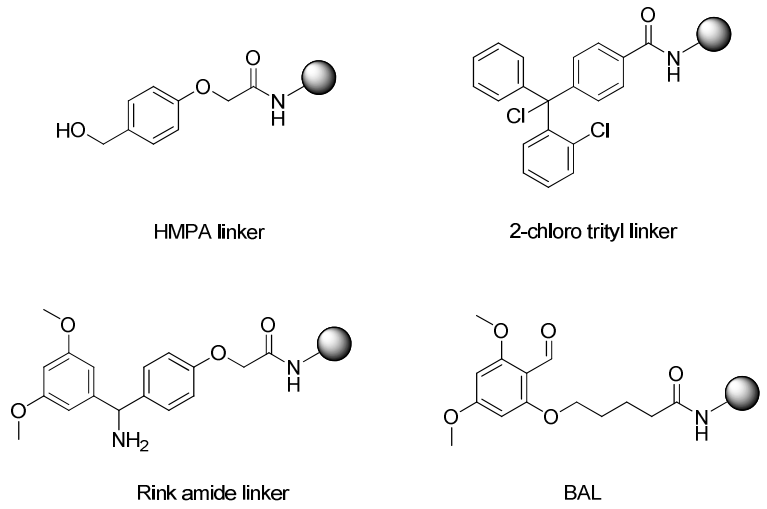


Figure 3.2. *Commonly used linkers in SPPS.*

Other common handles are the HMPA (4-hydroxymethyl-phenoxyacetic acid) linker[161] and the 2-chlorotrityl linker[162] to obtain *C*-terminal acids, and the BAL (backbone amide linker)[163,164] to obtained e.g. *C*-terminal aldehydes and thioesters (Figure 3.2).

3.2 CD spectroscopy

Circular dichroism (CD) spectroscopy plays an important role in the characterization of the novel PYY3-36 analogs described in this thesis. The present chapter gives a brief introduction to CD spectroscopy and its application to the study secondary structures of peptides and proteins.

3.2.1 Theory

CD spectroscopy is an excellent and fast technique to evaluate secondary structures and folding properties of peptides and proteins. CD spectroscopy is defined as the unequal absorption of left- and right-turned circularly polarized light. Electromagnetic radiation is associated with time-dependent electric (*E*) and magnetic (M) fields. The light source in CD spectroscopy is plane polarized light. Unpolarized light contains oscillations in all directions perpendicular to the direction of propagation, whereas plane polarized light only has oscillations in one plane. The plane polarized light is formed as the sum of two beams of left and right circularly polarized light (Figure 3.3.A).

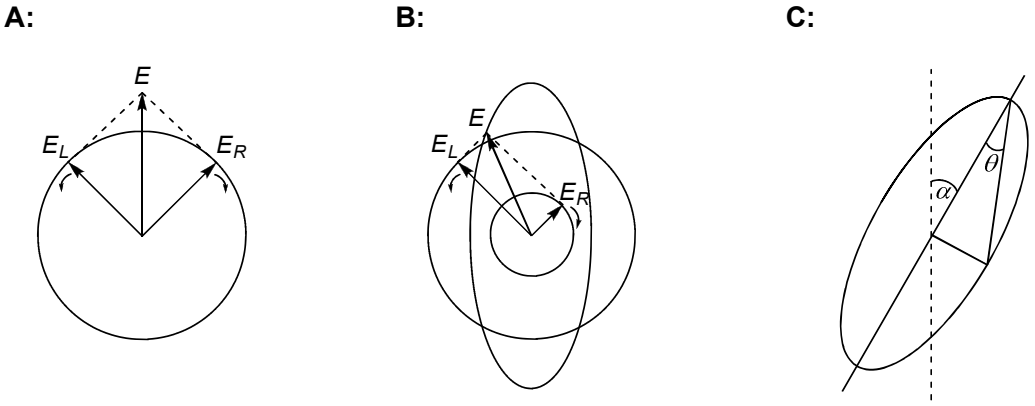


Figure 3.3. *Plane polarized light can be resolved into two circularly components (A). When the light interacts with a chiral compound the two components will absorb differently – thus the resultant vector follows an elliptical path (B). Definition of optical rotation, α, and ellipticity, θ (C).*

The two polarized beams form a clockwise and a counterclockwise screw in the direction of propagation. Hence, at a given time, t, two vectors can be formed from the two circular polarized waves, E_R and E_L, which are opposite in phase (Figure 3.3.A).

When applying the circular polarized light on a chromophore that is either chiral or is situated in a chrial environment the two vectors E_R and E_L interact differently. The properties of the resulting wave depend on the intensities and difference in phase of the components waves (circular birefringence). Chiral molecules absorb right- and left-turned circularly polarized light to different extents and degrees of refraction. The result of difference in absorbance is that the $E_R + E_L$ vectors results in a vector that traces out an ellipse (Figure 3.3.B). Difference in refraction index, a slow-down of the waves to different extents, causes a rotation of the axes by the angle α (Figure 3.3.C).

CD spectroscopic data are reported either in units of ΔE, the difference in absorbance of E_R and E_L by an asymmetric molecule, or in degrees of ellipticity, which is defined as the angle whose tangent is the ratio of the minor to the major axis of the ellipse (Figure 3.3.C), or when describing peptides and proteins, mean residue ellipticities (MRE):

$$[\theta] = \frac{\theta}{10 \cdot l \cdot c \cdot n}$$

Where θ [mdeg] is measured ellipticity, l [cm] is the path length, c [mol/L] is the concentration of the sample and n is the number of residues.

3.2.2 CD of peptides and proteins

The α-helix, β-sheet, and random coil type of protein secondary structure each have a characteristic CD-spectrum (Figure 3.4). α-helical peptides and proteins have negative bands at 222 nm and 208 nm and a positive band at 193 nm.[165] Peptides and proteins forming anti-parallel β-sheets have negative bands at 218 nm and positive bands at 195 nm,[166] whereas random coiled peptides and proteins have very low ellipticity above 210 nm and negative bands near 195 nm.[167]

The limitations of far-UV CD spectroscopic data are that sheets tend to be much less accurate than those of helices.[168] Moreover, secondary structure content can not be calculated accurately from circular dichroism (CD) spectra for proteins with mixed α-helix and β-sheet elements in their structure.[168]

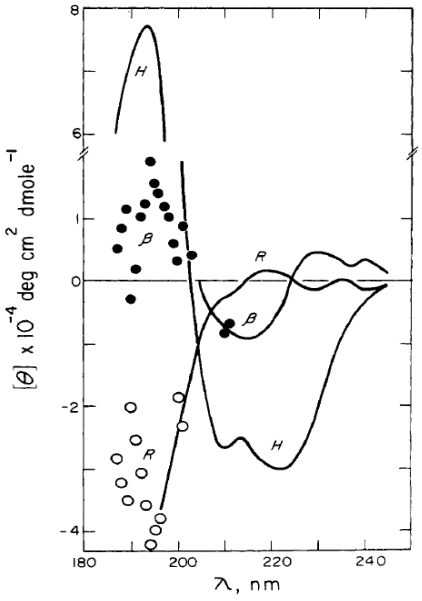


Figure 3.4. *Circular dichroism (CD) spectra of α-helix (H), β-sheet (β) and unordered (R) form computed from the CD of five proteins (myoglobin, lysozyme, lactate dehydrogenase, papain and ribonuclease).*[169]

3.2.3 Analysis of α-helices

The bands from β-sheets and random coil contribute very little to the CD signal at 222 nm (Figure 3.4). Thus, the fraction of α-helical structure, f_H, in a peptide or protein can be estimated:

$$f_H = \frac{[\theta]_{222}}{[\theta]_{H,222}}$$

However, the reference value $[\theta]_{H,222}$ is dependent on the number of residues, n:

$$f_H^n = [\theta]_H^\infty \left(1 - \frac{k}{n}\right)$$

Yang and co-workers obtained CD data from proteins with known structures and found $[\theta]_{H,222}^\infty$ = -39,500 deg cm^2 dmol^{-1} and k = 2.57.[169]

3.3 Assays

Radioligand binding studies and signal transduction assays are two important techniques used to evaluate novel peptide ligands towards membrane bound receptors such as GPCRs. Radioligand binding has been used to provide detailed information about receptor-ligand interactions on membrane preparations, whole cells, tissue slices, and even whole animals.

The most frequently used type is the radioligand competition binding assay in which one concentration of peptide radioligand and various concentrations of unlabeled peptide ligand compete for binding to their receptor.

3.3.1 Radioligand competition binding assay

The radioligand competition binding assay has been used to study the affinity of ligands of GPCRs over several years and has gained detailed information of the affinity of the PP-fold peptides towards the Y receptors.[107,117] In general, the radioligand competition binding assay is conducted on membrane preparations of Y receptor transfected human cell lines.[170] The actual binding assay can be performed in a buffered solution containing the radioligand (^{125}I labeled agonist), displacer (novel ligand) and membrane preparations. Subsequent to incubation the bound ligand is separated from unbound by filtration or centrifugation, and the radioactivity is determined using a γ-counter. The concentration of unlabeled drug that blocks half the radioligand binding is defined as the IC_{50} (inhibitory concentration 50%). The value of the IC_{50} is determined by three factors: (*i*) The K_d of the receptor for the competing ligand - the equilibrium dissociation constant for binding of the unlabeled ligand, (*ii*) the concentration of the radioligand, and (*iii*) the affinity of the radioligand for the receptor (K_m) - high affinity radioligand low K_m. The K_d can be calculated from the IC_{50} according to Cheng and Prusoff[171]:

$$K_d = \frac{IC_{50}}{1 + [radiol.] \cdot (K_m)^{-1}}$$

If the concentration of radioligand is much smaller than the K_m the K_d will approximately equal the IC_{50} value.[172]

The radioligand displacement assay used in to characterize the novel PYY3-36 analogs presented in this thesis was based on membranes from SK-N-MC and HEK293 Flp-In T-Rex cell lines expressing the human Y1, Y2 and Y4 receptor subtypes, respectively. The used radioligands were ^{125}I-[^{31}Leu,^{34}Pro]-pNPY for Y1 receptor, ^{125}I-hPYY3-36 for Y2 receptor and ^{125}I-hPP for Y4 receptor.

3.3.2 Signal transduction assay

The radioligand competition binding assay is a very strong tool to evaluate novel GPCR agonists, however, it reveals only the binding power of a ligands and not the ligands ability to activate the receptor. G-protein coupled receptors (GPCR) are activated by an extracellular signal that creates a conformational change in the receptor. Depending on the receptor subtype, activation is either linked to the cAMP or phosphatidylinositol signaling pathways (Figure 3.5). Two main types of cAMP signal transduction pathways exists; the stimulatory and inhibitory that leads either to stimuli or to inhibition of adenylyl cyclase

through the heterotrimeric G-proteins. Down stream effects of adenylyl cyclase is activation or deactivation of transcription factors. In the phosphatidylinositol signally parthway phospholipase Cβ (PLC-β) is activated after stimuli from a heterotrimeric G-protein. The lipase hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Figure 3.5 and 3.6). IP_3 activates Ca^{2+}-channels located in the membrane of the smooth endoplasmic reticulum as well as the mitochondria, and DAG will activate protein kinase C (PKC), which together will cause biological effects. The heterotrimeric G-proteins consist of the G_α and the tightly associated $G_{\beta\gamma}$ subunits. The family of the G_α subunit is divided into four main groups based on their sequence homology: $G_{\alpha s}$ (stimulate adenylyl cyclases), $G_{\alpha i/o}$ (inhibit adenylyl cyclases), $G_{\alpha q/11}$ (stimulate PLC-β), and $G_{\alpha 12/13}$ (associated with the regulation of a sodium proton exchange protein).[173] More than 70 combinations of G-protein subunits is possible,[174] which regulate more than 865 different GPCRs.[175]

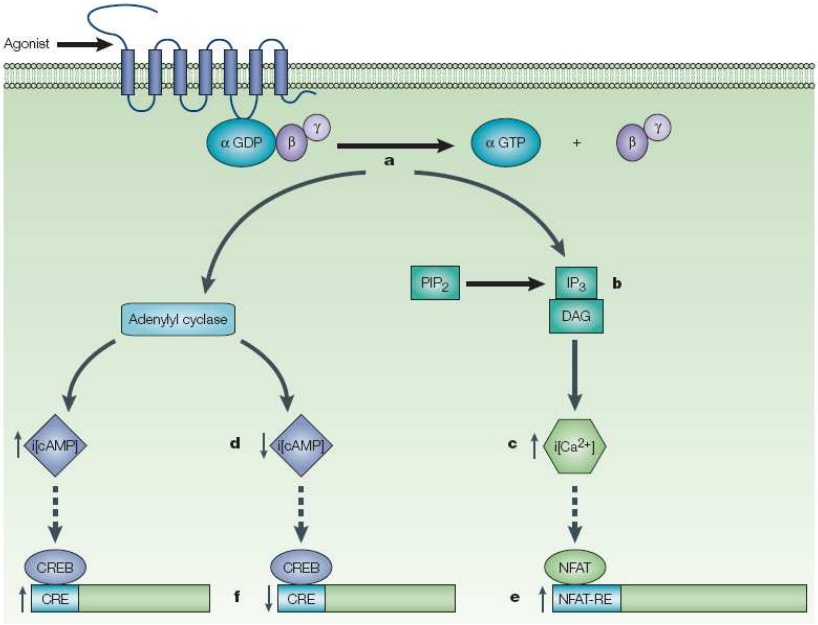


Figure 3.5. *The different GPCR signaling pathways. a) After GPCR activation the heterotrimeric G-proteins interact and facilitates its activation via exchange of GDP for GTP at the α-subunit. b) Hydrolysis of PIP_2 to IP_3 and DAG. c) IP_3 leads to increased Ca^{2+} levels. d) Adenylyl cyclase leads to changes in cAMP levels. e and f) Transcription factors is activated or deactivated.*[176]

Because GPCRs, *via* different G-proteins, regulate a variety of second messengers, it is not surprising that a range of assays are available to detect GPCR-dependent G-protein activation. Aiming at developing a 'universal' screening system in which any receptor can be assayed with a common assay endpoint, chimeric G-proteins has been intensively investigated.[174,177] Additionally, $G_{\alpha i/o}$ coupled GPCRs, such as the Y receptors, has limited downstream detection possibilities. However, stimulation of PLC or cAMP offers a series

of assay options that are widely applied in ligand screening and drug discovery (Figure 3.5).[176,178-180] Natural promiscuous G-proteins (G-proteins that can activate a wide range of GPCRs) exist, but the broadness of their applicability is still limited. Most natural promiscuous G-proteins, such as $G_{\alpha 16}$ ($G_{\alpha q}$ subtype), fail to activate $G_{\alpha i/o}$-selective GPCRs.[181-183] Replacing *C*-terminal amino acids of $G_{\alpha q}$ with the corresponding $G_{\alpha i}$ residues in addition to truncation of the *N*-terminus enabled interaction between G_i- and G_s-linked receptors with PLC-β and IP_3 release.[184-186] The G-protein mutant ($G_{\alpha\Delta 6qi4myr}$) provides a very robust signal response for selected $G_{\alpha i}$-coupled receptors, such as the Y receptors.[118,187] Co-transfecting a Y receptor subtype together with the chimeric $G_{\alpha\Delta 6qi4myr}$-protein into stable cell lines allows for measuring the activity of novel ligands towards the Y receptors via the PLC-β/IP_3 pathway. The lifetime of IP_3 is short thus the GPCR activation is monitored by the IP_3 degradation product, inositol monophosphate (IP_1), which accumulates in the cell in the presence of lithium chloride (Figure 3.6). Alternatively, a Ca^{2+} efflux from the endoplasmatic reticulum (ER) can be measured by fluorescence sensing (Figure 3.6).

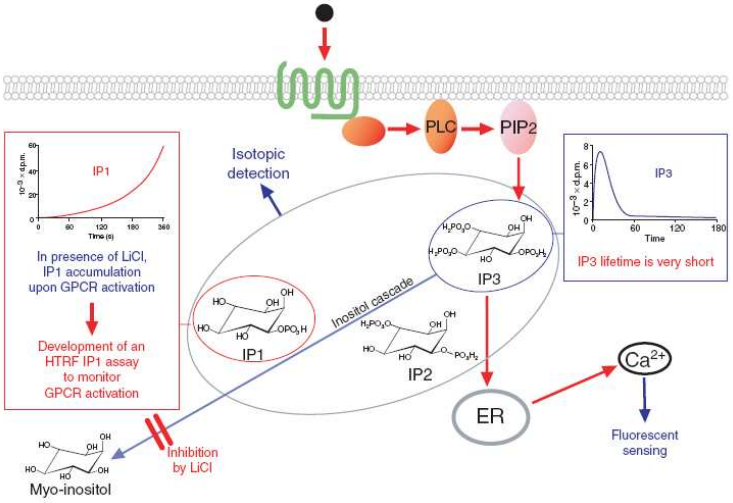


Figure 3.6. *Possible measuring techniques when the $G_{\alpha i}$ coupled receptor is activated though chimeric G proteins.*[188]

Major differences between affinities observed in competition binding studies and potencies observed from signal transductions studies may be obtained, which could be related to the fact that in heterologous competition binding studies, such as the competition binding studies used to characterized novel PYY3-36 analogs in this thesis, the radioligand differ chemically from the investigated ligand, which may allow the radioligand to trap the receptor in one conformation such that high concentration of the ligand is required to induce the conformational changes before binding.[189] Another possible explanation for a discrepancy between affinity and potency could be that the signal transduction assay allows for intracellular amplification of the response, which may increase the potency.

3.3.3 Animal model of obesity

The understanding of the regulation of food intake partly originates from animal studies, especially in rodents. Moreover, animal models have provided valuable knowledge of environmental effects, such as responses to high-energy diets, and in the development of peptide drug candidates for the treatment of obesity.

The initial animal models of obesity originate from spontaneous single-gene loss-of-function mutations, which are discovered by noticing an abnormal obese phenotype, followed by selective breeding to expose the responsible gene in homozygotes (having two copies of the gene). The *ob/ob* mouse model of obesity is a classic example of spontaneous single-gene loss-of-function mutations (loss of the leptin gene).[190] Despite the landmark discovery of the *ob/ob* mouse and the leptin gene, genetic screens of the human population have revealed small numbers of obese individuals that have loss-of-function mutations in this particular gene.[191]

It is generally accepted that human obesity is caused by both genetic as well as environmental factors, and that the genetic basis is poly- rather than monogenic. Hence, to study anti-obesity drugs animal models with a polygenic background disposed to an obesogenic environment are the animal models of choice. For example, chronic exposure of rodents such as C57BL/6J mice, NMRI mice, and Sprague-Dawley rats to high-fat diets results in increasing body-weight and an obese phenotype (Figure 3.7).[192-198]

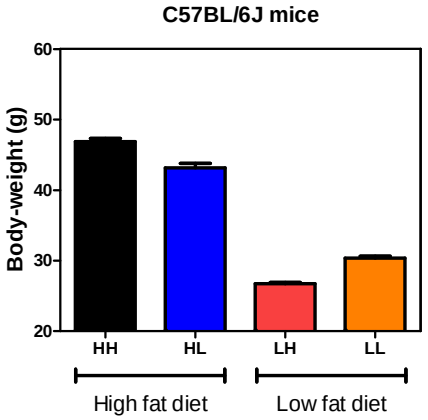


Figure 3.7. *C57BL/6J mice responses to different compositions of diet for 4 months. HH; high-fat, high-sucrose diet. HL; high-fat, low-sucrose diet. LH; low-fat, high-sucrose diet. LL; low-fat, low-sucrose diet.*[192]

Diet-induced obesity (DIO) has a late onset in mice, which is similar to common human obesity resulting from over-consumption of food and an inactive life-style. Depending on the animal strain, the gender, as well as the composition of the diet, and the duration of being exposed to a high-fat diet, DIO mice developed increased adiposity that results in

hyperleptinemia, peripheral leptin insensitivity and insulin resistance already after 8 weeks on high-fat diet.[197,198] Thus, the DIO mouse model is an excellent model of obesity. Moreover, outbreeding of Sprague-Dawley focusing on low and high weight gainers has enabled the development of a diet-induced rat model of human obesity and human leanness (obesity resistance).[196,199] The diet-induced obese (DIO) rats are prone to become obese when offered a diet with an energy content from fat that is very similar to human obesogenic environment (31% energy from fat).

Part III
‘Results and discussion’

Chapter 4. PYY3-36 project

The work presented in this thesis is divided into four projects (Figure 4.1) aiming at improving the Y receptor selectivity (Y2 over Y1), the Y2 receptor affinity and potency, or/and the metabolic stability. The basis of the designs has consistently been that the novel ligands should associate with the membrane prior to Y receptor activation - as hypothesized for the PP-fold peptides.[1-4] Recognizing that the α-helical segment is thought to control the secondary conformation of the receptor-recognizing *C*-terminal pentapeptide the native α-helix was generally not modified. PYY3-36 was primarily modified in the *N*-terminus by considerable modifications that could potentially lead to radical alteration in the secondary structure of PYY3-36.

The novel PYY3-36 analogs were mainly developed based on rational designs, except the *C*-terminal Tyr-36 modifications (project 1, Figure 4.1, chapter 4.1) that are based on a small combinatorial library of non-proteinogenic Phe analogs. A combinatorial design strategy was not the method of choice for the development of the majority of the novel Y2 receptor ligands because during PYY3-36 synthesis multiple byproducts emerge and as a result HPLC purification is required. Moreover, it was believed that the probability of discovering new high-affinity Y2 receptor ligands from a combinatorial perspective would be low due to a high number of futile compounds. Thus, it was projected that the time-benefit of combinatorial design would be unprofitable. With a rational design approach it was believed that the development of high-affinity compounds towards the Y2 receptor was more straightforward – higher probability of finding a hit. Furthermore, the rational design approach would provide compounds which were highly original.

The strategy was to develop novel ligands showing an enhanced pharmacologically profile compared to PYY3-36 before formulating it to be a longer-acting analog. Thus, after identification of a hit compound (i.e. in this context a novel high-affinity PYY3-36 analog with enhanced selectivity and *in vivo* efficacy), it was to be chemically modified by PEGylation or lipidation, which consequently should increase its metabolic stability. Only a few hits presented in this thesis were taken into this step of drug development.

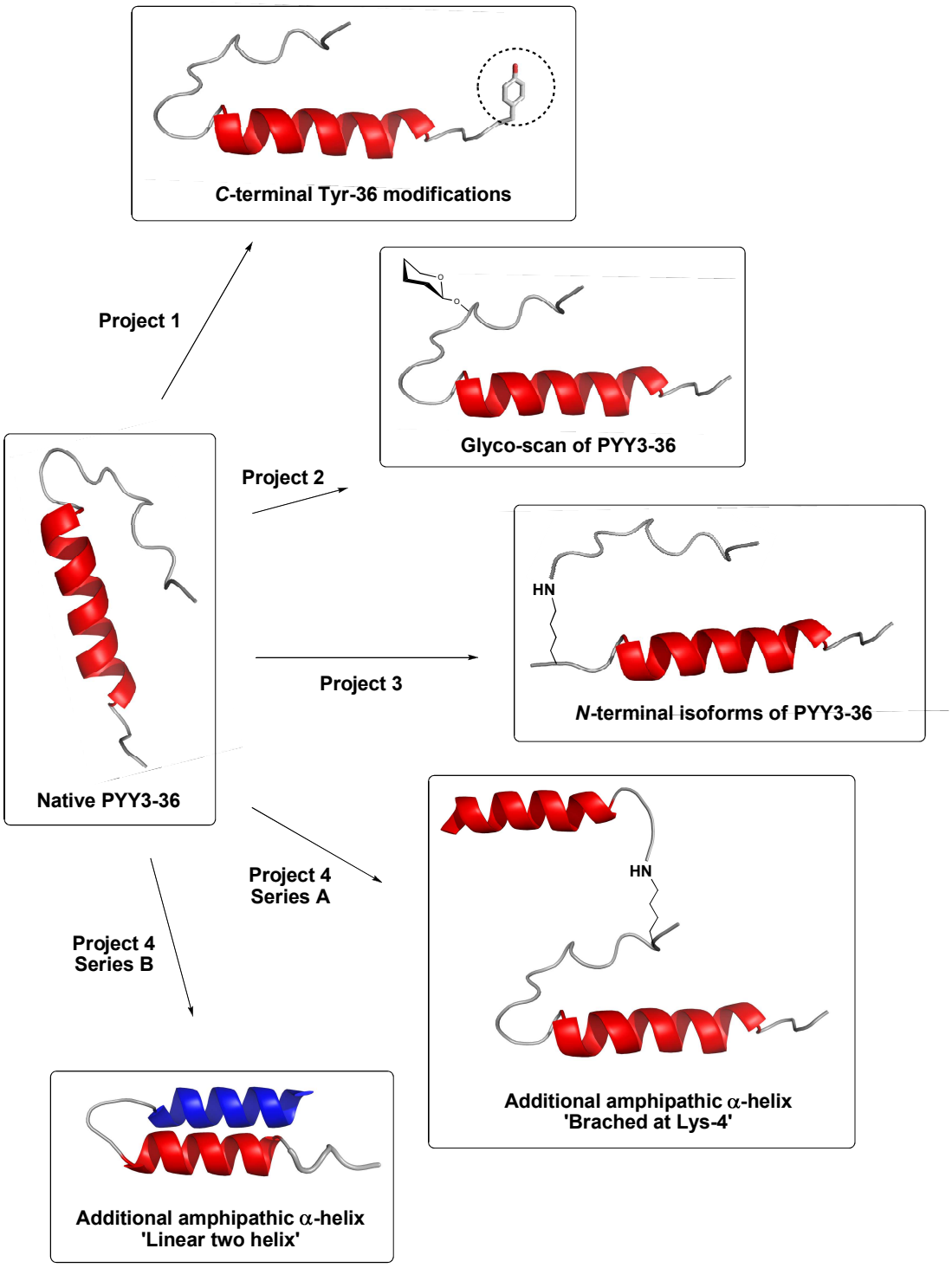


Figure 4.1. *Project overview.*

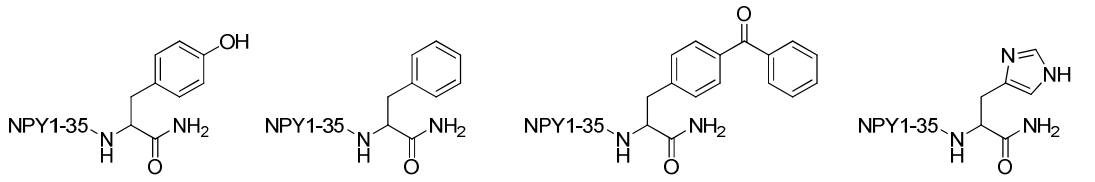
All novel ligands were made using SPPS, purified by RP-HPLC, characterized as well as quantified by LC-MS and the secondary structures were determined by CD spectroscopy. Moreover, the novel ligands were assayed by radioligand competition binding assays towards the Y1, Y2 and Y4 receptor subtypes. Assays for binding towards the Y5 receptor were not performed because it is primarily found in the CNS, and it was anticipated that

the novel ligands presented in this thesis do not cross the blood brain barrier and engage the Y5 receptor. Moreover, Y5 receptor antagonism does not induce weight-loss in overweight and obese adults suggesting the Y5 receptor to be less important in the control of energy homeostasis.[200] Hits were assayed either by signal transduction assay and/or by *in vivo* efficacy analysis in mouse models of obesity. Finally, several PYY3-36 analogs were analyzed for stability against peptidases in buffered solution and/or in mouse serum and the half-lives determined by RP-HPLC.

In the following chapter the main results of each project are given - the papers and manuscripts in the appendix section presents a thorough description of the design and the results of each project. Moreover, the experimental setup for each project is presented in the papers and manuscripts given in the appendix section.

4.1 Project 1: *C*-terminal Tyr-36 modifications

Tyr-36 of PYY3-36 has generally been assumed to be highly important for Y receptor activity due to the high conservativeness throughout species.[36,91] Tyr-36 is part of the *C*-terminal pentapeptide that is crucial for receptor recognition. The significance of Tyr-36 in Y2 receptor binding has been demonstrated in both NPY[107] and PYY3-36[108] Ala-scans that show an almost complete elimination of Y2 receptor affinity (>1000 fold decrease). However, a few of NPY analogs modified at residue 36 with different aromatic analogs showed altered Y receptor selectivity (Scheme 4.1).[80]



	NPY	[^{36}Phe]-NPY	[^{36}Bpa]-NPY	[^{36}His]-NPY
Y1R:	21 nM	2.6 nM	118 nM	470 nM
Y2R:	0.17 nM	0.42 nM	0.3 nM	

Scheme 4.1. *The effect of single amino acid replacement of the C-terminal Tyr-36 on binding affinity of NPY to the Y1 and Y2 receptors.*[80]

The Ala-scans as well as the [^{36}Phe]-NPY and [^{36}Bpa]-NPY analogs indicates that an aromatic group may by required for Y2 receptor affinity, however, not for Y1 receptor affinity. Moreover, the results of the [^{36}Bpa]-NPY analog indicates that there could be a unique pocket in binding site of the Y2 receptor which could be exploited to enhance the Y receptor selectivity. This led to the development of nine novel [^{36}X]-PYY3-36 analogs (a selection is shown in Table 4.1).

Table 4.1. Radioligand competition binding assay and signal transduction assay of PYY3-36 and [^{36}X]-PYY3-36 towards the Y receptor subtypes Y1, Y2, and Y4.

^{36}X	Binding assay, IC_{50} [nM]			Functional assay, EC_{50} [nM]	
	Y1R[a]	Y2R[b]	Y4R[b]	Y1R[c]	Y2R[c]
PYY3-36	7.8 ± 1.1	0.50 ± 0.09	255 ± 29	135 ± 32	7.6 ± 1.6
p-fluoro-Phe	> 1000	0.76 ± 0.02	>1000	737 ± 30	12 ± 1.0
p-chloro-Phe	198 ± 14	1.79 ± 0.22	>1000	>1000	5.2 ± 1.6
p-bromo-Phe	350 ± 101	1.67 ± 0.06	>1000	>1000	5.4 ± 2.1
p-iodo-Phe	>1000	1.65 ± 0.51	>1000	>1000	11 ± 4.0
p-amino-Phe	388 ± 175	0.96 ± 0.32	>1000	367 ± 98	11 ± 2.0

[a] SK-N-MC cells expressing the Y1R. [b] Y2R or Y4R transfected HEK293 Flp-In T-Rex cells. [c] Y1R or Y2R and GαΔ6qi4myr transfected COS-7 cells.

The novel Y2 receptor ligands showed increased Y receptor selectivity and especially analogs having a halogen or amino group in the *para* position of Phe-36 of PYY3-36 showed promising results due to the high Y2 receptor binding affinity and potency in addition to an almost complete abolishment of Y1 receptor affinity and potency (Table 4.1). Modifications of residue 36 of PYY3-36 have shown that conservative modifications in the *C*-terminal pentapeptide can lead to a huge increase of Y receptor selectivity. The results obtained in this study may be incorporated into metabolically stable PYY3-36 analogs to increase their Y receptor selectivity. This work was published in Journal of Peptide Science (Paper 1).[201]

4.2 Project 2: Glyco-scan of PYY3-36

The Ala-scan is a Gold standard to study the effect of point mutations in a peptide sequence. In an Ala-scan all amino acids are substituted with the smallest chiral amino acid, Ala. Here the glyco-scan concept has been formulated and introduced. The glyco-scan will allow the study of effects on one or more of the following properties (*i*) improvement in solubility, (*ii*) protecting against proteolytic cleavage (which consequently increases the half-life of the peptide and enhances the concentration at the pharmacological targets), (*iii*) modification of the clearance, (*iv*) alteration of the function (e.g. GPCR binding), and many more. Peptides has been previously been glycosylated to extent their half-lives and to improve solubility, to add specific recognition, etc.[202,203] A glyco-scan posses the opportunity for a broad degree of variation and diversity, which includes (*i*)

introduction of different carbohydrates (glycans), (*ii*) glycosylation of side-chains as O-, N-, and C-glycosides, and (*iii*) glycosylation of either a native or a non-proteinogenic amino acid.

In this particular glyco-scan of PYY3-36, hydroxyl side-chain functionalities of Ser and Tyr were glycosylated. The glyco-amino acids were synthesized according to procedures reported in literature[204-206] and incorporated into the PYY3-36 sequence using standard Fmoc SPPS. Novel PYY3-36 analogs having a glycan positioned in the water-membrane interface (according to the membrane compartment model of the PP-fold peptides) or in the *N*-terminus were assayed for Y receptor affinity and selectivity. Interestingly, the Y2 receptor could accommodate glycosylations very well in contrast to the Y1 and Y4 receptors. Especially, glycosylation of the α-helical region showed both increased Y receptor selectivity. The glycosylation of Tyr-21, located in the amphipathic α-helix of PYY3-36, leads to enhanced selectivity, originating from a reduced affinity towards the Y1 receptor (Table 4.2).

Table 4.2. The binding affinity towards the Y receptors as well as the degree of α-helicity in solution of PYY3-36 and the novel PYY3-36 analogs glycosylated at Tyr-21 is given. Native PYY3-36 sequence: H-IKPEAPGEDASPEELNRY[21]YASLRHYLNLVTRQRY-NH_2

	Binding affinity, IC_{50} [nM][a]			α-helicity[b]
Peptide	Y1R	Y2R	Y4R	[%]
PYY3-36	7.8 ± 1.1	0.50 ± 0.09	255 ± 29	26
[^{21}Y(β-D-Glc)]-PYY3-36	150 ± 44	0.84 ± 0.12	687 ± 104	47
[^{21}Y(β-D-Gal)]-PYY3-36	70 ± 17	0.51 ± 0.01	714 ± 222	34

[a] SK-N-MC cells expressing the Y1 receptor or HEK293 Flp-In T-Rex cells expressing the Y2 or Y4 receptors. [b] 10 mM phosphate buffer

Interestingly, CD spectroscopy of the glycosylated PYY3-36 analogs in solution showed no disturbance of the inherent PYY3-36 α-helix for the majority of the glyco-PYY3-36 analogs. The analogs glycosylated on a Tyr residue (position 20, 21, 27) even demonstrated a significant increase in stability of α-helix (Table 4.2 and Figure 4.2).

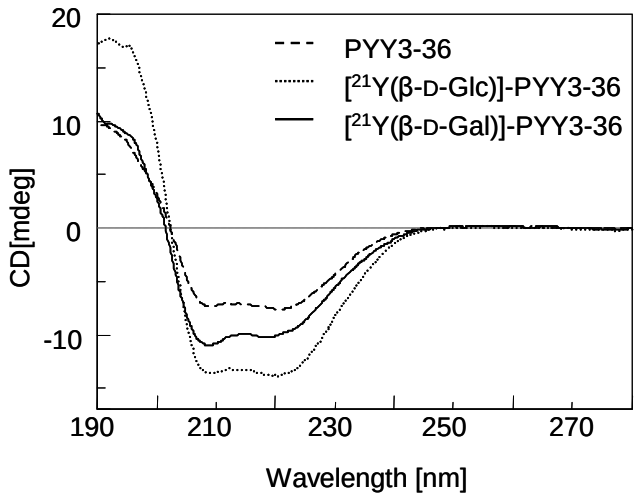


Figure 4.2. *UV CD spectra of PYY3-36, [^{21}Y(β-D-Glc)]-PYY3-36 and [^{21}Y(β-D-Gal)]-PYY3-36 in aqueous buffered solution (20 μM peptide in 10 mM phosphate buffer).*

Moreover, a similar metabolic digestion patterns for both PYY3-36 and glycosylated analogs were shown by LC-MS - serum carboxy-peptidases cleaves at the amino side of Arg-33 and Arg-35 terminating the physiological effects of native PYY3-36 as well as the glycosylated PYY3-36 analogs. This work has been accepted for publication in ChemBioChem (Paper 2).

4.3 Project 3: *N*-terminal isoforms of PYY3-36

Contrary to NPY, PP and PYY1-36 are backfolded in solution:[1-5] The Pro residues of the *N*-terminus of PYY and the Tyr side-chains of the α-helix intercalates in a zipper-type fashion.[207] Additionally, residues 10-14 of PYY1-36 plays a pivotal role in the formation of the backfolding, which has been demonstrated by the complete abolishment of backfolding of PYY1-36 when moving the Pro-14 to position 13.[207] Given the latter and that the *N*-terminus has been demonstrated to be extremely important for Y1 receptor affinity,[80,109,112-115] a number of isoformic branched PYY analogs were designed. The aim was to alter the native turn motif of PYY such that the Y receptor selectivity was increased. The *N*-terminus of NPY has previously been modified, by replacing the residues 5-24 with 6-aminohexanoic acid (Ahx), [Ahx5-24]-NPY, which consequently led to an abolishment of Y1 receptor affinity.[80]

Several different *N*-terminal PYY segments were introduced to the side-chain of an additional Lys-12 of [^{11}Ile,^{12}Lys]-PYY11-36 (Table 4.3). Even though peptides **1** (PYY1-36 isoform), **2** (PYY2-36 isoform), and **3** (PYY3-36 isoform) were branched isoforms of PYY they resulted in Y1 and Y2 receptor potencies equivalent to those for PYY3-36. Peptide **3** gave a slight, but insignificant, decrease in Y1 receptor potency compared to PYY3-36. Exchanging the GEDA turn with the less flexible GPRRP turn

motif (peptides **4** and **5**) resulted in five- to sevenfold increase in Y2 receptor potencies compared to PYY3-36. The Y1 receptor potencies of peptide **4** and **5** only dropped 2.5-fold compared to peptides **1** and **3**, respectively, and the Y1 receptor potency of peptide **5** (PYY3-36 isoform) was only reduced twofold compared to PYY3-36. Thus, the introduction of the GPRRP turn motif resulting in major increases in Y2/Y1 receptor selectivity.

Table 4.3. Potencies of PYY3-36, and the Lys-12 branched [^{11}Ile,^{12}Lys]-PYY11-36 analogs towards the Y1 and Y2 receptors.

		Y1R[a]	Y2R[a]
Peptide	Lys-12 branch	EC_{50} [nM]	EC_{50} [nM]
PYY3-36		90 ± 11	5.6 ± 2.1
1	H-YPIKPEAPGEDA-	12 ± 7	5.6 ± 3.2
2	H-PIKPEAPGEDA-	80 ± 22	4.3 ± 2.9
3	H-IKPEAPGEDA-	118 ± 19	4.6 ± 2.4
4	H-YPIKPEAPGPRRP-	4.5 ± 3.3	1.1 ± 0.3
5	H-IKPEAPGPRRP-	46 ± 23	0.7 ± 0.3

[a] Transfected Y1 or Y2 receptors as well as GαΔ6qi4myr in COS-7 cells.

The stability of PYY3-36 and peptides **1**-**5** were investigated in mouse serum which showed the primary sites for cleavage were in the *C*-terminus between residue Thr-32 and Arg-33 as well as between Gln-34 and Arg-35. PYY3-36 and peptides **1**-**3** resulted in equal half-lives in contrast to peptide **4** and **5** which were digested > 10 times faster. The reason for the increased digestion is still inconclusive. Due to the latter peptides **4** and **5** were not investigated *in vivo*.

Peptides **2** and **3** were tested for efficacy in an acute mouse model obesity (NMRI mice) showing at least to be as good as PYY3-36 in the ability to reduce body-weight (Figure 4.3).

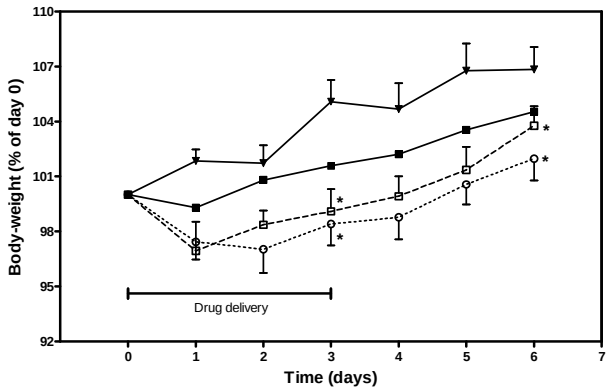


Figure 4.3. *Acute minipump experiment in lean male NMRI mice (3 days). Vehicle (▼), 0.25 µmol/kg/day PYY3-36 (□), 0.25 µmol/kg/day peptide* ***2*** *(■) and 0.25 µmol/kg/day peptide* ***3*** *(○). Values are means ± SEM (n = 7). One way ANOVA followed by a "Dunnett's multiple comparison test": (*) P < 0.05 compared to vehicle.*

One analog, peptide **3**, an isoform of PYY3-36, was further tested chronically in DIO mice (Figure 4.4), resulting in a reduction in body-weight similarly to PYY3-36. Interestingly, the isoformic branched PYY3-36 analog resulted in beneficial effects on the fat-cell metabolism shown by its ability to increase adiponectin levels significantly compared to PYY3-36 (Figure 4.5).

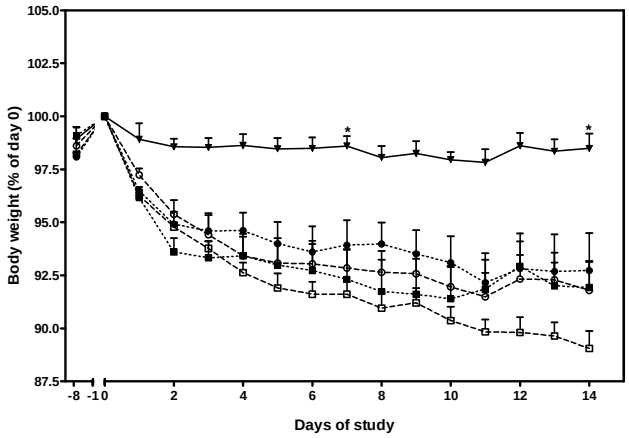


Figure 4.4. *Chronic minipump experiment in male DIO C57BL/6J mice (2-wk). Vehicle (▼), 0.25 µmol/kg/day PYY3-36 (□), 1.0 µmol/kg/day PYY3-36 (■), 0.25 µmol/kg/day peptide* ***3*** *(○), 1.0 µmol/kg/day peptide* ***3*** *(●). Values are means ± SEM (n = 9). One way ANOVA with "Dunnett's Multiple Comparison Test": (*) P < 0.05 compared to vehicle.*

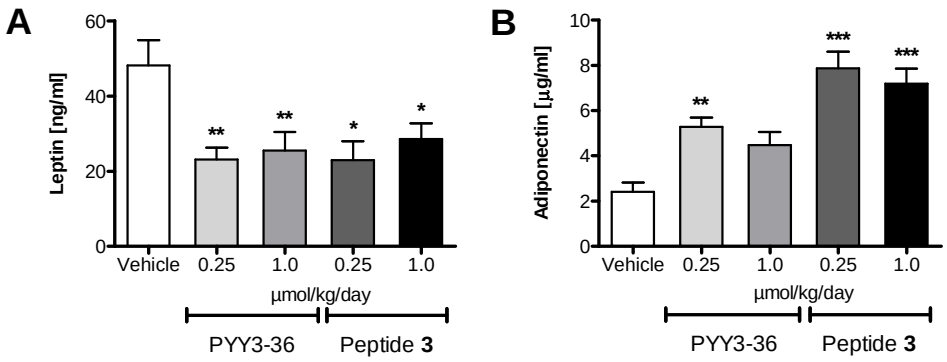


Figure 4.5. *Leptin levels on day 14 (A) and adiponectin levels on day 14 (B) of PYY3-36 and peptide **3**. Values are means ± SEM (n = 9). One way ANOVA with "Dunnett's Multiple Comparison Test": (*) P < 0.05, (**) P < 0.01, (***) P < 0.001 compared to vehicle.*

Increased adiponectin levels can indicate a positive remodeling of the adipose tissue, however, the effect is somewhat surprising because peptide **3** showed equal affinity, potency, and selectivity towards the Y receptors compared to PYY3-36. Moreover, the stability and pharmacokinetics are also similar between the two ligands. It could be speculated whether the beneficial effect could originate from an activation of an orphan receptors, such as GPR83 (GIR).[208]

Project 3 has resulted in a patent application (WO2009/080608) and one manuscript aimed for publication in Organic and Biomolecular Chemistry (Paper 3).

4.4 Project 4: Additional amphipathic α-helix

Given that the PP-fold family of peptides interact with the membrane prior to receptor interaction[1-5] and that truncating the *N*-terminus of PYY and NPY has proven important for Y receptor selectivity,[80,109,112-115] an array of novel PYY3-36 analogs modified in the *N*-terminus by introducing an amphipathic α-helix has been designed. The amphipathic nature of the additional α-helix, and thereby a possible increase in affinity towards the cell membrane, should lead to an accumulation of peptide near the Y receptors. In other words, bringing the peptide from the three-dimensional space of solution on to the two-dimensional plane of the cell membrane should increase the probability of the peptide finding the receptor.

The project is divided into two series based on their difference in architecture (Figure 4.6). Series A, an α-helix was introduced as a branch at Lys-4 of native PYY3-36. Two different amphipathic sequences were investigated, (*i*) PYY17-31, the native α-helix of PYY, and

(*ii*) its retro counterpart, PYY31-17, which is also predicted to form an α-helix. Moreover, different turn motifs between the branch point and new helix have been investigated.

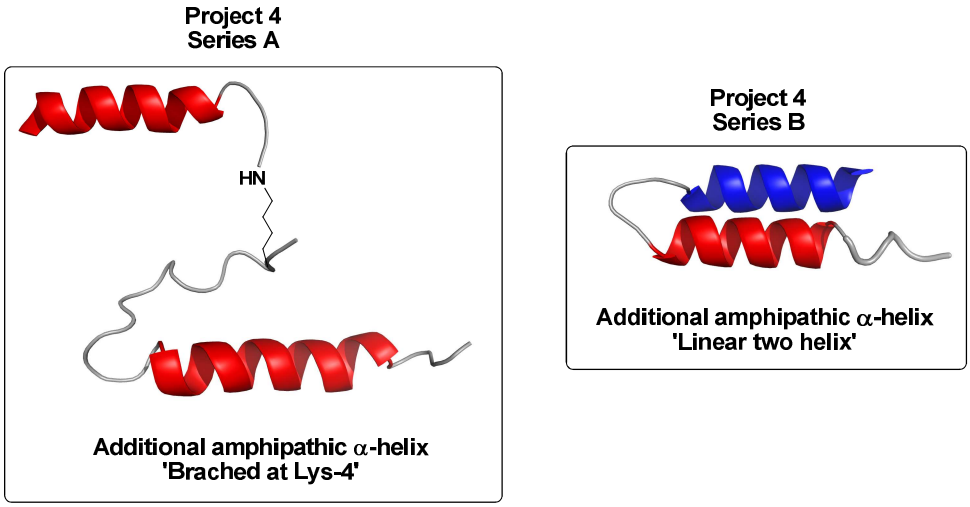


Figure 4.6. *Overview of project 4.*

Series B, here an amphipathic α-helix was introduced in the *N*-terminus of PYY13-36. Several different amphipathic heptad repeat α-helices were investigated. The novel analogs were designed to form helix-loop-helix type of structures in solution - the hydrophobic sides of the two α-helices may assemble. Furthermore, the novel analogs may self-associate into di- or tetramers in solution.

4.4.1 Series A

Several of the novel Y2 receptor ligands showed improved Y receptor selectivity (Y2 over Y1) in the radioligand competition binding assay (Table 4.4) and furthermore, two analogs showed to be superior to PYY3-36 in their ability to reduce body-weight in acute mice studies (Figure 4.7).

Adding an amphipathic α-helix to the side-chain of Lys-4 of PYY3-36 has only moderate impact on binding affinity towards the Y2 and Y4 receptors. The binding data showed a slight tendency that the peptides containing the retro sequence were better accepted by the Y2 receptor than the natural sequence, however, the nature of the turn region led to more significant differences in binding affinities towards the Y2 receptor (Table 4.4). The GPRRP turn motif (peptides **8** and **9**), adapted from Hill and Degrado,[209] were the best accepted sequence and gave equivalent to enhanced Y2 receptor binding affinities. The GPRRP turn motif limits the flexibility of the additional α-helix more than the other turn motifs, which may guide the *C*-terminal in an optimal conformation for Y2 receptor binding and/or prevents Y1 receptor binding. Interestingly, the analogs with a GPRRP turn motif (peptides **8** and **9**) were also well tolerated towards the Y4 receptor and led to almost a threefold increase in binding affinity (Table 4.4). The binding affinities towards the Y1

receptor were to a great extent affected by the addition of an additional amphipathic α-helix. However, no turn (peptides **6** and **7**) and the PE turn motif (peptide **11**) showed to have little effect on the binding affinity towards the Y1 receptor. Peptides **8**-**10** and **12**-**13** showed a very low Y1 receptor affinity, which consequently leads to improved Y2/Y1 and Y4/Y1 receptor selectivity's compared to PYY3-36 (Table 4.4).

Table 4.4. PYY1-36, PYY3-36 and H-I[4]KPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2 analogs binding affinity towards the Y receptor subtypes Y1, Y2, and Y4, respectively.

	Lys-4 branched sequences		Nature of α-helix	Y1R[a]	Y2R[b]	Y4R[b]
Peptide	α-helix	Turns		IC_{50} [nM]	IC_{50} [nM]	IC_{50} [nM]
PYY1-36				4.0±1.7	3.8±1.6	141±79
PYY3-36				7.8±1.1	2.1±0.2	255±29
6	H-LNRYYASLRHYLNLV	-	PYY17-31	11.7±3.7	6.2±1.1	395±121
7	H-VLNLYHRLSAYYRNL	-	PYY31-17	7.9±0.5	5.4±0.3	496±91
8	H-LNRYYASLRHYLNLV	GPRRP-	PYY17-31	>1000	2.1±0.2	173±60
9	H-VLNLYHRLSAYYRNL	GPRRP-	PYY31-17	>1000	0.4±0.1	101±16
10	H-LNRYYASLRHYLNLV	GG-	PYY17-31	>1000	14.1±4.5	567±40
11	H-VLNLYHRLSAYYRNL	PE-	PYY31-17	17.2±1.8	7.8±2.2	276±5
12	H-LNRYYASLRHYLNLV	SP-	PYY17-31	>1000	7.6±0.4	298±15
13	H-VLNLYHRLSAYYRNL	SP-	PYY31-17	>1000	5.6±1.8	362±127

[a] SK-N-MC cells expressing the Y1 receptor. [b] Transfected Y2 or Y4 receptors in HEK293 Flp-In T-Rex cells.

Only peptides **8** and **12** showed significant body-weight lowering effects in acute *in vivo* studies (Figure 4.7). In contrast to the results obtained by the binding experiments, the orientation of the additional amphipathic α-helix, *i.e.* forward vs. retro sequence, was important for *in vivo* efficacy - only forward sequences showed efficacy. Finally, the *in vivo* experiments generally confirmed the results from the affinity studies, i.e. the turn motif was of central importance.

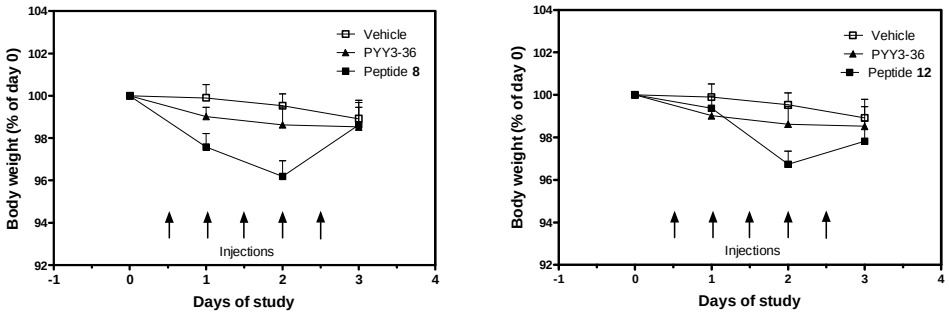


Figure 4.7. *Acute effect of peptides **8** and **12** in lean male NMRI mice. The mice were given either 0.9% saline as vehicle (□), 1.0 µmol/kg/day PYY3-36 (▲) or 1.0 µmol/kg/day branched peptide (■) two times a day for two days, shown by and arrow, with a total of five injections. The body-weight were calculated every morning for four days and given as mean body-weight (percent of day zero). There was seven mice in each group (n=7).*

4.4.2 Series B

It is anticipated that the novel analogs of series B in association with a membrane are unfold in a position where the two amphipathic helices are located on the surface of the lipid bilayer analogous to the PP-fold peptides (Figure 4.8). Because the peptides of series B are designed to have increased affinity to the membrane, an increased potency towards the Y receptor could follow. Moreover, the additional α-helix may contribute to guide the *C*-terminal α-helix into a conformation favorable for Y2 receptor affinity and activity.

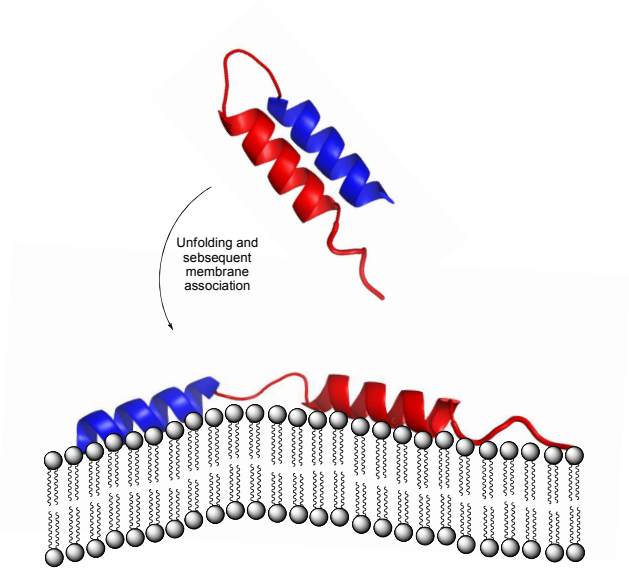


Figure 4.8. *Schematic representation of the hypothesized membrane association of the PYY analogs with an additional N-terminal α-helix. The inherent PYY13-36 segment is shown in red and the novel heptad repeat α-helical sequence is shown in blue.*

More than 20 PYY analogs of series B have been designed, synthesized, characterized, and assayed. Several showed increased Y receptor selectivity (Y2 over Y1) and a few even showed increased Y2 receptor affinity and potency. A selection of the most selective compounds is given in Table 4.5. Especially peptides **15-18** showed to be very potent Y2 receptor agonists with a two- to fivefold increase in potency compared to PYY3-36. The increase in Y2/Y1 receptor selectivity mainly originates from an increase in Y2 receptor potency, thus somewhat unexpected the addition of an α-helix instead of the *N*-terminus of PYY did not disturb the activation of the Y1 receptor. Peptide **17** is currently being investigated in a fluorescence-based single liposome assay. Here, the binding affinity towards the membrane and compound density vs. vesicle size can be established.[210] The aim with this study is to establish a correlation between increased membrane binding affinity and receptor activation. Moreover, the fluorescence-based single liposome assay can further support a membrane compartment model for PYY3-36.[1-5] Finally, the secondary structure of peptide **17** is being studied by CD in the presents of liposomes to establish the degree of α-helicity when associated to a membrane.

Table 4.5. PYY3-36 and R^1-NRYYASLRHYLNLVTRQRY-NH_2 (R^1-PYY18-36) binding to Y receptor subtypes Y1, Y2, and Y4, in addition to functional Y1 and Y2 receptor evaluation of the most potent Y2R agonists.

		Binding affinity assay, IC_{50} [nM]			Signal transduction assay, EC_{50} [nM]	
Peptide	*N*-terminal, R^1	Y1R[a]	Y2R[b]	Y4R[b]	Y1R[c]	Y2R[c]
PYY3-36	H-IKPEAPGEDASPEEL-	7.8 ± 1.1	0.50 ± 0.09	255 ± 29	90 ± 12	5.6 ± 1.1
14	H-AEELLKKLEELLKKASPEEL-	424 ± 2	6.1 ± 1.8	>1000	141 ± 11	32 ± 13
15	H-YESLLKKLSELLKKASPEEL-	239 ± 124	4.9 ± 2.7	>1000	94 ± 33	3.9 ± 2.8
16	H-YLERKLKELERKLKELSPEEL-	11 ± 1.4	0.9 ± 0.4	>1000	113 ± 21	2.3 ± 1.2
17	H-YLERELKKLERELKKLSPEEL-	>1000	3.2 ± 2.6	>1000	97 ± 31	3.1 ± 1.8
18	H-YLKALKEALKALKEALKSPEEL-	>1000	4.3 ± 0.5	664 ± 276	63 ± 27	1.1 ± 1.0
19	H-NLEELKKKLEELKGSPEEL-	27 ± 0.4	2.1 ± 0.5	532 ± 354	-	-

[a] SK-N-MC cells expressing the Y1R. [b] Y2R or Y4R transfected HEK293 Flp-In T-Rex cells. [c] Y1R or Y2R and GαΔ6qi4myr transfected COS-7 cells.

The work of project 4 has resulted in two manuscripts - one for each series. Series A: Manuscript submitted for publication in ChemMedChem (Paper 4). Series B: Manuscript under preparation (Paper 5).

Chapter 5. Concluding remarks and perspective

The increasing research in gut-brain interactions are resulting in an increasing understanding of the control and regulation of food intake and energy homeostasis. New targets and ligands are still appearing, however, GLP-1 and PYY3-36 are still among the most interesting biopharmaceuticals as anti-obesity therapeutics. Thus, the thesis aim of developing PYY3-36 analogs, which are more selective, highly potent, and metabolically stable, is still highly relevant. In the present thesis, numerous novel Y2 receptor ligands with increased affinity and potency as well as enhanced Y receptor selectivity were designed, synthesized, characterized and assayed. The peptides were synthesized using standard Fmoc SPPS at room temperature. Especially the *de-novo* designed PYY analogs of project 4, series B (linear two helix), showed to be complicated to synthesize and purify – typically long coupling times (2 × 2.5 hours) as well as multiple purifications by RP-HPLC were required. The glyco-peptides were also somewhat difficult to synthesize. The resultant yields of the glyco-peptides were generally low, possibly because of high steric hindrance in the coupling steps of the glyco-amino acids.

As part of the Ph.D.-project and in close cooperation with Rheoscience scientists and technicians, the radioligand competition binding assay was set up and further improved to enhance the performance of the assay. The radioligand competition binding assay was used as first line of evaluation in the pursuit of potent and selective Y2 receptor agonists.

Four successful novel peptide designs of PYY3-36 have been explored: *i*) Modifying the conserved *C*-terminal Tyr-36 by non-proteinogenic phenylalanine analogs has led to several highly potent and selective Y2 receptor of which especially the halogenated analogs showed to be prosperous. Phe-36 analogs have the potential to improve the Y receptor selectivity in other PYY3-36 agonists, where the *C*-terminus is intact. *ii*) Using PYY3-36 as a model peptide, the glyco-scan concept has been formulated and introduced. The glyco-scan concept has the potential for a broad applicability in drug discovery alongside the Ala-scan. A glyco-scan can, as shown by PYY3-36, provide novel compounds with unique physical properties that can led to altered affinities towards a given target. *iii*) Alteration of the turn motif of PYY has resulted in analogs giving sub-nanomolar affinity towards the Y2 receptor as well as animal experiments showing a significant reduction in body-weight and food intake. One analog showed beneficial effects on the fat-cell metabolism. The reason for these unexpected results is speculative, but activation of orphan receptors in the adipose tissue or in the hypothalamus could be an explanation. *iv*) An additional amphipathic α-helix was introduced either at, or instead of,

the *N*-terminus of PYY3-36 to increase the affinity towards the membrane prior to receptor recognition. Both series showed interesting results, i.e. increased affinity, potency and selectivity towards the Y receptors. Moreover, two analogs of series A reduced body-weight in acute mouse studies. The novel analogs of series B resulted in highly potent and selective analogs, which via their design partly confirm the membrane compartment model for the PP-fold peptides. The introduction of an additional α-helix also led to an improved Y2/Y1 receptor selectivity, mainly as result of the increase in Y2 receptor potency. Generally, the affinity towards the Y1 receptor was unaffected which was somewhat unexpected because the *N*-terminus of PYY previously has been shown to be of great importance for Y1 receptor activity. Further studies are currently been performed to establish the binding affinity of peptide **17** to the membrane and to determine its secondary structure in the presents of liposomes. These studies will hopefully support and confirm the underlying principle of the design of the analogs of series B.

The rational design has resulted in several highly potent and selective Y2 receptor agonists from a fairly small set of novel analogs. The hit rate has been reasonably high, which support the basis for doing rational designs on medium sized peptide hormones such as PYY3-36. Besides providing several highly potent and selective compounds the rational designs has lead to valuable knowledge about ligand-receptor and ligand-membrane interactions, which can be used in the design of new Y2 receptor agonists.

Second-generation PYY analogs could involve introducing *de novo* heptad repeat sequences as the branch at Lys-4 of PYY3-36. Selected *de novo* heptad repeat sequences showed very attractive results in the series B of project 4, and could lead to enhanced pharmacological effects of the branched analogs (series A). Moreover, altering the linear two helix analogs (series B) in the hinge (turn) region, for example by the GPRRP turn or as a branch at an additional Lys, could lead to increased selectivity. Moreover, *p*-bromo-Phe could be introduced instead of the *C*-terminal Tyr of analogs of projects 2, 3, and 4 to further increase Y receptor selectivity. Finally, the highly potent linear two helix (series B) analogs should be either lipidated or PEGylated to increase their half-lives.

From a physiological perspective, the energy balance is regulated by numerous hormones that act in concert. In response to a reduction in food intake and adiposity the body mounts a counter-regulatory response to stop further weight-loss which might limit the effectiveness of single-agent weight-loss therapies. Consequently, the effects of combining two or more anorectic agents that act on different receptor systems have been reported to give additive effects (e.g. combined administration of native GLP-1 and PYY3-36).[44] Novel long-acting, highly potent and selective analogs of PYY3-36 should further improve the effect of dual therapeutics. Recently, a new series of novel peptides with agonism at both the glucagon and GLP-1 receptors has been published.[211]

The results gained in this thesis provide a greater understanding of the pharmacology of PYY3-36 and the pharmacology of the Y receptors, which hopefully can lead to new and more efficacious therapeutics against obesity.

Part IV
‘References’

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Appendix 1

Paper 1:

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Modifying the conserved *C*-terminal tyrosine of the peptide hormone PYY3-36 to improve Y2 receptor selectivity

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The Y2 selective PYY derived peptide PYY3-36 was recently shown to play a role in appetite regulation. Novel PYY3-36 analogs with high selectivity for the Y2 receptor could be potential drug candidates for the treatment of obesity. The *C*-terminal pentapeptide segment of PYY3-36 is believed to bind to the Y receptors. Tyr-36 is highly conserved across species and only few successful modifications of Tyr-36 have been documented. PYY3-36 analogs were prepared using solid-phase peptide chemistry and tested for binding to the Y1, Y2 and Y4 receptor subtypes by radioligand displacement assay. The Y2 receptor agonists with the best affinity and selectivity were further investigated for activity towards the Y1 and Y2 receptor subtypes. Unexpectedly, modifications of Tyr-36 were well-tolerated, and the analogs of PYY3-36 in which the Tyr-36 hydroxyl group was substituted with a halogen or an amino group were particularly well tolerated and yielded an improved selectivity and approximately equipotent affinity to the Y2 receptor. These modifications could be used to design new potential drug candidates for the treatment of obesity.

Keywords: PYY; PYY3-36; Y2 receptor agonist; gut hormone; tyrosine analogs; obesity

Introduction

More than 35 peptides with either stimulatory or inhibitory effects on appetite have been identified to date. [1] A number of these peptides are produced by endocrine cells lining the gut and are released during ingestion and digestion of a meal. One well-known gut hormone recently implicated in appetite regulation is the 36 amino acid linear 'peptide tyrosine tyrosine' (PYY) first isolated from porcine intestine. [2] PYY is secreted by intestinal L-cells following ingestion of a meal. [3] After secretion, PYY is *N*-terminally truncated by dipeptidyl peptidase IV (DPP-IV) to produce PYY3-36. PYY and PYY3-36 exert a number of actions on gastrointestinal functions including inhibition of pancreatic exocrine secretion, [4–7] inhibition of gastrointestinal motility, [8–11] inhibition of gastric acid secretion [12,13] and stimulation of gallbladder contraction [14]. Recently, PYY3-36 was shown to inhibit food intake in rats and humans. [15,16] Subsequently, several studies in rats and mice using intraperitoneal [17–19] or intravenous [20,21] administration of PYY3-36 have shown acute reductions of food intake. Chronic administration of PYY3-36, in different animal models, has been shown to reduce body weight, [19,22] further supporting a role of PYY3-36 in appetite and weight control.

PYY belongs to the PP-fold family of peptides together with NPY and pancreatic polypeptide (PP). PP is a circulating peptide hormone produced and released from the endocrine pancreas in response to ingestion of food. [23] The peptide hormone alters biliary function and inhibits pancreatic secretion, as well as gastric and intestinal motility [24–26] and was recently shown to inhibit food intake in humans. [15,16] The second family member, NPY, acts as a neurotransmitter and is abundant in the central and peripheral nervous system. [27] The physiological functions of NPY are numerous, e.g. related to feeding, memory, blood pressure, cardiac contractility and intestinal secretion. [28] All three peptides bind to the family of Y receptors; the Y1, Y2, Y4 and Y5 subtypes. While PP is a potent Y4 receptor agonist, it displays very low affinity to the Y1, Y2 and Y5 receptors. NPY and PYY1-36 have very similar binding profiles, and their orexigenic effects are believed to be predominantly mediated via a nanomolar affinity to hypothalamic Y1 receptor. PYY3-36 is believed to be the endogenous ligand for the Y2 receptor to which it binds with sub-nanomolar affinity, however, it also binds with sub-micromolar affinity to both Y1 and Y4 receptors. [29] The appetite suppressing properties of PYY3-36 is believed to be mediated by central Y2 receptors. [16] Interestingly, recent data from rodents and humans suggests that PYY3-36 administration could interact with Y1 and/or Y5 receptors, and thereby counteract the anorectic effects of Y2 stimulation. [30,31] If this is indeed the case, novel highly Y2 selective compounds should show more anorectic and body-weight lowering potential.

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Table 1. Sequences of human NPY, human PP, human PYY1-36 and [^{36}X]-PYY3-36

Peptide	Sequence	*C*-terminal[a]
PP	H-APLEPVYPGDNATPEQMAQYAADLRRYINML	TRPRY-NH_2
NPY	H-YPSKPDNPGEDAPAEDMARYYSALRHYINLI	TRQRY-NH_2
PYY1-36	H-YPIKPEAPGEDASPEELNRYYASLRHYLNLV	TRQRY-NH_2
[^{36}X]-PYY3-36[b]	H-IKPEAPGEDASPEELNRYYASLRHYLNLV	TRQR**X**-NH_2

[a] Conserved pentapeptide.
[b] Human PYY3-36: X = Y.

Peptide Design

Since the discovery of the PP-fold peptides, a vast number of studies have investigated the PP-fold peptide structure and their Y receptor affinities. The *C*-terminal pentapeptide sequences of native NPY, PYY1-36 and PP are highly conserved and play a pivotal role for the affinity to the binding pocket of the Y receptors (Table 1). [32,33] Diverse interaction between acidic Y receptor residues and the *C*-terminal arginines at position 33 and 35 of the PP-fold of peptides are most likely leading to differences in the docking mode of the ligands to the Y receptors, which could be one reason for the affinity differences. [34] An exception to the conserved *C*-terminal is Pro-34 of PP, which could partly explain its differentiated Y receptor selectivity. The *C*-terminal Tyr is assumed to be essential for NPY binding and changes will lead to dramatic reduction in affinity to both the Y1 and Y2 receptor subtypes. [33] Ala scans of NPY [35] and PYY3-36 [36] both report complete loss of affinity to the Y1 and Y2 receptors when replacing Tyr-36 with Ala. These results strongly indicate that the effect from the aromatic side-chain of Tyr-36 is extremely important for the binding to the Y receptor subtypes. [35,36] NPY has additionally been point-substituted at position 36 by replacing Tyr with Phe, which lowered the affinity (13-fold for Y1R and 11-fold for Y2R) but had no impact on selectivity. [35] However, incorporation of the large, hydrophobic residue 4-benzoyl-phenylalanine, [^{36}Bpa]-NPY, gave a 590-fold decrease in affinity to the Y1 receptor and a moderate eightfold-drop in binding to the Y2 receptor. [37] The steric prerequisite for receptor binding was further explored by introducing a D–Tyr at position 36 of NPY, which resulted in a highly reduced affinity to the Y1 receptor (100-fold), compared to modest 10-fold drop to the Y2 receptor. [38] These results indicate that the Y receptors favour an aromatic side-chain at residue 36, and the size could lead to Y2 receptor selectivity due to steric limitations of the Y1 receptor.

Here we describe a number of [^{36}X]-PYY3-36 analogs; we aimed at increasing Y2 receptor selectivity while maintaining its potency. This series included peptides of unnatural Phe or Tyr analogs, which could aid in understanding the structural requirements in the receptor binding pocket.

Materials and Methods

Materials

The organic solvents and reagents for peptide synthesis were all of analytical reagent grade and were obtained from Iris Biotech GmbH (Germany), except for DMF which was obtained from Sigma-Aldrich (Denmark). TentaGel S Rink Amide resin was obtained from Novabiochem. Milli–Q (Millipore) water was used for RP–HPLC analyses and purifications. Human Embryonic Kidney (HEK) 293 cells (HEK293 Flp-In T-Rex) and pcDNA3.1 vector were purchased from Invitrogen. Dulbecco's Modified Eagle's Medium (D-MEM), FCS, penicillin–streptomycin solution, phosphate buffered saline (PBS), sucrose and 99% glycerol were obtained from Sigma-Aldrich. FuGENE 6 transfection reagent, complete protease inhibitor cocktail tablets and BSA were purchased from Roche. 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS), $MgCl_2 \cdot 6H_2O$, $CaCl_2$ and NaCl were obtained from AppliChem GmbH. (*2S*,*3S*)-1,4-bis-sulfanylbutane-2,3-diol (DTT) was obtained from GE Healthcare, and radioactive labeled agonists were purchased from Phoenix pharmaceuticals. 96-Well filtration multiscreen HTS, DV plates were obtained from Millipore. [^{3}H]-*myo*-inositol (PT6-271) was purchased from Amersham.

Peptide Synthesis

The peptides were prepared by automated peptide synthesis on a Syro II peptide synthesiser (MultiSynTech) by standard SPPS on TentaGel S Rink Amide resin with Fmoc for protection of N^{α}-amino groups. Side-chain protecting groups were *tert*-butyl (Ser, Thr, Tyr), 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf, for Arg), and trityl (Trt, for Asn, Gln, His). N^{α}-Fmoc amino acids (4.0 equiv) were coupled using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide HBTU (3.8 equiv), 1-HOBt (4.0 equiv) and *N*,*N*-DIEA (8.0 equiv) as coupling agents in DMF for 45 min, except unnatural amino acids which reacted for 120 min. N^{α} -Fmoc deprotection was performed using piperidine-DMF (2 : 3) for 3 min, followed by piperidine-DMF (1 : 4) for 12 min. The peptide amides were released from the solid support by treatment with TFA-triethylsilane (TES)-H_2O (95 : 2 : 3) for 2 h. The TFA solutions were concentrated by nitrogen flow and the compounds were precipitated with diethylether to yield the crude materials as white powders. Purification was accomplished by preparative RP-HPLC (Dionex Ultimate 3000 system) on a preparative column (FeF Chemicals, 300 Å 5 μm C4 particles, 2.1 × 200 mm) using the following solvent system: solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA. B gradient elution (0–50 min: 10–60%) was applied at a flow rate of 10 ml min^{-1} and column effluent was monitored by UV absorbance at 215 and 254 nm simultaneously. Identification was carried out by ESI-MS (MSQ Plus Mass Spectrometer, Thermo). The peptides were analysed by analytical HPLC (Dionex Ultimate 3000 system equipped with a PDA UV detector or Dionex P580 pump equipped with Waters 996 PDA and Waters 717plus autosampler) using 'orthogonal' eluent systems, first A–B (solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA, as above), then eluent system C–D (solvent C, 10 mM NH_4OAc; solvent D, 10% 100 mM NH_4OAc in AcN). First analysis; eluent system A–B was applied on a C4 analytical column (Phenomenex, Jupiter, 300 Å 5 μm C4 particles, 3.9 × 150 mm) where a B gradient elution (0–14 min: 5–100%) was applied at a flow rate of 1.0 ml min^{-1}. Second analysis; eluent system C–D was applied on a C18 analytical column (Phenomenex, Gemini, 110 Å 3 μm C18 particles, 4.60 × 50 mm) where a C gradient elution (0–14 min: 5–100%) was applied at a flow rate of 1.0 ml min^{-1}. Quantification and characterisation data are given in Table 2.

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Table 2. Quantification and characterisation data of PYY3-36 and H-IKPEAPGEDASPEELNRYYASLRHYLNLVTRQR^{36}**X**-NH_2 analogs

No	^{36}X	ESI-MS (m/z)[a] Calculated MS	Found	Assigned	Purity (%) Eluent system A-B[b]	Eluent system C-D[c]
PYY3-36	Tyr	4049.46	1012.76	$[M+4H]^{4+}$	98	99
			810.21	$[M+5H]^{5+}$		
1	*homo*-Tyr	4063.49	1016.87	$[M+4H]^{4+}$	99	99
			813.36	$[M+5H]^{5+}$		
2	*D*-Tyr	4049.46	1013.03	$[M+4H]^{4+}$	97	97
			810.40	$[M+5H]^{5+}$		
3	*p*-fluoro-Phe	4052.44	1013.51	$[M+4H]^{4+}$	99	99
			810.83	$[M+5H]^{5+}$		
4	*p*-chloro-Phe	4061.91	1017.19	$[M+4H]^{4+}$	99	99
			814.17	$[M+5H]^{5+}$		
5	*p*-bromo-Phe	4112.36	1028.66	$[M+4H]^{4+}$	99	100
			823.19	$[M+5H]^{5+}$		
6	*p*-iodo-Phe	4160.35	1040.09	$[M+4H]^{4+}$	95	95
			832.35	$[M+5H]^{5+}$		
7	*p*-nitro-Phe	4078.46	1019.81	$[M+4H]^{4+}$	96	95
			816.18	$[M+5H]^{5+}$		
8	*p*-amino-Phe	4048.48	1013.12	$[M+4H]^{4+}$	99	96
			810.16	$[M+5H]^{5+}$		
9	*m*-nitro-Tyr	4094.16	1024.15	$[M+4H]^{4+}$	98	98
			819.20	$[M+5H]^{5+}$		

[a] Identified by ESI-MS on an MSQ Plus Mass Spectrometer (Dionex).
[b] Quantified by RP-HPLC at 215 nm. Eluent A: 0.1% TFA in H_2O. Eluent B: 0.1% TFA in AcN.
[c] Quantified by RP-HPLC at 215 nm. Eluent C: 10 mM NH_4OAc. Eluent D: 10% 100mM NH_4OAc in AcN.

Radioligand Displacement Assay

Cell culture and receptor expression: The SK-N-MC cells were cultured in a 1 : 1 mix of HAM F12 and D-MEM 1885, containing 15% (v/v) FCS, 1% non-essential amino acids and 1% (v/v) penicillin–streptomycin in humidified atmosphere of 5% carbon dioxide and 95% air, at 37 °C. The HEK-derived (293 Flp-In T-Rex) cell line was cultured in D-MEM, containing 10% (v/v) FCS and 1% (v/v) penicillin–streptomycin. Cells were grown as monolayers in humidified atmosphere of 5% carbon dioxide and 95% air, for 48 h at 37 °C. Using serum-free D-MEM and FuGENE, six transfection reagent HEK293 Flp-In T-Rex cells were transiently transfected by pcDNA3.1 vectors which encode either the human Y2 or Y4 receptor (FuGENE 6/pcDNA3.1, 3 : 1). The transfected HEK293 Flp-In T-Rex cells were set to express the receptors in humidified atmosphere of 5% carbon dioxide and 95% air, for another 48 h at 37 °C.

Preparation of membrane fractions

The SK-N-MC and the transiently transfected HEK293 Flp-In T-Rex cells were washed with PBS and homogenized in cold homogenisation buffer (50 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.0 mM DTT, complete protease inhibitor cocktail (one tablet per 50 ml buffer)). An equal amount of 0.6 M sucrose was added to the cell-mixture. The homogenate was centrifuged (10,000 g, 10 min at 4 °C). Cell pellets were washed in washing buffer (50 mM TRIS (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 ml buffer), 1.0% (w/w) BSA fraction V), and subsequently the suspensions were centrifuged (10,000 g, 10 min at 4 °C). The pellets were re-suspended in glycerol containing binding buffer [50 mM TRIS (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 ml buffer), 1.0% (w/w) BSA fraction V, 20% (v/v) glycerol] and concentration was adjusted to an OD600 of 1.6.

Binding affinity

All binding experiments were performed in 96-well filtration MultiScreen HTS, DV plates and every concentration point was performed as triplicates. The unlabeled peptide (25 µl) at concentrations between 10 pM and 10 µM, cell membrane suspension (3.5 µl), binding buffer (61.5 µl, 50 mM TRIS, pH 7.5, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail [one tablet per 50 ml buffer), 1.0% (w/w) BSA fraction V)], radioligand solution (10 µl; Y1R: ^{125}I-[^{31}Leu,^{34}Pro]-pNPY; Y2R: ^{125}I-PYY3-36; and Y4R: ^{125}I-PP; specific activity of 800–1000 Ci/mol). After 1–2 h of incubation, the assay was terminated by filtration. Finally, the membrane-receptor ligand complexes were washed twice in cold TRIS buffer (50 mM TRIS, pH 7.5, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$) containing 5.0 % (w/w) BSA fraction V. Bound radioactivity was determined as counts per minute (Wallac 1470 Wizard™ Automated Gamma Counter). Binding data were analysed with GraphPad Prism 5.0 (GraphPad Software, Inc. San Diego, CA, USA). The displacement experiments were replicated until the standard error of mean (S.E.M.) of $-\log IC_{50}$ was below 0.1 ($n = 2–5$).

Functional Assay

Transfections and tissue culture: COS-7 cells were grown in D-MEM 1885 supplemented with 10% FCS, 2 mM glutamine and 0.01 mg/ml gentamicin. Cells were transfected with 10 µg cDNA

of wild type Y1 or Y2 receptors and 10 μg cDNA of a Gα Δ6qi4myr [39], using the calcium phosphate precipitation method with chloroquine addition. The chimeric G-protein allow the Gαi coupled receptors to signal through the signal transduction pathways known for the Gαq coupled receptors. Receptors from the PP-fold family of peptides – the Y1 and Y2 receptors – were cloned from a human cDNA library and expressed in a pcDNA3.1 vector.

Phosphatidylinositol turnover

One day after transfection, COS-7 cells were incubated for 24 h with 5 μCi of [^{3}H]-*myo*-inositol in 1 ml medium, washed twice in buffer, 20 mM HEPES, pH 7.4, and were subsequently incubated in 0.5 ml buffer supplemented with 10 mM LiCl at 37 °C for 30 min. After stimulation with various concentrations of PYY3-36 or the synthetic analogs for 45 min at 37 °C, cells were extracted with 10% ice-cold formic acid followed by incubation on ice for 30 min. The generated [^{3}H]-inositol phosphate was purified on Bio-Rad AG 1-X8 anion-exchange resins. Determinations of each measuring point were made in duplicates. The functional assays were replicated three times (except PYY3-36 which was replicated four times).

Results and Discussion

Peptide Synthesis

All peptides were assembled using the Fmoc/*t*-Bu strategy by automated SPPS on Rink Amide TentaGel resin. The N^{α}-Fmoc-protected amino acids were coupled using HBTU as coupling reagent, DIEA as base and HOBt as additive, in DMF. Coupling times were generally 45 min, except for the unnatural amino acid derivatives which were coupled for 2 h. The peptides were side-chain deprotected and simultaneously cleaved from the solid support by a TFA cocktail containing TES and water as scavengers. Finally, the peptides were purified by RP-HPLC and characterized orthogonally by analytical HPLC and mass spectrometry. The final products were obtained with >95% purity (Table 2).

Affinity and Activity Evaluation

To characterize the influence of substituting Tyr-36 with unnatural amino acid analogs, the binding affinity of the novel PYY3-36 analogs were tested using a radioligand displacement assay (competition binding assay) based on membranes from SK-N-MC and HEK293 Flp-In T-Rex cell lines expressing the human Y1, Y2 and Y4 receptor subtypes, respectively. ^{125}I-[^{31}Leu,^{34}Pro]-pNPY for Y1R, ^{125}I-hPYY3-36 for Y2R and ^{125}I-hPP for Y4R were used as radioligands. The analogs with the highest affinity and potency for the Y2 receptor were additionally tested for their ability to activate the Y1 and Y2 receptors. Binding affinities (IC_{50} values) and activation potencies (EC_{50} values) for the [^{36}X]-PYY3-36 analogs as well as native PYY3-36 are summarized in Table 3.

Structure–Affinity Relationship

PYY3-36 is highly potent but only moderately selective, whereas all the novel peptides in this series had an increased Y receptor selectivity. First, extending the length of the Tyr-36 side-chain by an extra methylene moiety moves the phenol group further away from the peptide backbone, which potentially influences the aromatic $\pi-\pi$ interaction and the hydroxyl hydrogen bonding interactions between the receptor and the ligand in a positive manner. [^{36}*homo*Tyr]-PYY3-36 (Table 3, peptide **1**) lowered the affinity more than 100-fold for the Y1 receptor, whereas only a modest threefold drop in affinity to the Y2 receptor was observed. These results could indicate steric restrictions in the Tyr-36 binding pocket of the Y1 receptor, compared to the Y2 receptor. Other explanations for the decreased Y1 receptor affinity could be due to the side-chain of *homo*-Tyr having one more rotatable bond, compared to Tyr, and hence the possibility that it binds to another region of the Y1 receptor binding pocket.

The binding affinities of the Y-receptors were poorer when the orientation of the side-chain of Tyr-36 was converted to the corresponding D-analog. The binding affinity of [^{36}D-Tyr]-PYY3-36 (Table 3, peptide **2**) to the Y2 receptor decreased sevenfold and basically had no affinity to the Y1 receptor (>100-fold drop). The relative Y2 receptor affinity corresponds to that of [^{36}D-Tyr]-NPY, [38] but [^{36}D-Tyr]-PYY3-36 (Table 3, peptide **2**) was far more

Table 3. PYY3-36[a] and [^{36}X]-PYY3-36 binding to Y receptor subtypes Y1, Y2, and Y4, in addition to functional Y1 and Y2 receptor evaluation of the most potent Y2R agonists

		Binding assay			Functional assay	
Peptide	^{36}X	Y1R[b] IC_{50} [nM]	Y2R[c] IC_{50} [nM]	Y4R[c] IC_{50} [nM]	Y1R[d] EC_{50} [nM]	Y2R[d] EC_{50} [nM]
PYY3-36		7.8 ± 1.1	0.50 ± 0.09	255 ± 29	135 ± 32	7.6 ± 1.6
1	*homo*-Tyr	>1000	1.63 ± 0.44	>1000	>1000	51 ± 6.0
2	*D*-Tyr	>1000	3.50 ± 0.15	>1000	n.d.	36 ± 13
3	*p*-fluoro-Phe	>1000	0.76 ± 0.02	>1000	737 ± 30	12 ± 1.0
4	*p*-chloro-Phe	198 ± 14	1.79 ± 0.22	>1000	>1000	5.2 ± 1.6
5	*p*-bromo-Phe	350 ± 101	1.67 ± 0.06	>1000	>1000	5.4 ± 2.1
6	*p*-iodo-Phe	>1000	1.65 ± 0.51	>1000	>1000	11 ± 4.0
7	*p*-nitro-Phe	>1000	8.27 ± 2.85	>1000	n.d.	55 ± 4.0
8	*p*-amino-Phe	388 ± 175	0.96 ± 0.32	>1000	367 ± 98	11 ± 2.0
9	*m*-nitro-Tyr	246 ± 87	12.0 ± 5.9	>1000	n.d.	50 ± 11

[a] Native peptide.
[b] SK-N-MC cells expressing the Y1R.
[c] Y2R or Y4R transfected HEK293 Flp-In T-Rex cells.
[d] Y1R or Y2R and Gα Δ6qi4myr transfected COS-7 cells.

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selective. The results above show that the position and orientation of the aromatic side-chain relative to the peptide backbone is very important for binding to the Y1 and Y4 receptors, but the Y2 receptor seems more tolerant of the conformational changes.

Replacing the hydroxyl group with electron-withdrawing substituents at the *para* position of residue Phe-36 (Table 3, peptides **3–7**) resulted in a moderate to significant decrease in the affinity to the Y1 receptor, in the affinity order Cl ≈ Br > F ≈ I ≈ NO_2, but only moderate decreases in the affinity to the Y2 receptor, in the affinity order F ≈ Cl ≈ Br ≈ I ≫ NO_2. The fact that the introduction of electron-withdrawing substituents in the *para* position of Phe-36 negatively affects Y1 receptor binding could be due to steric interactions in the active site as binding affinity decreased with size of the substituents. As [^{36}Phe(*p*-Cl)]-PYY3-36 (Table 3, peptide **4**), [^{36}Phe(*p*-Br)]-PYY3-36 (Figure 1 and Table 3, peptide **5**) and [^{36}Phe(*p*-I)]-PYY3-36 (Table 3, peptide **6**) all bound with nanomolar affinity to the Y2 receptor, and neither chlorine, bromine nor iodine participates in hydrogen bonding, shows that the interaction between residue 36 of PYY3-36 and the Y2 receptor could be influenced by van der Waals forces and $\pi - \pi$ bonding. Interestingly, the fluorinated compound, [^{36}Phe(*p*-F)]-PYY3-36, both increased Y1/Y2 receptor selectivity and maintained a high affinity to the Y2 receptor (Figure 1 and Table 3, peptide **3**). Hydroxyl and fluorine share several properties, in particular polarity. [40,41] Fluorine is not a sterically demanding substituent, given its small van der Waals radius (1.35 Å) which resembles the one of hydrogen (1.20 Å). [42] Fluorine cannot donate a hydrogen bond but may rather, due to its electronegative properties, accept them. [41] Finally, fluorine-containing compounds cause the substituent to be more resistant to metabolic degradation, because of the high carbon-fluorine bond energy. [42] The fact that [^{36}Phe(*p*-F)]-PYY3-36 (Figure 1 and Table 3, peptide **3**) is a good agonist for the Y2 receptor indicates that interactions with the active site resembles that of PYY3-36.

Exchanging the hydroxyl group of Tyr-36 with an amine, such as that found in [^{36}Phe(*p*-NH_2)]-PYY3-36 (Table 3, peptide **8**), resulted in sub-nanomolar affinity to the Y2 receptor. This amino group is a hydroxyl isostere because the *anilino* amino group of phenylalanine is not protonated at physiological pH. [^{36}Phe(*p*-NH_2)]-PYY3-36 may introduce a minor steric disturbance, as the amino group is slightly larger than the hydroxyl group and a reduced affinity to the Y1 and Y4 receptor subtypes was observed (Table 3, peptide **8**).

None of the peptides in this series showed improved Y4 binding or even an affinity comparable with that of the native PYY3-36. All [^{36}X]-PYY3-36 analogs resulted in poor Y4 affinities above 1000 nM. The receptor sequence homology between the Y1 and Y4 receptor is high (42%) but comparatively it is low between the Y1 and Y2 receptor (31%). [43] Thus, a loss in Y1 receptor affinity is also likely to lead to a decrease in Y4 receptor affinity, which is indeed what is observed (Table 3).

Structure–Activity Relationship

All the PYY3-36 analogs were investigated for Y2 receptor activity and the most potent analogs were additionally tested for Y1 receptor activity (Figure 2 and Table 3). The decreases in affinity observed in competition binding studies for the PYY3-36 analogs, compared to the native PYY3-36, were also mirrored in the functional assay for the Y2 receptor. Only [^{36}*homo*Tyr]-PYY3-36 (Table 3, peptide **1**) was slightly more affected in potency compared to binding, as a sevenfold reduction in Y2 receptor potency was observed compared to a threefold decrease in affinity. This difference could be explained by a steric interference of *homo*-Tyr with the conformational changes to the active state of the receptor independent of the high affinity. [^{36}*homo*Tyr]-PYY3-36 shows a small drop in Y2 receptor activity, however, it also results in a considerable reduction in Y1 receptor activity (Table 3, peptide **1**), which confirms that the introduction of one more rotatable bond increases the Y1/Y2 receptor selectivity considerably.

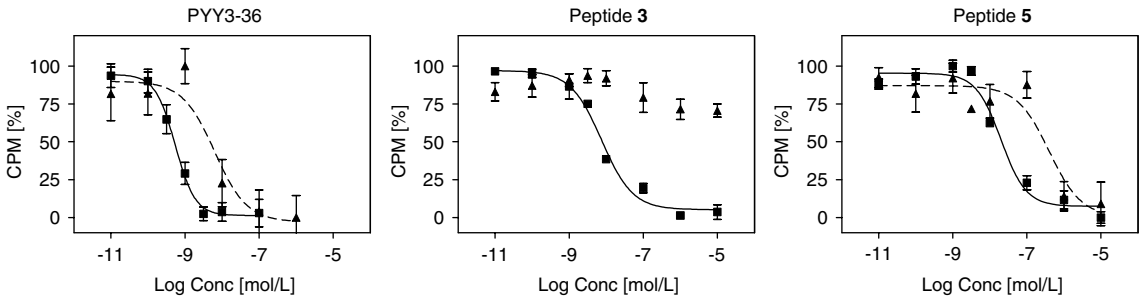

Figure 1. Radioligand binding curves. The binding of PYY3-36, [^{36}Phe(*p*-F)]-PYY3-36 (**3**) and [^{36}Phe(*p*-Br)]-PYY3-36 (**5**) towards the Y1 (▲) and Y2 (■) receptors.

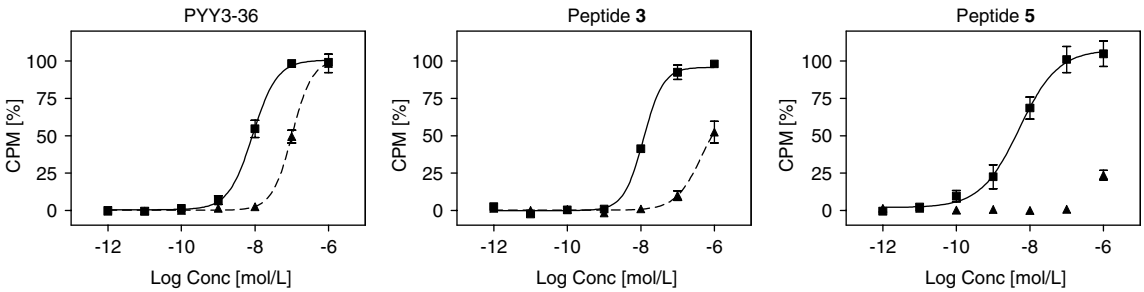

Figure 2. Functional assay. The activity of PYY3-36, [^{36}Phe(*p*-F)]-PYY3-36 (**3**) and [^{36}Phe(*p*-Br)]-PYY3-36 (**5**) towards the Y1 (▲) and Y2 (■) receptors.

[^{36}D-Tyr]-PYY3-36 (Table 3, peptide **2**), [^{36}Phe(*p*-NO_2)]-PYY3-36 (Table 3, peptide **7**) and [^{36}Tyr(*m*-NO_2)]-PYY3-36 (Table 3, peptide **9**) resulted in four- to sevenfold reduction in Y2 receptor activity, as a result these analogs were not investigated for Y1 receptor activity.

Functional data demonstrate that [^{36}Phe(*p*-NH_2)]-PYY3-36 was slightly better accepted by the Y1 receptor than initially shown by the binding assay. The potency of the *anilino* analog was only modestly decreased for the Y1 receptor leading to a small increase in Y1/Y2 receptor selectivity. The halogenated compounds (Figure 2 and Table 3, peptide **3–6**) as well as the *anilino* analog (peptide **8**, Table 3) gave Y2 receptor activities equivalent to PYY3-36, which shows that the Y2 receptor was fairly flexible towards substition in the *para* position of Phe-36. The activity of the Y1 receptor appears to be intolerant to even small changes in the *para* position of Phe-36, as shown by the large drop in affinity for [^{36}Phe(*p*-F)]-PYY3-36 with the small 4-fluoro substituent (Table 3, peptide **3**). The functional activity studies of the chlorine, bromine and iodine analogs (Figure 2, Table 3, peptide **4–6**) confirmed that hydrogen bonding was very important in the ability to activate the Y1 receptor. All halogenated PYY3-36 analogs were poor Y1 receptor activators and resulted in major increases in Y1/Y2 receptor selectivity.

Conclusion

Previously, a significant number of PYY and NPY analogs have been synthesized and analysed, however, the present results show that novel compounds with Y receptor selectivity can be developed by careful modifications of Tyr-36 in the pentapeptide part of PYY3-36, which binds to the receptors. Selectivity was improved using unnatural amino acid derivatives such as *para* substituented phenylalanines. Analogs which were halogenated as well as aminated in the *para* position of Phe-36 of PYY3-36 (**3–6** and **8**) maintained the high Y2 receptor potency shown by native PYY3-36 and all the novel PYY3-36 compounds gave rise to an increased Y1/Y2-receptor selectivity. Most profound were the analogs where the Tyr-36 hydroxyl group was substituted with a halogen (**3–6**), because of their major decreases in Y1 receptor potency, while maintaining an excellent Y2-receptor affinity and activity. *C*-terminal Tyr modified PYY3-36 analogs with sub-nanomolar affinity to the Y2 receptor and simultaneously high Y1/Y2 receptor selectivity have not, to our knowledge, been reported in literature. We believe the insights gained in this study will be useful for the development of potential drug candidates derived from PYY3-36 for the treatment of obesity.

Acknowledgement

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Appendix 2

Paper 2:

Søren L. Pedersen, Catharina Steentoft, Niels Vrang and Knud J. Jensen. Glyco-scan: Varying glycosylation in the sequence of the peptide hormone PYY3-36 and its effect on receptor selectivity, *ChemBioChem*, **2009**, accepted.

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Glyco-scan: Varying glycosylation in the sequence of the peptide hormone PYY3-36 and its effect on receptor selectivity

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The increasing prevalence of obesity worldwide calls for safe and highly efficacious satiety drugs. PYY3-36 has been implicated in food intake regulation and novel peptide analogs with high Y2 receptor subtype selectivity and potency, have potential as drugs for the treatment of obesity. It has been hypothesized that PYY3-36 associates with the plasma membrane prior to receptor activation such that the amphipathic α-helix of PYY3-36 possibly guides the C-terminal pentapeptide into the correct conformation for receptor activation. Ala-scans are used routinely to study the effect of individual amino acids in a given peptide sequence. Here we report the glyco-scan of the peptide hormone PYY3-36 in which hydroxyl side-chain functionalities were glycosylated; in addition new glycosylation sites were introduced. An array of novel PYY3-36 analogs having a glycan positioned in the water-membrane interface or in the N-terminal were screened for Y receptor affinity and selectivity as well as metabolic stability. Interestingly, the Y2 receptor readily accommodated glycosylations in contrast to the Y1 and Y4 receptors. Especially glycosylations in the α-helical region of PYY3-36 were favorable both in terms of Y receptor selectivity and in terms of endopeptidase resistance. We thus report several PYY3-36 analogs with enhanced Y receptor selectivity. Our results can be used in the design of novel PYY analogs for the treatment of obesity. The glyco-scan concept, as systematically demonstrated here, has the potential for a wider applicability.

Introduction

Peptide tyrosine tyrosine (PYY) is a member of the PP-fold family of peptides that also consists of pancreatic polypeptide (PP) and neuropeptide Y (NPY).[1] The physiological actions of the PP-fold family of peptides occur through their activation on a group of G-protein coupled receptors (GPCR), the so-called Y receptors (Y1, Y2, Y4 and Y5), which belong to the rhodopsin-like superfamily (class A) of receptors.[2]

PYY (PYY1-36) is secreted from the endocrine L-cell following the ingestion of a meal.[3] Following secretion PYY1-36 is *N*-terminally cleaved by dipeptidyl peptidase IV (DPP-IV) to yield PYY3-36.[4] The major blood-circulating form of PYY, PYY3-36, has a high affinity towards the Y2 receptor, and to a lesser extent the Y1 receptor.[5] PYY1-36 and PYY3-36 exert several actions on gastrointestinal functions including inhibition of pancreatic exocrine secretion,[6] inhibition of gastrointestinal motility,[7] inhibition of gastric acid secretion[8] and stimulation of gallbladder contraction.[9] Moreover, PYY3-36 reduces food intake.[10-15] The anorectic properties of PYY3-36 has been proposed to be mediated by centrally located presynaptic Y2 receptors located in the hypothalamic arcuate nucleus.[15] Activation of these Y2 receptors would inhibit NPY release leading to a diminished feeding.[15,16] It should be noted, however, that Y2 receptors are also expressed in several other brain areas including the caudal brainstem, areas that could play a role in PYY3-36 mediated appetite suppression.[17] Several studies in both rats and mice using intraperitoneal[11,12,18] or intravenous[13,14] administration of PYY3-36 have shown acute reductions of food intake. Even though Tschop *et al*. questioned the initial findings,[19] chronic administration of PYY3-36, in different animal models, has shown reductions in body-weight,[12,13] supporting the initial findings by Batterham *et al.*[15] that PYY3-36 is involved in appetite and weight control. A role of PYY3-36 in human appetite regulation is further supported by findings that 90 minutes infusion of PYY3-36 to lean or obese humans leads to significant 24-hour reductions in buffet intake.[15,20]

Native PYY3-36 as a pharmaceutical drug candidate suffers from low selectivity and metabolic stability. The half-life of intravenous administrated PYY3-36 has been reported to be 13 minutes in mouse[21] and 19 minutes in rabbit,[22] making metabolically stable and longer-acting analogs of PYY3-36 highly desirable. Several PEGylated versions of full-length and truncated PYY3-36 have shown half-lives that are enhanced up to 24 hours. However, the increased metabolic stability of

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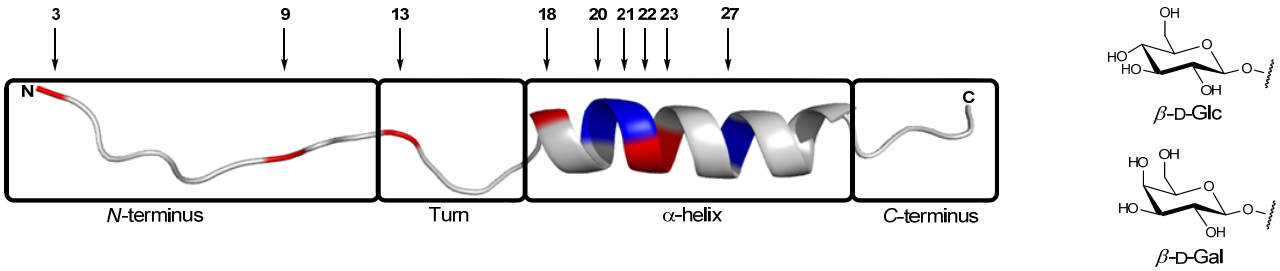

Figure 1. Illustration of the PYY3-36 glycosylation sites. Either β-D-Glc or β-D-Gal are *O*-linked to Ser (red) or Tyr (blue) residues positioned in the flexible *N*-terminal or in the amphipathic α-helix.

PEGylation leads to significant decreases in receptor potency for all Y receptor subtypes and show only minor *in vivo* efficacy superiorities over PYY3-36.[23] Longer-acting PYY3-36 analogs showing better Y2 receptor affinity and selectivity are therefore in demand.

Peptides have previously been glycosylated to extend their half-lives, to improve solubility, to add specific recognition, etc.[24] Also, numerous peptides have been synthesized for studies on recognition, e.g. of mucin glycopeptides.[25] Whereas Ala-scans are generally regarded as the gold standard to study the effect of individual amino acids in a peptide sequence only one very recent study[26] used systematic variation of glycosylation in a peptide sequence for structure function studies. Here we introduce the concept of glyco-scan of a peptide. While in an Ala-scan all amino acids are substituted with the smallest chiral amino acid, Ala, a glyco-scan has wider potentials, including (i) introduction of different carbohydrates (glycans), (ii) glycosylation of side-chains as *O*-, *N*-, and *C*-glycosides, and (iii) glycosylation of either a native or a mutated amino acid. A peptide glyco-scan will allow the study of the effect of one or more of the following properties (i) improvement in solubility, (ii) protection against proteolytic cleavage (which can increase the half-life of the peptide and enhances the concentration at the pharmacological targets), (iii) modification of the clearance, (iv) binding to carbohydrate specific receptors, (v) alteration of the function (e.g. GPCR binding), and many more. A limitation in the application of the glyco-scan concept is that glycopeptides most often are only accessible by chemical synthesis, as current molecular biology allows only some control of *N*-glycosylation but not full control of location of *O*-glycosylation in a sequence. However, reliable protocols for the solid-phase synthesis of glycopeptides have been developed and a range of glycosylated amino acids are commercially available. Hence, a glyco-scan not only offers great possibilities for variation, but can also potentially address more parameters than an Ala-scan. Given the large numbers of possibilities for glycosylation, we anticipate that a subset of structural possibilities will most often be addressed in the glyco-scan of a peptide.

Here we report the application of the glyco-scan concept for the study of the peptide hormone PYY3-36 by the introduction of *O*-glycosides (Figure 1).

Results and Discussion

Starting from the hypothesis by Zerbe and co-workers[27,28] that PYY3-36 binds to the membrane prior to receptor binding, we did not include side-chains from the hydrophobic face but chose amino acids from the hydrophilic side of the helix as well as from the *N*-terminal end. We included residues, such as Tyr, which could be located at the membrane-water interface and where the hydrophobic side of a monosaccharide could potentially interact with the membrane. Glycopeptides are believed to be able to enter the CNS by the glucose transporter (GLUT-1).[29] *C*-linked, *N*-linked and α-*O*-linked sugar conjugates are not expected to promote transport since the glucose transporter is specific for *β*-*O*-linked glucosides.[29,30] Therefore *β*-*O*-linked glycosylated PYY3-36 analogs have the potential of enhancing the CNS permeability and thereby increasing the agonist concentration at the hypothalamic Y2 receptors. As the *C*-terminal pentapeptide of PYY3-36 is highly important for Y receptor potency, no carbohydrates were incorporated into this section of the peptide hormone. Additionally, it has been shown that the α-helix of PYY3-36 is vital for Y receptor activation. It has been hypothesized that the α-helix is guiding the *C*-terminus into the correct conformation for receptor activation[31] and that the amphipathic nature of the PP-fold peptides are leading to an association with the membrane prior to receptor activation.[27,32] The Tyr residues of the α-helix are likely to be located in the water-membrane interface and glycosylation of these residues could potentially stabilize the amphipathic α-helix of PYY3-36. Galactose itself can participate in van der Waals interactions and stacking with aromatic moieties, due to the orientation of hydroxyls in or above the pyranosyl $^4C_1$ plane (Figure 1).[33] Hence, galacto-PYY3-36 could lead to an enhanced amphipathic nature of PYY3-36. It has previously been hypothesized that the Y receptor selectivity could originate from at least two different lateral positions of the α-helix tube, when being docked into the active site of the Y receptor.[34] Hence, pre-organizing the α-helix and the *C*-terminal pentapeptide, both when bound to the lipid bilayer and in the receptor, could disfavour one of the conformations and thereby increase Y receptor selectivity. Thus, the carbohydrate residues will function as a lateral support of the α-helix tube - like an outrigger canoe - preventing it from turning into the unfavoured conformation. Mutations of Tyr-20 and Tyr-27 have been reported to destabilize back-folding in solution more than mutations at Tyr-21. The side-chains of Tyr-20 and Tyr-27 are part of the hydrophobic core in the backfolded structure in the absence of membranes, however Tyr-21 also shows a major impact on backfolding.[35] Thus, *O*-glycosylation of these Tyr side-chains are likely to selectively destabilize the back-folded conformers in solution due to steric repulsion. This may also make these particular PYY3-36 glycoforms more susceptible to proteolytic degradation.

Thus, PYY3-36 was glycosylated at the following Ser and Tyr side-chain hydroxyls: (i) The native Ser and Tyr in human PYY3-36, (ii) position 18 that is Ser in porcine PYY3-36, (iii) residue 3 (Ser) and 22 (Tyr) which were adapted from the NPY sequence, (iv) Gly-9 was mutated to Ser(*β*-D-Gal) (the only uncharged polar residue in the *N*-terminal of PYY3-36). This particular glyco-scan

of PYY3-36 thus called for the synthesis of 15 novel glycoforms of the peptide hormone. Given the possibility that PYY3-36 in pharmacological doses affecting appetite and food intake, also engage central orexigenic Y1 receptors and the poor metabolic stability of native PYY3-36, we aimed at developing more selective (Y2 over Y1) PYY3-36 analogs with the possible additive effect of enhanced metabolic stability.

Peptide synthesis

All peptides were prepared using the Fmoc/*t*-Bu strategy by automated solid-phase peptide synthesis on Rink Amide TentaGel resin (0.1 mmol scale). The N^{α}-Fmoc-protected amino acids were coupled using HBTU, HOBt and DIPEA as coupling reagents, in DMF, with coupling times of 45 minutes, except for the glycosylated N^{α}-Fmoc-protected Ser and Tyr pentafluorophenyl esters which were coupled using HOBt as auxilliary nucleophile in DMF for 2 hours. The glycosylated amino-acid derivatives; N^{α}-Fmoc-Ser(Bz_4-β-D-Glc)-OPfp, N^{α}-Fmoc-Ser(Ac_4-β-D-Gal)-OPfp, N^{α}-Fmoc-Tyr(Bz_4-β-D-Glc)-OPfp and N^{α}-Fmoc-Tyr(Ac_4-β-D-Gal)-OPfp were synthesized according to the literature.[36-38] The Fmoc group was removed using piperidine-DMF (1:4) for 3 + 12 minutes. After completion, the carbohydrates were deprotected on-resin using 1.0% $NaOCH_3$ in MeOH (apparent pH 9.5) for 1 hour. Subsequently, the peptides were side-chain deprotected and simultaneously cleaved from the solid support using TFA-TES-H_2O (95:2:3). Finally, the peptides were purified by preparative RP-HPLC, quantified by analytical RP-HPLC and characterized by mass spectrometry. The final products were obtained with >95% purity (Table 3).

Circular dichroism.

UV CD of PYY3-36 and peptide **1**-**15** (20 μM) in 10 mM phosphate buffer (pH 7.4) were obtained using a JASCO J-810 circular dichroism spectrometer. The spectra acquired were typical for the α-helical structure, showing characteristic minima at 208 nm and 222 nm (Figure 2). The results of PYY3-36 are in agreement with the literature.[39] The degree of α-helicity of the glyco-peptides were between 23 and 41% (Table 1), except peptides **7** (Figure 2) and **12** which gave 47 and 15%, respectively (Table 1). Unexpectedly, the [Tyr(β-D-Glc)]-PYY3-36 analogs (peptide **6** and **7**) and to a lesser extent [Tyr(β-D-Gal)]-PYY3-36 analogs (peptide **13**-**15**) exhibited a higher degree of α-helicity, indicating a stabilization of the α-helix (Table 1 and Figure 2). The introduced carbohydrates were well tolerated and did not, besides peptide **12**, result in disruption of the secondary structure. Given that peptide **4** (28%) and **12** (15%) have significant differences in their degree of α-helicity, and that neither the gluco- nor galacto-PYY3-36 analogs lead to significant differences in Y receptor affinity, we conclude that in this particular case there is no correlation between the degree of α-helicity and Y receptor affinity or selectivity (Table 1).

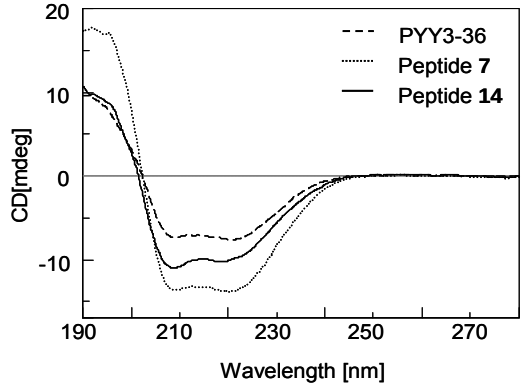

Figure 2. UV CD spectra of PYY3-36, peptide **7** and peptide **14** in aqueous buffered solution (20 μM peptide in 10 mM phosphate buffer).

Structure affinity relationship.

The consequence of glycosylation of PYY3-36 for receptor binding was evaluated using a radioligand displacement assay (competition binding assay) based on membranes from SK-N-MC and HEK293 Flp-In T-Rex cell lines expressing the human Y1, Y2 and Y4 receptor subtypes, respectively. ^{125}I-[^{31}Leu,^{34}Pro]-pNPY for Y1 receptor, ^{125}I-hPYY3-36 for Y2 receptor and ^{125}I-hPP for Y4 receptor were used as radioligands. Binding affinities (IC_{50} values) for PYY3-36 and glycosylated PYY3-36 analogs (peptide **1-15**) are summarized in Table 1 and binding curves of PYY3-36 and peptide **7** are given in Figure 3.

In general, all the glycosylated PYY3-36 analogs only showed a slight (2-3 fold) decrease in Y2 receptor binding affinity. At position 3 an Ile was substituted with a glycosylated Ser (peptide **1** and **8**) leading to an increase in polarity of the PYY3-36 *N*-terminal segment, however, the potency was only slightly affected and equivalent to the Ala-substitution.[40] The *N*-terminally modified analogs (peptide **1**, **2** and **8**-**10**) generally showed 10-fold decreases in Y1 receptor affinity, except [^{13}S(β-D-Gal)]-PYY3-36 which demonstrated a 20-fold drop in potency towards the Y1 receptor. However, due to the concurrent loss of Y2 binding the Y2/Y1 receptor selectivity remained almost unchanged for these compounds. Interestingly, mutation of Gly-9 to Ser(β-D-Gal) gave only a small reduction in Y2 receptor affinity (Table 1, peptide **9**) even though Gly in that position may be critical for forming the

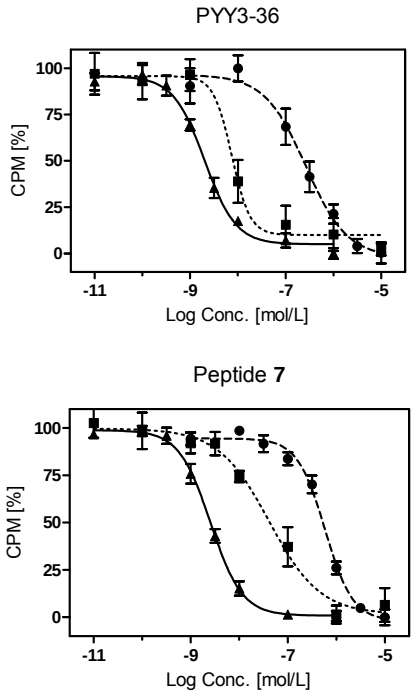

Figure 3. Binding of PYY3-36 and peptide **7** towards the Y1 (■), the Y2 (▲) and Y4 (●) receptors. The curves are given as means of the replicates for each peptide.

Table 1. The binding affinity of PYY3-36 and the novel glyco-PYY3-36 analogs towards the Y receptor subtypes Y1, Y2, and Y4, respectively. Moreover, the degree of α-helicity in solution is given in percent. Sequence of native PYY3-36: H-^{3}IKPEAP^{9}GEDA^{13}SPEEL^{18}NR^{20}Y^{21}Y^{22}A^{23}SLRH^{27}YLNLVTRQRY-NH_2.

		Y1R[a]	Y2R[b]	Y4R[b]	Selectivity				α-helicity[d]
Peptide		IC_{50} [nM]	IC_{50} [nM]	IC_{50} [nM]	Y2/Y1	Rel.	Y2/Y4	Rel.	[%]
PYY3-36		7.8 ± 1.1	0.50 ± 0.09	255 ± 29	0.064	1.0	0.0020	1.0	26
1	[^{3}S(β-D-Glc)]-PYY3-36	57 ± 18	1.53 ± 0.00	>1000	0.027	2.4	< 0.0015	> 1.3	39
2	[^{13}S(β-D-Glc)]-PYY3-36	44 ± 11	1.96 ± 0.37	>1000	0.045	1.4	< 0.0020	> 1.0	28
3	[^{18}S(β-D-Glc)]-PYY3-36	29 ± 10	0.83 ± 0.01	639 ± 89	0.029	2.2	0.0013	1.5	37
4	[^{22}S(β-D-Glc)]-PYY3-36	63 ± 18	1.03 ± 0.01	>1000	0.016	4.0	< 0.0010	> 2.0	28
5	[^{23}S(β-D-Glc)]-PYY3-36	76 ± 20	0.58 ± 0.14	456 ± 123	0.008	8.0	0.0013	1.5	28
6	[^{20}Y(β-D-Glc)]-PYY3-36	20 ± 4	0.83 ± 0.21	>1000	0.042	1.5	< 0.0008	> 2.5	41
7	[^{21}Y(β-D-Glc)]-PYY3-36	150 ± 44	0.84 ± 0.12	687 ± 104	0.006	10.7	0.0012	1.7	47
8	[^{3}S(β-D-Gal)]-PYY3-36	-	1.20 ± 0.01	>1000	-	-	< 0.0012	> 1.7	-
9	[^{9}S(β-D-Gal)]-PYY3-36	92 ± 15	1.03 ± 0.25	>1000	0.011	5.8	< 0.0010	> 2.0	25
10	[^{13}S(β-D-Gal)]-PYY3-36	168 ± 84[c]	1.44 ± 0.35	>1000	0.009	7.1	< 0.0014	> 1.4	31
11	[^{22}S(β-D-Gal)]-PYY3-36	238 ± 124[c]	0.91 ± 0.27	519 ± 80	0.004	16	0.0018	1.1	23
12	[^{23}S(β-D-Gal)]-PYY3-36	8.1 ± 0.5	1.14 ± 0.35	>1000	0.141	0.5	< 0.0011	> 1.8	15
13	[^{20}Y(β-D-Gal)]-PYY3-36	40 ± 23[c]	0.99 ± 0.19	>1000	0.024	2.7	< 0.0010	> 2.0	26
14	[^{21}Y(β-D-Gal)]-PYY3-36	70 ± 17	0.51 ± 0.01	714 ± 222	0.007	9.1	0.0007	2.8	34
15	[^{27}Y(β-D-Gal)]-PYY3-36	10 ± 2	0.72 ± 0.07	>1000	0.072	0.9	< 0.0007	> 2.8	31

[a] SK-N-MC cells expressing the Y1 receptor. [b] Transfected Y2 or Y4 receptors in HEK293 Flp-In T-Rex cells. [c] S.E.M of –log IC50 was only below 0.2. [d] 10 mM NaH_2PO_4.

PP-fold structure.[35] The flexible Gly-9 residue has the ability to adopt certain backbone angles which is not possibly for other amino acids.

All our analogs showed at least a twofold drop in binding affinity towards the Y4 receptor. Glycosylation of the *N*-terminus caused the most profound changes in Y4 receptor affinity and it can be hypothesized that this is due to a disturbance of a low affinity binding between the agonist and the *N*-terminal domain of the receptor prior to activation, similar to the interaction between PP and the *N*-terminal domain of the Y4 receptor.[41] Changing the carbohydrate in a specific position only gave small variations in Y2 receptor binding affinity, however, two analogs showed major changes towards the Y1 receptor: Peptide **5**, [^{23}S(β-D-Glc)]-PYY3-36, dropped almost 10-fold in affinity towards the Y1 receptor compared to peptide **12**, [^{23}S(β-D-Gal)]-PYY3-36. No obvious pattern appears and the affinity variations are sequence and position specific. In other positions the Y1 receptor binding affinity demonstrates a decrease of the galactose (peptide **11**) over the glucose (peptide **4**) substituents (4-fold).

Glycosylation of the amphipathic α-helix of PYY3-36 gave the most interesting changes in affinity (Table 1 and Figure 3 and 4). The binding affinities towards the Y2 receptor were preserved, however, major changes in Y1 and Y4 receptor binding affinity was shown. As mentioned, all analogs with glycosylation in the helix were designed to place the glycan in the membrane-water interface or on the hydrophilic side (Figure 4). The observed

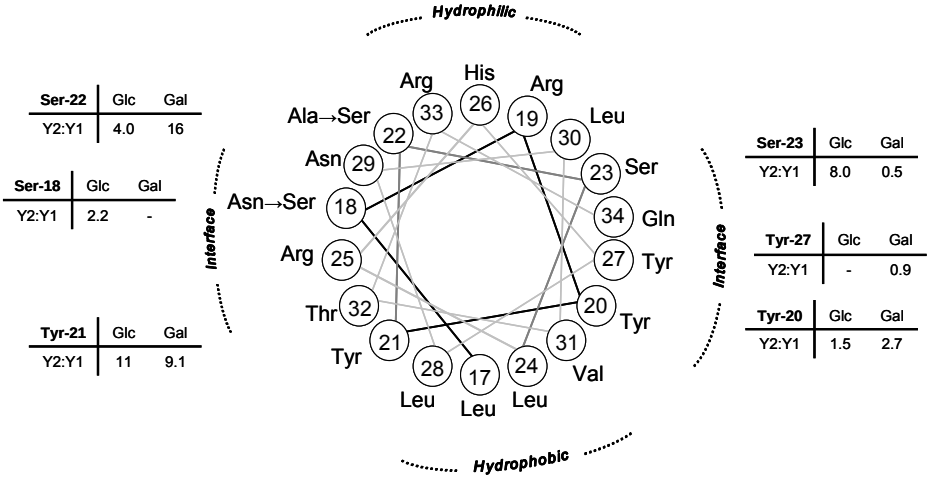

Figure 4. Glycosylation of the α-helix tube of PYY3-36 and the relative Y2:Y1 receptor ratio. The highest selectivity originates from glycosylation of the Tyr-21 and Ser-22 on the same side of the amphipathic α-helix.

increase in relative Y2/Y1 receptor selectivity ratio for novel analogs having a glycan in the α-helix of PYY3-36 originates from a decrease in Y1 receptor potency. One exception was [^{23}S(β-D-Gal)]-PYY3-36 (peptide **12**) which was equipotent towards the Y1 receptor compared to PYY3-36. None, or minor decreases in Y1 receptor affinity was observed for peptide **6**, **12** and **15**, however, significant drops in Y1 receptor affinity was observed for peptide **4**, **7**, **11** and **14** (Table 1 and Figure 3).

The latter were all positioned at the same side of the α-helix (Figure 4), thus we speculate that the glycan may guide a movement of the α-helical tube leading to a more or less favourable conformation for Y1 receptor binding. Menten *et al.* have previously hypothesized two different orientations of the α-helix in the Y2/Y5 and Y1/Y4 receptor subtypes for NPY and PP.[34] Beck-Sickinger *et al.* reported that residue Asp$^{6.59}$ of the Y2 receptor primarily interacts with Arg-33 of NPY and Asp$^{6.59}$ of the Y1 and Y4 receptors interacts with Arg-35 of NPY.[34] Addition of a glycan may more or less prevent the α-helix tube to be turned into a conformation where Arg-35 of PYY3-36 and Asp$^{6.59}$ of Y1 receptor can interact. Another possibility is steric interaction between the carbohydrate and the Y1 and Y4 receptors, in contrast to the Y2 receptor which may be more receptive to bulky substituents in that region. Peptide **7**, [^{21}Y(β-D-Glc)]-PYY3-36, and peptide **11**, [^{22}S(β-D-Gal)]-PYY3-36, were the glyco-PYY3-36 analogs giving the highest Y2 receptor selectivity (19 and 38 fold increase) and potency (below a twofold decrease) (Table 1, Figure 3 and 4).

Stability studies.

Several studies have shown that glycosylation could reduce the susceptibility of a peptide to proteolytic digestion and result in extended peptide activity.[26,29,42] The metabolic stability of native PYY3-36 and glycosylated analogs were evaluated by testing their resistance against trypsin and proteinase K catalyzed digestion. Monitoring by RP-HPLC analysis during the incubation time showed that in the presence of trypsin or proteinase K, PYY3-36 was gradually digested and the half-life of PYY3-36, was 233 and 196 minutes, respectively (Table 3). Degradation curves were performed to all the glycosylated PYY3-36 analogs, except peptide **6**, **8**, **11** and **15**.

Trypsin catalyzed cleavage of glycosylated PYY3-36 analogs generally demonstrated equivalent or reduced degradation half-lives compared to PYY3-36. However, peptide **7**, [^{21}Y(β-D-Glc)]-PYY3-36, demonstrated an increase in stability towards trypsin (Table 2). Trypsin is a serine protease and predominantly cleaves at the carboxyl side of Lys and Arg, except when either is followed by proline. Residue Tyr-21 is positioned at the carboxyl side of Arg-20 and glycosylation of Tyr-21 could therefore prevent trypsin from cleaving at this position leading to the observed changes in half-life.

Proteinase K (endopeptidase K) is a promiscuous serine proteinase predominantly cleaving at the carboxyl side of aliphatic and aromatic amino acids with blocked α-amino groups. Peptide **5** and **7** showed increases in stability towards proteinase K (Table 2), which further confirms that glycosylation of Tyr-21 can prohibit certain types of endopeptidases from cleaving, hence increasing the half-life of the glycopeptide.

Mouse serum degradation of the glyco-peptides and native PYY3-36 showed no significant differences during incubation

Table 2. Trypsin and proteinase K catalyzed cleavage studies, as well as metabolic digestion in mouse serum of native and glycosylated PYY3-36.

	Trypsin[a]	Proteinase K[b]	Mouse serum[c]
Peptide	Half life [min]	Half life [min]	Undigested peptide after 300 min [%]
PYY3-36	233	196	69
1	176	168	73
2	153	214	69
3	188	206	69
4	168	151	73
5	176	271	63
7	269	231	66
9	217	171	54
10	154	200	64
12	-	150	-
13	197	188	-
14	225	210	-

[a] 12.5 M peptide and 625 ng mL^{-1} trypsin in 0.1 M NH_4HCO_3 at RT. [b] 12.5 M peptide and 313 ng mL^{-1} proteinase K in 3.0 mM $CaCl_2$ at RT. [c] Water-mouse serum (40:1) at 37°C.

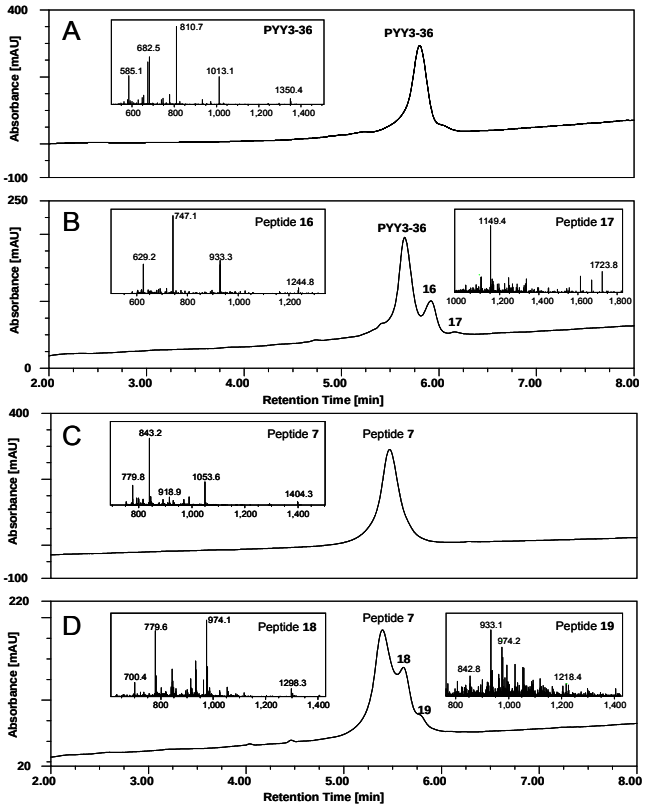

Figure 5. Peptide metabolism in diluted mouse serum. LC-MS analysis of the initial crude mixture of PYY3-36 (A) and after 300 min incubation (B), as well as glyco-peptide **7** after 0 min (C) and 300 min (D) incubation. Serum to peptide (107 μM) ratio 1:40. After 300 min incubation both PYY3-36 and peptide **7** were metabolized to Des Arg35-Tyr36 (peptide **16** and **18**) and Des Arg33-Gln-Arg-Tyr36 (peptide **17** and **19**).

after 30, 60, 180 (not shown), and 300 min (Table 2) at 37°C. Native PYY3-36 was primarily digested by carboxy-peptidases cleaving at the amino side of Arg-33, to give peptide **16**, and Arg-35, to give peptide **17** (Figure 5). Glycosylation of Tyr-21 did not increase the half-life of peptide **7** in mouse serum and gave the same degradation pattern as for PYY3-36 (Figure 5). Identical half-lives were observed for all the novel glyco-PYY3-36 analogs (Table 2). Even though the glyco-scan did not provide a PYY3-36 analog with a significantly longer half-life in serum the results from trypsin and proteinase K digestion studies show that glycosylation may prevent endopeptidases, located in different tissues, from digesting glyco-PYY3-36 analogs *in vivo*. Medeiros and Turner have reported that PYY is indeed metabolized by endopeptidases in human kidney and jejunum.[43]

Conclusion

In conclusion, we have introduced the concept of a glyco-scan of a peptide. Using glycosylation of human PYY3-36 at selective positions peptide sequence, which have different roles in peptide-receptor interaction we show that (i) glycosylation in most positions is relatively well tolerated with regard to Y2 receptor binding, (ii) the glyco-scan provided novel PYY3-36 analogs with improved Y2/Y1 selectivity, and (iii) the introduction of monosaccharides in this system did not increase metabolic stability. We report that especially glycosylation of Tyr-21, located in the amphipathic α-helix of PYY3-36, leads to enhanced Y2 receptor selectivity originating from a reduced affinity towards the Y1 receptor. We have recently reported *C*-terminal modified PYY3-36 analogs with sub-nanomolar affinity to the Y2 receptor and simultaneously high relative Y2/Y1 receptor ratio.[44] We believe that insights gained in this study will be useful for the development of potential drug candidates derived from PYY3-36. The glyco-scan concept, as systematically demonstrated here, has the potential for wider applicability in peptide biochemistry and in peptide drug design.

Experimental Section

Materials. The organic solvents and reagents for peptide synthesis were all of analytical reagent grade and were obtained from Iris Biotech GmbH (Germany), except for DMF which was obtained from Sigma Aldrich (Denmark). TentaGel S Rink Amide resin was obtained from Novabiochem or Rapp Polymere GmbH. Milli-Q (Millipore) water was used for RP-HPLC analyses and purifications. Human Embryonic Kidney 293 cells (HEK293 Flp-In T-Rex), and pcDNA3.1 vector were purchased from Invitrogen. Dulbecco's Modified Eagle's Medium (D-MEM), fetal calf serum, penicillin-streptomycin solution, phosphate buffered saline (PBS), sucrose, and 99% glycerol were all obtained from Sigma-Aldrich. FuGENE6 transfection reagent, complete protease inhibitor cocktail tablets and bovine serum albumin (BSA) were purchased from Roche. 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS), $MgCl_2 \cdot 6H_2O$, $CaCl_2$, and NaCl were obtained from AppliChem GmbH. (*2S,3S*)-1,4-bis-sulfanylbutane-2,3-diol (DTT) was obtained from GE Healthcare and radioactive labeled agonists were purchased from Phoenix pharmaceuticals. 96-Well filtration MultiScreen HTS, DV plates were obtained from Millipore.

Circular dichroism. UV CD spectra were recorded on a JASCO J-810 circular dichroism spectrometer using rectangular Hellma quartz cells with a light path of 1 mm. The peptide solutions were approximately 20 μM in 10 mM NaH_2PO_4, pH 7.4. The absolute concentration was determined spectroscopically (tyrosine absorption at 274 nm, using ε = 1420 $cm^{-1}M^{-1}$). The mean residue ellipticity (MRE) was calculated according to Yang and co-workers.[45] The number *n* refers to the number of residues. The helicity was calculated according to a formula by Yang and co-workers.[45]

Peptide synthesis. The peptides were prepared by automated peptide synthesis on a Syro II peptide synthesizer (MultiSynTech) by standard solid-phase peptide synthesis (SPPS) on TentaGel S Rink Amide resin (0.24 mmol/g) with 9-fluorenylmethyloxycarbonyl (Fmoc) for protection of N^{α}-amino groups. Side-chain protecting groups were *tert*-butyl (Glu, Asp, Ser, Thr, Tyr), 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf, for Arg), and trityl (Trt, for Asn, Gln, His). N^{α}-Fmoc-Ser(Bz_4-β-D-Glc)-OPfp, N^{α}-Fmoc-Ser(Ac_4-β-D-Gal)-OPfp were synthesized according to procedures reported by Meldal and Jensen,[37] as well as Sjölin and Kihlberg,[38] moreover N^{α}-Fmoc-Tyr(Bz_4-β-D-Glc)-OPfp, N^{α}-Fmoc-Tyr(Ac_4-β-D-Gal)-OPfp were synthesized according to procedures reported by Jensen *et al*.[36] The peptides syntheses were conducted in 0.1 mmol scale. N^{α}-Fmoc amino acids (4.0 equiv) were coupled using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide HBTU (3.8 equiv), 1-hydroxybenzotriazole (HOBt) (3.6 equiv), 1-hydroxy-7-azabenzotriazole (HOAt) (0.4 equiv) and *N,N*-diisopropylethylamine (DIEA) (7.8 equiv) as coupling reagents in DMF for 45 min. However, the glycosylated amino acid derivatives (2.0 equiv) were coupled using HOAt (2.0 equiv) as coupling reagent in DMF for 120 min. D-glucose was either β-O-glycosidically linked to Ser (residue 3, 13, 18, 22 or 23) or Tyr (residue 20 or 21), furthermore D-galactose was either β-O-glycosidically linked to Ser (residue 3, 9, 13, 22 or 23) or Tyr (20, 21 or 27). N^{α}-Fmoc deprotection was performed using piperidine-DMF (2:3) for 3 min, followed by piperidine-DMF (1:4) for 12 min. The carbohydrates were deprotected on-resin using 1.0% $NaOCH_3$ in anhydrous methanol (pH 9.5) for 1 hour. The glyco-peptide were released from the solid support by treatment of

Table 3. LC-MS data of PYY3-36 and peptide **1-15**.

	ESI-MS (m/z)[a]		Purity[b]
Peptide	Calcd. MS	Found, $[M+4H]^{4+}$	[%]
PYY3-36	4049.5	1012.8	98
1	4185.5	1046.6	100
2	4211.6	1053.7	95
3	4184.6	1047.0	100
4	4227.6	1057.3	100
5	4211.6	1053.5	99
6	4211.6	1053.3	99
7	4211.6	1053.3	96
8	4185.5	1046.9	100
9	4241.6	1061.1	100
10	4211.6	1053.3	98
11	4227.6	1057.1	100
12	4211.6	1053.4	96
13	4211.6	1053.3	95
14	4211.6	1053.3	100
15	4211.6	1053.8	100

[a] Identified by ESI-MS on a MSQ Plus Mass Spectrometer, Thermo.

[b] Quantified by RP-HPLC at 215 nm. Eluent A: 0.1% TFA in H_2O. Eluent B: 0.1% TFA in AcN.

trifluoroacetic acid (TFA)-triethylsilane (TES)-H_2O (95:2:3) for 2 hours. The TFA solutions were concentrated by nitrogen flow and the compounds were precipitated with diethylether to yield the crude materials as white powders. Purification was accomplished by preparative RP-HPLC (Dionex Ultimate 3000 system) on a preparative column (FeF Chemicals, 300 Å 5 μm C4 particles, 2.1×200 mm) followed by a second purification on a preparative C18 column (FeF Chemicals, 200 Å 10 μm C18 particles, 2.1×200 mm) using the following solvent system: solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA. B gradient elution (0-50 min: 10% to 60%) was applied at a flow rate of 10 mL min^{-1} and column effluent was monitored by UV absorbance at 215 and 254 nm simultaneously. Identification was carried out by ESI-MS (MSQ Plus Mass Spectrometer, Thermo). The peptides were quantified by analytical HPLC (Dionex Ultimate 3000 system equipped with a PDA UV detector) using the eluent system A-B (solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA). Eluent system A-B was applied on a C4 analytical column (phenomenex, Jupiter, 300 Å 5 μm C4 particles, 3.9×150 mm) where a B gradient elution (0-14 min: 5% to 100%) was applied at a flow rate of 1.0 mL min^{-1}. Quantification and characterization data are given in Table 3.

Radioligand displacement assay. Cell culture and receptor expression: The SK-N-MC cells were cultured in a 1:1 mix of HAM F12 and Dulbecco's Modified Eagle's Medium (D-MEM 1885), containing 15% (v/v) fetal calf serum (FCS), 1% non essential amino acids and 1% (v/v) penicillin-streptomycin in humidified atmosphere of 5% carbon dioxide and 95% air, at 37°C. The human embryonic kidney (HEK293 Flp-In T-Rex) derived cell line was cultured in Dulbecco's Modified Eagle's Medium (D-MEM), containing 10% (v/v) fetal calf serum (FCS) and 1% (v/v) penicillin-streptomycin. Cells were grown as monolayers in humidified atmosphere of 5% carbon dioxide and 95% air, for 48 hours at 37°C. Using serum-free D-MEM and FuGENE6 transfection reagent HEK293 Flp-In T-Rex cells were transiently transfected by pcDNA3.1 vectors which encode either the human Y2 or Y4 receptor (FuGENE6-pcDNA3.1, 3:1). The transfected HEK293 Flp-In T-Rex cells were set to express the receptors in humidified atmosphere of 5% carbon dioxide and 95% air, for another 48 hours at 37°C.

Preparation of membrane fractions: The SK-N-MC and the transiently transfected HEK293 Flp-In T-Rex cells were washed with phosphate buffered saline (PBS) and homogenized in cold homogenization buffer (50 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.0 mM DTT, complete protease inhibitor cocktail (one tablet per 50 mL buffer)). An equal amount of 0.6 M sucrose was added to the cell mixture. The homogenate was centrifuged (10,000 g, 10 min at 4°C). Cell pellets were washed in washing buffer (50 mM TRIS (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 mL buffer), 1.0% (w/w) BSA fraction V), and subsequently the suspensions were centrifuged (10,000 g, 10 min at 4°C). The pellets were re-suspended in glycerol containing binding buffer (50 mM TRIS (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 mL buffer), 1.0% (w/w) BSA fraction V, 20% (v/v) glycerol) and concentration was adjusted to an OD600 of 1.6.

Binding affinity: All binding experiments were performed in 96-well filtration MultiScreen HTS, DV plates at room temperature and every concentration point was performed as triplicates. The unlabeled peptide (25 µL) at concentrations between 10 pM and 10 µM, cell membrane suspension (3.5 µL), binding buffer (61.5 µL, 50 mM TRIS, pH 7.5, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 mL buffer), 1.0% (w/w) BSA fraction V)), radioligand solution (10 µL; Y1R: ^{125}I-[^{31}Leu,^{34}Pro]-pNPY; Y2R: ^{125}I-PYY3-36; and Y4R: ^{125}I-PP; specific activity of 800-1000 Ci/mmole). After 1-2 hours of incubation the assay was terminated by filtration. Finally, the membrane-receptor-ligand complexes were washed twice in cold TRIS buffer (50 mM TRIS, pH 7.5, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$). Bound radioactivity was determined as counts per minute (Wallac 1470 Wizard™ Automated Gamma Counter). Binding data were analyzed with GraphPad Prism 5.0 (GraphPad Software, Inc. San Diego, CA, USA). The displacement experiments were replicated until the standard error of mean (S.E.M.) of –log IC_{50} was below 0.1 (n = 2-5), except when otherwise stated.

Trypsin and protease K catalyzed cleavage. PYY3-36 and the glycosylated analogs were dissolved in 175 µL protease free water to a final peptide concentration of 50 µM. Trypsin; to the peptide solution 20 µL of 1.0 M NH_4HCO_3 and 5 µL of 25 µg mL^{-1} trypsin (Sigma Aldrich) was added. Protease K; to the peptide solution 20 µL of 30 mM $CaCl_2$ and 5 µL of 12.5 µg mL^{-1} protease K (Sigma Aldrich) was added. Samples (25 µL) were directly injected into the HPLC for every 75 min over a period of 6 hours. The half-life was established based on the assumption of first order catalysis. The percentage of digested peptide was monitored by RP-HPLC (Dionex Ultimate P580 pump equipped with a Waters 996 PDA UV detector and 717 autosampler) using eluent system C-D (solvent C, water containing 0.1% TFA; solvent D, MeOH containing 0.1% TFA) applied on a C18 analytical column (phenomenex, Gemini, 110 Å 3 μm C18 particles, 4.60×50 mm) where a C gradient elution (0-14 min: 5-100%) was applied at a flow rate of 1.0 mL min^{-1}.

***In vitro* metabolism of PYY3-36 and the glycosylated analogs in mouse serum.** PYY3-36 and the glycosylated analogs were dissolved in 195 µL protease free water to a final peptide concentration of 107 µM. To the peptide solution 5 µL of freshly prepared mouse serum (male NMRI mice) was added. After incubation at 37°C for 0, 30, 60, 180, and 300 min 20 µL of diluted serum deproteinated with 60 µL of ethanol. After centrifugation (14,000 rpm, 4°C, 15 min) the supernatant was analyzed by LC-MS (Dionex Ultimate 3000 system equipped with a PDA UV detector, MSQ Plus Mass Spectrometer, Thermo) using eluent system A-B (solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA) applied on a C18 analytical column (phenomenex, Gemini, 110 Å 3 μm C18 particles, 4.60×50 mm) where an A gradient elution (0-12 min: 5-100%) was applied at a flow rate of 1.0 mL min^{-1}.

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Keywords: Glyco-scan · Glycosylated amino acids · Glyco-peptide · PYY3-36 · Y2 agonist · Metabolic stability

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Appendix 3

Paper 3:

Søren L. Pedersen, Pottayil G. Sasikumar, Shekar Chelur, Birgitte Holst, Andreas Artmann, Knud J. Jensen, and Niels Vrang. Re-design of a peptide hormone: *N*-terminally branched PYY3-36 isoforms give improved lipid and fat cell metabolism in diet-induced obese mice, *in manuscript* (Organic and Biomolecular Chemistry).

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Re-design of a peptide hormone: *N*-terminally branched PYY3-36 isoforms give improved lipid and fat cell metabolism in diet-induced obese mice

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The prevalence of obesity is increasing with an alarming rate worldwide and there is a need for efficacious satiety drugs. PYY3-36 has been shown to play a role in hypothalamic appetite regulation and novel analogs targeting the Y2 receptor have potential as drugs for the treatment of obesity. We have designed a new series of PYY3-36 analogs, by introducing different *N*-terminal segments of PYY as a branch at position 12 of the native sequence. We hypothesized that such modifications would alter the back-folding of PYY, due to changes in the turn motif, which could change the binding mode to the Y receptor subtypes and possibly also alter metabolic stability. In structure-affinity/activity relationship experiments one series of PYY isoforms displayed equipotency towards the Y receptors. However, an increased Y2 receptor potency for the second series of PYY isoforms resulted in enhanced Y receptor selectivity compared to PYY3-36. Additionally, acute as well as chronic mice studies showed body-weight lowering effects for one of the PYY isoforms, which was also reflected in a reduction of circulating leptin levels. Interestingly, while the stability and pharmacokinetic profile of PYY3-36 and the *N*-terminally modified PYY3-36 analog were identical, only mice treated with the branched analog showed marked increases in adiponectin levels as well as reductions in non-esterified free fatty acids and triglycerides.

Introduction

The native Y2 receptor agonist PYY3-36 plays a role in appetite regulation and Y2 receptor agonists are potentially a novel class of compounds for the treatment of obesity.[1] Indeed a number of studies in rodents have shown that chronic Y2 agonism lowers body-weight.[2-4]

PYY is a member of the PP-fold family of peptides that also consist of pancreatic polypeptide (PP) and neuropeptide Y (NPY).[5] The PP-fold peptides bind and activate a family of G-protein coupled receptors, the so-called Y receptors (Y1, Y2, Y4 and Y5).[6] Full length PYY is released postprandially from endocrine L-cells lining the gut[7] and subsequent to secretion PYY1-36 is *N*-terminally truncated by dipeptidyl peptidase IV (DPP-IV) to produce PYY3-36.[8,9] PYY1-36 is a powerful but nonselective agonist stimulating both the Y1 and Y2 receptors,[6,10] leading to numerous actions especially on the gastrointestinal function.[11] The *N*-terminally truncated form of PYY1-36 - PYY3-36 - has a high affinity towards the Y2 receptor, and to a lesser extent the Y1 receptor.[10] Moreover, the anorectic and body-weight lowering effects of peripherally administered PYY3-36 is believed to be mediated by central Y2 receptors, possibly located in the hypothalamic arcuate nucleus.[1]

Herein, we present data from a novel class of PYY isoforms, which displays changes in the selectivity towards the Y receptors, body-weight lowering effects in different mice models, and finally additional beneficial effects on circulating lipids as well as on fat-cell metabolism. While the body-weight lowering effects of PYY3-36 and a PYY3-36 isoform (peptide **4**) was similar, unexpectedly, 14-days infusion of peptide **4** to DIO-mice lead to significantly higher adiponectin levels compared to PYY3-36, which is an additional beneficial effects on fat cell metabolism.

Peptide design.

The sequences of the PP-fold peptides consist of a *C*-terminal receptor-recognizing segment, an amphipathic α-helix and a more flexible *N*-terminus. It has been hypothesized that all members of the PP-fold family of peptides bind to the membrane prior to interaction with the receptors.[12] The

Table 1. Sequences of human NPY, PP, PYY1-36, PYY3-36, as well as main chain of the PYY isoforms.

Peptide	Sequence
PP	H-APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-NH_2
NPY	H-YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-NH_2
PYY1-36	H-YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2
PYY3-36	H-IKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2
Isoforms[a]	H-I**K**SPEELNRYYASLRHYLNLVTRQRY-NH_2

[a] Novel analogs: *N*-terminal sequences branches from the ε-amine of an additional Lys (bold) positioned at the amino end of PYY13-36.

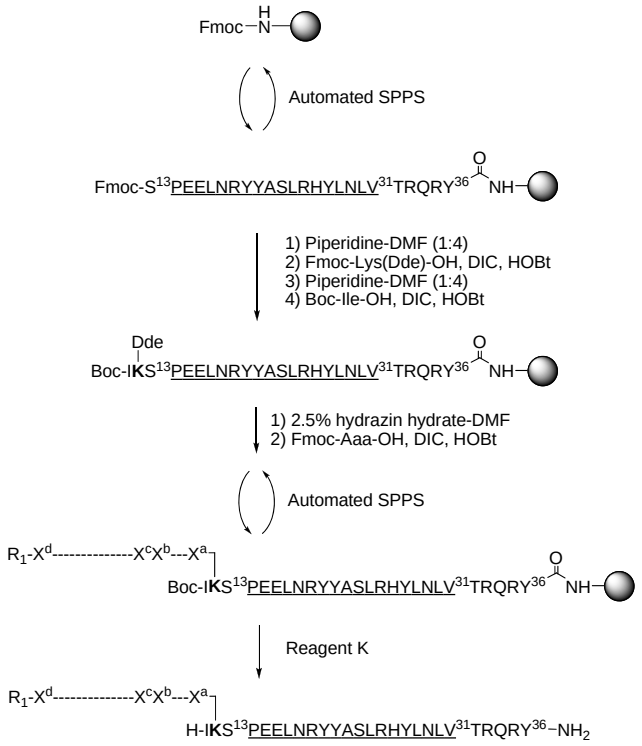
N-terminal segments (residue 1-12) have proven very important for the Y receptor selectivity and essential for Y1 receptor activity, which has been shown by *N*-terminally truncating both PYY1-36 and NPY.[13,14] Pro-14 of the turn motif of PYY1-36 is highly important for controlling back-folding in solution; moving Pro-14 to position 13, as seen in the NPY sequence, abolishes all hairpin structure.[15] Consequently, the [^{13}Pro,^{14}Ala]-PYY1-36 mutant shows a destabilized *C*-terminal α-helix.[15] Moreover, Ser-14 has also been shown to be important for PYY1-36 back-folding in solution.[15] Given the importance of the turn motif of PYY for back-folding and α-helix stability, we designed two series of branched PYY analogs: Series 1 were branched at an ε amine of an additional Lys positioned before Ser-13 of the native sequence. This additional *N*-terminal amine (Ile N^{α}) near the native PYY α-helix provides an additional positive charge. In series 2 - which were branched similarly to series 1 - we replaced the inherent turn motif of PYY with a GPRRP turn to guide the branch into a favorable Y2 receptor binding conformation via a tighter back-folding, consequently altering the binding mode towards the Y receptors or increasing the affinity towards the membrane, which possibly leads to an enhanced concentration of ligand in the vicinity of the Y receptors.

Results and discussion.

Peptide synthesis.

Human PYY1-36, PYY3-36 and the PYY isoforms were assembled using the Fmoc/*t*-Bu strategy by automated solid-phase peptide synthesis on Rink Amide TentaGel resin. The N^{α}-Fmoc-protected amino acids were coupled using DIC and HOBt as coupling reagent and additive, in DMF, with coupling times of 2 hours. The Fmoc group was removed using 1:4 piperidine-DMF, for 3 + 17 min. The sequences of PYY1-36, PYY3-36 and PYY13-36 were assembled using standard Fmoc/*t*-Bu chemistry as described above. The sequence of the resin-bound PYY13-36 was additionally modified by coupling of the Dde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl) side-chain protected Lys followed by Fmoc removal and coupling of a N^{α}-Boc-protected Ile (Scheme 1). The Dde group was removed by 2.5 % (v/v) hydrazine hydrate in DMF making the ε-amino group available for elongation of the peptide branch (Scheme 1, a-d), which additionally was performed by standard Fmoc/*t*-Bu chemistry.

Series 1 maintained the turn motif from native PYY (PYY9-12, GEDA), however series 2 the turn motif (X^a-X^b, Scheme 1) was exchanged with the less flexible GPRRP turn (adapted from helix-loop-helix structures originally designed by Degrado *et al.*).[16] The branched *N*-terminal segments (X^c-X^d, Scheme 1), either originated from PYY1-8, PYY2-8, or PYY3-8 and the branched *N*-terminus was either free amine or lipidated (R_1, Scheme 1). After completion, the peptides were side-chain deprotected and simultaneously cleaved from the solid support using reagent K. Finally, the peptides were purified by preparative RP-HPLC, quantified by analytical RP-HPLC and characterized by mass spectrometry. The final products were obtained with > 95 % purity (Table 5).



Scheme 1. Reaction scheme describing the solid-phase synthesis of the PYY isoforms. Underlined sequences represent the inherent α-helical segment. Residues X^a-X^b shows the position of the turn motifs and residues X^c-X^d illustrates the position of the branched *N*-terminal segments derived from the native PYY sequence. R_1 represents modifications to the branched N^{α}-amino group of the PYY isoforms.

Circular dichroism.

The secondary structures of PYY1-36, PYY3-36, and peptides **1-7** in solution were assessed by circular dichroism (CD) in 10 mM phosphate buffer. The spectra obtained were typical for the α-helical structure, showing characteristic minima at 208 nm and 220 nm (Figure 1). The degree of α-helicity of the analyzed PYY analogs were between 22 and 29%, furthermore PYY1-36 and PYY3-36 had an α-helicity of 27 and 26%, respectively, which shows that the branch was well tolerated and did not disturb the overall secondary structure of the inherent α-helix.

In vitro pharmacology.

To characterize the influence of the alteration of secondary structure of the PYY isoforms, both the novel and inherent ligands were assayed by radioligand displacement binding assay (competition assay) based on membranes from SK-N-MC and HEK293 Flp-In T-Rex cell lines expressing the human Y1, Y2 and Y4 receptor subtypes, respectively. ^{125}I-[^{31}Leu,^{34}Pro]-pNPY for Y1R, ^{125}I-hPYY3-36 for Y2R and ^{125}I-hPP for Y4R were used as radioligands. The non-lipidated analogs were additionally evaluated for activity in a signal transduction assay (functional assay). Peptide **1**, branched version of PYY1-36, showed higher Y2 receptor affinity and

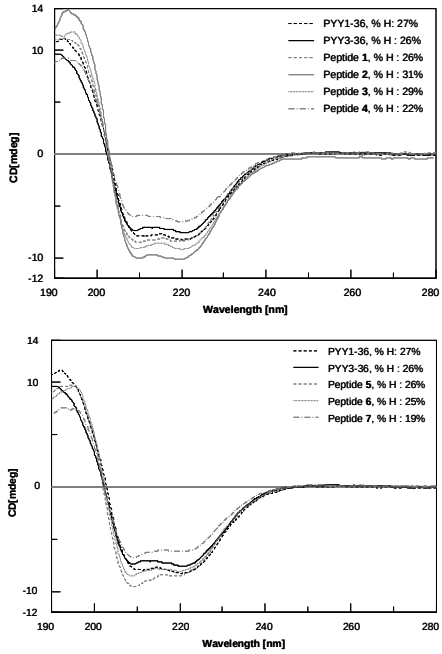


Figure 1. Circular dichroism (CD) of PYY1-36, PYY3-36, peptides **1**-**7** in phosphate buffer as well as degree of α-helicity for each compound.

better selectivity compared to PYY1-36 - a fourfold increase in Y2 receptor affinity and an almost threefold drop in Y1 receptor affinity compared to PYY1-36 and equal affinity compared to PYY3-36 – however, the signal transduction assay only show equipotency towards the Y2 receptor (Table 2, entry 1). Nevertheless, it is very interesting that branching of full length PYY1-36 can lead to an analog performing approximately as native PYY3-36 towards the Y2 receptor. The lipidated analog (peptide **2**) resulted in decreased (3.5-fold) Y1 receptor affinity compared to PYY3-36 but also an inauspicious threefold decrease in Y2 receptor affinity. However, compared to PYY1-36 peptide **2** showed a 2.5-fold improvement in Y2 receptor affinity and a 9-fold decrease in affinity towards the Y1 receptor (Table 2, entry 2). Moreover, peptide **2** also results in a drop in Y4 receptor affinity. Peptide **3** (the branched version of PYY2-36) had a Y receptor binding profile very similar to PYY3-36, as did peptide **4** (branched version of PYY3-36). Although binding showed peptide **4** to have a higher selectivity (Y2/Y1) than native PYY3-36, the functional assay only gave a marginal increase in selectivity (Table 2, entry 4). The affinities towards the Y4 receptor were unaffected for all the novel analogs except peptides **2** and **4**, which gave a twofold decrease in binding.

The second series of PYY analogs (containing the GPRRP modified turn motif) were able to displace the Y2 receptor selective radioligand ^{125}I-hPYY3-36 with high affinity and 3-(peptide **5**) and 2.5-fold (peptide **6**) improvement in IC_{50} value was observed compared to the native peptide. These affinities correspond well with the potencies observed in the functional characterization. Surprisingly, these two peptides displaced the Y1 selective radioligand ^{125}I-[^{31}Leu,^{34}Pro]-pNPY with very low apparent affinity – the IC_{50} was shifted 50- and 12-fold respectively for peptides **5** and **6**, compared to the native peptide. However, the potencies obtained from the functional study show that these peptides actually activated the Y2 receptor with a high potency. The peptide **5** displayed a 5-fold improved potency compared to the native peptide (PYY3-36) and peptide **6** showed 8-fold improved potency compared to PYY3-36. The explanation for this discrepancy between apparent affinity observed in competition binding studies and the potency observed in functional characterization is most likely related to the fact that a heterologous competition binding study has been used. In heterologous competition studies the radioligand differ chemically from the investigated ligand, which allow the radioligand to trap the receptor in one conformation and high concentration of the ligand is required to induce the conformational changes before binding.[17] Another possible explanation for the discrepancy between affinity and potency, is that the functional assay allow for intracellular amplification of the response, which may increase the potency.

It is well known that *N*-terminally truncated versions of both NPY, such as NPY3-36 and NPY13-36, and *N*-terminally truncated versions of PYY, such as PYY3-36, PYY13-36, improves the Y2/Y1 receptor specificity.[14] Recently, even shorter versions of PYY, the *N*-terminal PEGylated PYY22-36

Table 2. Binding affinities of PYY1-36, PYY3-36, and the novel PYY isoforms towards the Y receptors and potencies of PYY3-36 and the non-lipidated PYY isoforms. The sequence of the isoforms main chain: H-l**K**SPEELNRYYASLRHYLNLVTRQRY-NH_2

Peptide	Branch sequences	Binding affinity, IC_{50} [nM]			Potency, EC_{50} [nM]	
		Y1R[a]	Y2R[b]	Y4R[b]	Y1R[c]	Y2R[c]
PYY1-36		4.0 ± 1.7	3.8 ± 1.6	141 ± 79	-	-
PYY3-36		24.6 ± 15	0.50 ± 0.09	255 ± 29	90 ± 11	5.6 ± 2.1
1	H-YPIKPEAPGEDA-	10 ± 1.5	0.88 ± 0.05	239 ± 15	12 ± 7	5.6 ± 3.2
2	$CH_3(CH_2)_4CO$-YPIKPEAPGEDA-	36 ± 7	1.43 ± 0.09	627 ± 204	-	-
3	H-PIKPEAPGEDA-	25 ± 16	0.90 ± 0.01	277 ± 29	80 ± 22	4.3 ± 2.9
4	H-IKPEAPGEDA-	19 ± 5	0.95 ± 0.07	617 ± 24	118 ± 19	4.6 ± 2.4
5	H-YPIKPEAPGPRRP-	229 ± 22	0.17 ± 0.02	275 ± 80	4.5 ± 3.3	1.1 ± 0.3
6	H-IKPEAPGPRRP-	535 ± 182	0.20 ± 0.05	524 ± 155	46 ± 23	0.7 ± 0.3
7	$CH_3(CH_2)_4CO$-IKPEAPGPRRP-	174 ± 5	0.15 ± 0.04	571 ± 52	-	-

[a] SK-N-MC cells expressing the Y1 receptor. [b] Transfected Y2 or Y4 receptors in HEK293 Flp-In T-Rex cells. [c] Transfected Y1 or Y2 receptors as well as GαΔ6qi4myr in COS-7 cells.

Table 3. Metabolic digestion in diluted mouse serum[a] of native PYY3-36 and peptide **1-7**.

	Undigested peptide[b] [%]		
Peptide	After 60 min	After 120 min	After 300 min
PYY3-36	> 95	93	69
1	88	89	n.d.
2	> 95	86	66
3	> 95	83	53
4	94	88	69
5	~ 50	< 5	< 5
6	> 95	< 5	< 5
7	> 95	< 5	< 5

[a] Water-mouse serum (40:1) at 37°C. [b] Analyzed by analytical RP-HPLC.

analogs, were shown to retain some Y2 activity while greatly reducing Y1 activity.[18] Interestingly, however, while PEGylation and *N*-terminal truncation also reduces Y2 affinity (10-50 fold) our *N*-terminal branched peptides retain high affinity towards the Y2 receptor.

Mouse serum stability.

The serum stability of native PYY3-36 and the PYY isoforms was evaluated after 60, 120, and 300 min by analytical RP-HPLC. The first series of PYY analogs showed no significant variation in serum stability compared to PYY3-36 during a 300 min incubation period at 37°C (Table 3, entry 1-4). PYY3-36 and peptides **1-4** were primarily digested by carboxy-peptidases cleaving at the amino side of Arg-33 and Arg-35 (Figure S1-S8). Likewise, peptides **5-7** were digested in a similar mode however, unexpectedly rapidly (> 10 times as fast). We speculate that the additional Arg residues bind with high affinity to an in-active site of the carboxy-peptidase, which then may induce the *C*-terminal of PYY into the active site of the peptidase leading to an increased digestion. Due to the unexpected low half-life in serum peptides **5-7** were not further investigated.

Body-weight lowering effects in acute mouse study.

The effect of three days of subcutaneous minipump administration of PYY3-36, peptides **3** and **4** on body-weight in male NMRI mice was investigated. The two novel PYY analogs were selected for *in vivo* investigation based on their potency and serum stability. Pre-treatment analysis showed PYY3-36 and peptide **4** to be stable in 0.9% saline over a period of 14 days (data not shown) similar to what has been reported previously for PYY3-36.[3] On the first day of the study the animals were implanted with an Alzet osmotic minipump providing a continuous administration of either vehicle (0.9% saline), PYY3-36, peptide **3**, or **4** in peptide concentrations of 0.25 µmol/kg/day. A reduction in body-weight gain was observed at day 3 for animals treated with PYY3-36 (~ 6%), peptide **3** (~ 4%) and **4** (~ 7%), as well as at day 6 for peptide **4** (~ 4%) treated animals.

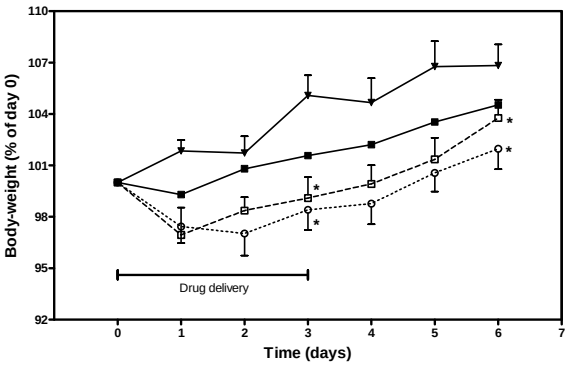


Figure 2. Acute minipump experiment in lean male NMRI mice (3 days). Vehicle (▼), 0.25 µmol/kg/day PYY3-36 (□), 0.25 µmol/kg/day peptide **3** (■) and 0.25 µmol/kg/day peptide **4** (○). Values are means ± SEM (n = 7). One way ANOVA followed by a "Dunnett's multiple comparison test": (*) $P < 0.05$ compared to vehicle.

Pharmacokinetic analysis of PYY3-36 and peptide 4.

Based on affinity, potency and the acute *in vivo* data peptide **4** was further investigated. The pharmacokinetic profile of PYY3-36 and peptide **4** were examined following an intravenous bolus injection. The calculated half-life of both compounds was approximately 35 min.

Chronic body-weight reduction in DIO-mice.

We next examined the effect of 14 days of administration of PYY3-36 and peptide **4** by osmotic minipumps in male diet-induced obese C57BL/6J mice. Mice were randomized according to body-weight and implanted subcutaneously in the back with an Alzet osmotic minipump delivering either vehicle (0.9% saline), PYY3-36 (0.25 or 1.0 µmol/kg/day) or peptide **4** (0.25 or 1.0 µmol/kg/day) (Figure 3). Both doses of PYY3-36 and peptide **4** reduced food intake and body-weight dose-dependently. A one-way ANOVA test followed by a "Dunnett's multiple comparison test" at day 7 and 14 showed PYY3-36 and peptide **4** reduced body-weight significantly compared to vehicle (Figure 3A).

The reduction in body-weight was correlated to a reduction in food intake during the first week of the study (Figure 3B and 3C), which was decreased similarly for all for treated animal groups compared to vehicle (Figure 3B and 3C). The degree of body-weight loss (~ 10%) during the first seven

Table 4. Amount of free fatty acids, triglycerides and cholesterol on day 14. Values are means ± SEM (n = 9).

	Peptide conc. [µmol/kg/day]	Free fatty acids [mmol/L]	Triglycerides [mg/dL]	Total cholesterol [mg/dL]
Vehicle		1.26 ± 0.27	217 ± 21	153 ± 56
PYY3-36	0.25	1.07 ± 0.26	191 ± 37	136 ± 53
	1.0	1.00 ± 0.18	201 ± 27	104 ± 27
Peptide **4**	0.25	0.92 ± 0.15 (*)	199 ± 26	111 ± 22
	1.0	0.86 ± 0.22 (**)	187 ± 26 (*)	96 ± 30

One way ANOVA with "Dunnett's Multiple Comparison Test": (*) $p < 0.05$, (**) $p < 0.01$ compared to vehicle.

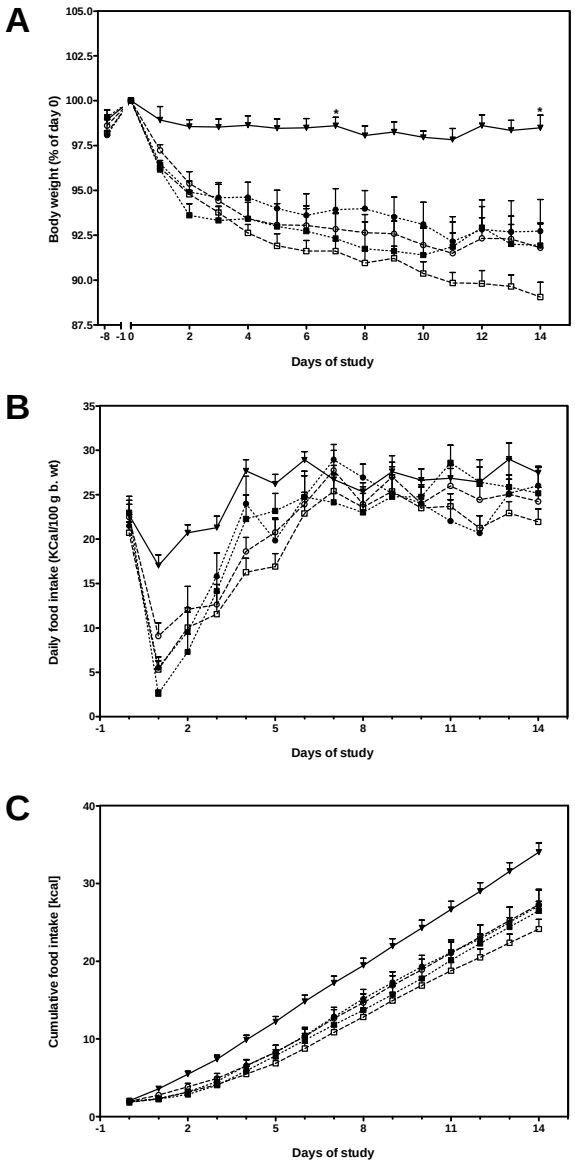


Figure 3. Chronic minipump experiment in male DIO C57BL/6J mice (2-wk). Body weight (A), daily food intake (B) and cumulative feed intake (C). Vehicle (▼), 0.25 µmol/kg/day PYY3-36 (□), 1.0 µmol/kg/day PYY3-36 (■), 0.25 µmol/kg/day peptide **4** (○), 1.0 µmol/kg/day peptide **4** (●). Values are means ± SEM (n = 9). One way ANOVA with "Dunnett's Multiple Comparison Test": (*) $P < 0.05$ compared to vehicle.

days was compatible to previous reports using PYY3-36 analogs.[1-3,9,19]

After the final dosing at day 14, blood was collected to measure leptin, non-esterified free fatty acids, triglycerides, cholesterol and adiponectin. In accordance with the body-weight loss both PYY3-36 and peptide **4** treated mice had lower levels of circulating leptin at termination (Figure 4A). Interestingly, adiponectin levels were increased to a larger extent in peptide **4** treated mice when compared to PYY3-36 (Figure 4B). Also, peptide **4** led to higher reductions in triglycerides and free fatty acids than PYY3-36, suggesting that peptide **4** exert additionally beneficial effects on fat cell metabolism. Although it can be speculated that the differential

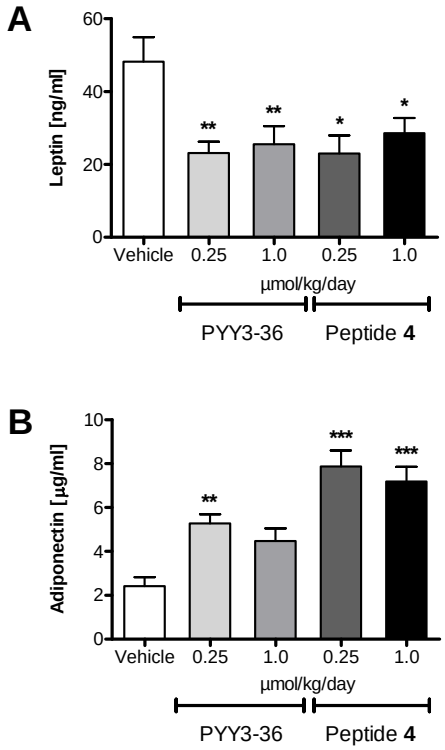


Figure 4. Leptin levels on day 14 (A) and adiponectin levels on day 14 (B). Values are means ± SEM (n = 9). One way ANOVA with "Dunnett's Multiple Comparison Test": (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ compared to vehicle.

effects of peptide **4** on adiponectin levels and plasma lipids could be due to an alteration in Y2/Y1 selectivity, the change in this selectivity ratio is rather modest and therefore other factors should be considered. First, the PYY3-36 isoform exemplified by peptide **4** may have an altered back-folding; the branch and the additional *N*-terminal amino group could lead to a changed secondary structure in the solution and membrane bound state.

It is also possible that peptide **4** activated other receptors than the Y receptors. The orphan GPCR, glucocorticoid-induced receptor (GIR, GPR83), has previously been speculated to be a target for PYY3-36. GIR is found in brain regions such as hypothalamus, however, the pharmaceutical action of the receptor is awaiting.[20]

In conclusion, we have characterized a new series of branched PYY analogs with remarkable lipid lowering and adiponectin stimulating effects in a mouse model of diet induced obesity. These analogs could be explored further in the search for novel drugs for the treatment of the metabolic syndrome and obesity.

Materials and methods.

Materials.

Human Embryonic Kidney 293 cells (HEK293 Flp-In T-Rex), and pcDNA3.1 vector were purchased from Invitrogen. Dulbecco's Modified Eagle's Medium (D-MEM), fetal calf serum, penicillin-streptomycin solution, phosphate buffered

saline (PBS), sucrose, and 99% glycerol were all obtained from Sigma-Aldrich. FuGENE 6 transfection reagent, complete protease inhibitor cocktail tablets and bovine serum albumin (BSA) were purchased from Roche. 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS), $MgCl_2 \cdot 6H_2O$, $CaCl_2$, and NaCl were obtained from AppliChem GmbH. (*2S,3S*)-1,4-bis-sulfanylbutane-2,3-diol (DTT) was obtained from GE Healthcare and radioactive labeled agonists were purchased from Phoenix pharmaceuticals. 96-Well filtration MultiScreen HTS, DV plates were obtained from Millipore. [^{3}H]-*myo*-inositol (PT6-271) was purchased from Amersham.

Peptide synthesis.

The peptides were prepared by automated peptide synthesis on a Symphony parallel synthesizer (PTI) by standard solid-phase peptide synthesis (SPPS) on TentaGel S Rink Amide resin (0.24 mmol/g loading) with 9-fluorenylmethyl-oxycarbonyl (Fmoc) for protection of N^{α}-amino groups except for the *N*-terminal Ile of the main-chain, where Boc (*tert*-butoxycarbonyl) was used. Side-chain protecting groups were *tert*-butyl (Ser, Thr, Tyr, Asp, Glu), *tert*-butoxycarbonyl (Lys), 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde, for branching at Lys), 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf, for Arg), and trityl (Trt, for Asn, Gln, His). N^{α}-Fmoc amino acids (6.0 equiv) were coupled using diisopropylcarbodiimide (DIC) (6.0 equiv) as coupling agent and 1-hydroxybenzotriazole (HOBt) (6.0 equiv) as additive in DMF for 120 min. N^{α}-Fmoc deprotection was performed using piperidine-DMF (1:4) for 3 min, followed by piperidine-DMF (1:4) for 17 min. The Lys(Dde) residue was side-chain deprotected with 2.5% (v/v) hydrazine hydrate in DMF at room temperature for 15 min. The peptide amides were released from the solid support by treatment of reagent K (trifluoroacetic acid (TFA)-phenol-thioanisol-1,2-ethanedithiol (EDT)-H_2O (82.5:5:5:2.5)) for 2.5 hours. The TFA solutions were concentrated by nitrogen flow and the compounds were precipitated with diethylether to yield the crude materials as white powders.

Purification was accomplished by RP-HPLC (Agilent 1200 series) on a preparative column (Zorbax-Eclipse XDB-C18, 7 μm particles, 21.2×250 mm) using the following solvent system: solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA. B gradient elution (0-2 min 5%, 2-5 min 5-25%, 5-50 min 25-60%) was applied at a flow rate of 20 mL min^{-1} and column effluent was monitored by UV absorbance at 220 nm. Identification and quantification were carried out by LC-MS (Agilent Technologies LC/MSD VL) using the eluent system A-B (solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA). The eluent system A-B was applied on a C18 analytical column (Zorbax Eclipse XDB, 80 Å 5 μm 4.6×150 mm) where the B gradient elution (0-25 min: 5-50%) was applied at a flow rate of 1.0 mL min^{-1}. Quantification and characterization data are given in Table 5.

Table 5. Quantification and characterization data of inherent PYY1-36, PYY3-36 and peptide isoforms.

	ESI-MS (m/z)[a]		Purity[b]
Peptide	Calcd. MS	Found, $[M+4H]^{4+}$	[%]
PYY1-36	4309.8	1078.1	98
PYY3-36	4049.5	1012.8	98
1	4551.2	1138.4	96
2	4650.3	1162.0	95
3	4388.0	1097.7	95
4	4291.0	1073.4	97
5	4743.5	1186.4	95
6	4482.2	1121.4	95
7	4581.2	1145.3	96

[a] Identified by ESI-MS on a Agilent Technologies LC/MSD VL. [b] Quantified by RP-HPLC at 215 nm. Eluent A: 0.1% TFA in H_2O. Eluent B: 0.1% TFA in AcN.

Circular dichroism.

UV CD spectra were recorded on a JASCO J-810 circular dichroism system using rectangular Hellma quartz cells with a light path of 1 mm. The peptide solutions were approximately 20 μM in 10 mM phosphate buffer, pH 7.4. The absolute concentration was determined spectroscopically (tyrosine absorption at 274 nm, using ε = 1420 cm^{-1}M^{-1}). The mean residue ellipticity (MRE) was calculated according to Yang *et al*. in which the number *n* refers to the number of residues.[21]

Radioligand displacement assay.

Cell culture and receptor expression: The SK-N-MC cells were cultured in a 1:1 mix of HAM F12 and Dulbecco's Modified Eagle's Medium (D-MEM 1885), containing 15% (v/v) fetal calf serum (FCS), 1% non essential amino acids and 1% (v/v) penicillin-streptomycin in humidified atmosphere of 5% carbon dioxide and 95% air, at 37°C. The human embryonic kidney (HEK293 Flp-In T-Rex) derived cell line was cultured in Dulbecco's Modified Eagle's Medium (D-MEM), containing 10% (v/v) fetal calf serum (FCS) and 1% (v/v) penicillin-streptomycin. Cells were grown as monolayers in humidified atmosphere of 5% carbon dioxide and 95% air, for 48 hours at 37°C. Using serum-free D-MEM and FuGENE 6 transfection reagent HEK293 Flp-In T-Rex cells were transiently transfected by pcDNA3.1 vectors which encode either the human Y2 or Y4 receptor (FuGENE 6/pcDNA3.1, 3:1). The transfected HEK293 Flp-In T-Rex cells were set to express the receptors in humidified atmosphere of 5% carbon dioxide and 95% air, for another 48 hours at 37°C.

Preparation of membrane fractions. The SK-N-MC and the transiently transfected HEK293 Flp-In T-Rex cells were washed with phosphate buffered saline (PBS) and homogenized in cold homogenization buffer (50 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.0 mM DTT, complete protease inhibitor cocktail (one tablet per 50 mL buffer)). An equal amount of 0.6 M sucrose was added to the cell mixture. The homogenate was centrifuged (10,000 g, 10 min at 4°C). Cell pellets were washed in washing buffer (SK-N-MC cells: 50 mM TRIS (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.2% (w/w) BSA fraction V. HEK293-Flp-In-T-Rex: 50 mM TRIS (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 mL

buffer), 1.0% (w/w) BSA fraction V), and subsequently the suspensions were centrifuged (10,000 g, 10 min at 4°C). The pellets were re-suspended in glycerol containing binding buffer (50 mM TRIS (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 mL buffer), 1.0% (w/w) BSA fraction V, 20% (v/v) glycerol) and concentration was adjusted to an OD600 of 1.6.

Binding affinity. All binding experiments were performed in 96-well filtration MultiScreen HTS, DV plates and every concentration point was performed as triplicates. The unlabeled peptide (25 µL) at concentrations between 10 pM and 10 µM, cell membrane suspension (3.5 µL), binding buffer (61.5 µL, 50 mM TRIS, pH 7.5, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 mL buffer), 1.0% (w/w) BSA fraction V)), radioligand solution (10 µL; Y1R: ^{125}I-[^{31}Leu,^{34}Pro]-pNPY; Y2R: ^{125}I-PYY3-36; and Y4R: ^{125}I-PP; specific activity of 800-1000 Ci/mmole). After 1-2 hours of incubation at room temperature the assay was terminated by filtration. Finally, the membrane-receptor-ligand complexes were washed twice in cold TRIS buffer (50 mM TRIS, pH 7.5, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$) containing 5.0% BSA fraction V. Bound radioactivity was determined as counts per minute (Wallac 1470 Wizard™ Automated Gamma Counter). Binding data were analyzed with GraphPad Prism 5.0 (GraphPad Software, Inc. San Diego, CA, USA). The displacement experiments were replicated until the standard error of mean (S.E.M.) of -log IC_{50} was below 0.1 (n = 2-5).

Functional assay.

Transfections and tissue culture. COS-7 cells were grown in Dulbecco's modified Eagle's medium 1885 supplemented with 10 % fetal calf serum, 2 mM glutamine and 0.01 mg/mL gentamicin. Cells were transfected with 10 µg cDNA of wild type Y1 or Y2 receptors and 10 µg cDNA of a GαΔ6qi4myr[22] using the calcium phosphate precipitation method with chloroquine addition. The chimeric G-protein allow the Gαi coupled receptors to signal through the signal transduction pathways known for the Gαq coupled receptors. Receptors from the PP fold family of peptides - the Y1 and Y2 receptors - were cloned from a human cDNA library and expressed in a pcDNA3.1 vector.

Phosphatidylinositol turnover. The assay was performed as previously described[23] and only described in brief in the following. One day after transfection, COS-7 cells were incubated for 24 hours with 5 µCi of [^{3}H]-*myo*-inositol in 1 mL medium, washed twice in buffer, 20 mM HEPES, pH 7.4, and were subsequently incubated in 0.5 mL buffer supplemented with 10 mM LiCl at 37˚C for 30 min. After stimulation with various concentrations of PYY3-36 or the synthetic analogs for 45 min at 37˚C, cells were extracted with 10 % ice-cold formic acid followed by incubation on ice for 30 min. The generated [^{3}H]-inositol phosphate was purified on Bio-Rad AG 1-X8 anion-exchange resins. Determinations of each measuring point were made in duplicates. The functional assays were replicated 3 times.

***In vitro* metabolism of PYY3-36 and PYY isoforms.**

Mouse serum stability. PYY3-36 and peptide **1-7** were dissolved in 195 µL protease free water to a final peptide concentration of 107 µM. To the peptide solution 5 µL of freshly prepared mouse serum (male NMRI mice) was added. After incubation at 37°C for 0, 60, 120, and 300 min 20 µL of diluted serum deproteinated with 60 µL of ethanol. Subsequent to centrifugation (14,000 rpm, 4°C, 15 min) the supernatant was analyzed by LC-MS (Dionex Ultimate 3000 system equipped with a PDA UV detector, MSQ Plus Mass Spectrometer, Thermo), using eluent system A-B (solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA) applied on a C18 analytical column (phenomenex, Gemini, 110 Å 3 μm C18 particles, 4.60×50 mm) where an A gradient elution (0-12 min: 5-100%) was applied at a flow rate of 1.0 mL min^{-1}.

Saline stability. PYY3-36 and peptide **4** (10 µM) were incubated in 0.9% saline at 37°C for 14 days. The samples were analyzed by analytical RP-HPLC at day 2, 7, and 14.

***In vivo* experiments.**

All experiments were conducted in accordance with internationally accepted principles for the care and use of laboratory animals and the protocols were approved by the Danish Committee for Animal Research (intravenous PK study and acute mice study) or the Institutional Animals Ethics Committee of Aurigene Discovery Technologies Ltd, Bangalore, India (chronic mice study).

Intravenous pharmacokinetic study.

Animals. Fifty-two male NMRI mice (Charles River), 8-9 weeks old at the time of arrival at the In Vivo Pharmacology Department of Rheoscience are used. Upon arrival (day -7), the animals were kept on a 12:12-h light-dark cycle (lights on at 03:00 AM) in a temperature-controlled environment (22-24°C). The mice have ad libitum access to standard chow (Altromin 1324, Brogaarden ApS, Denmark).

General procedure. At day -5 the mice are weighted and randomized according to body weight and divided into dosage groups (n = 3) which were terminated at t = 0, 2, 5, 15, 30, 60, 120, and 240 min. PYY3-36 and peptide **4** were investigated. The compounds (or vehicle) are administered by intravenous route (*i.v.*) at t = 0 min. Dosage for both peptides were 0.25 µmol/kg (2 mL/kg). Exact concentration of the peptide solutions are determined spectroscopically (tyrosine absorption at 274 nm, using ε = 1420 cm^{-1}M^{-1}). At time of termination, eye-blood was collected in cold EDTA tubes (containing 0.6 TIU (trypsin inhibitor unit) of aprotinin pr. mL of blood) and the mice are immediately euthanized by decapitation. Immediately after collection, the blood in the EDTA tubes were gently mixed several times to inhibit the activity of proteases.

Bioanalysis. The blood samples were centrifuged at 1600 X g for 15 min at 40°C, plasma was collected and stored at -70°C until analysis. Plasma concentrations of PYY3-36 and peptide **4** were determined by PYY ELISA kit (for human PYY, Phoenix Pharmaceuticals, lot # 600448; LLoQ 10 pg/mL). The interference from endogenous PYY was corrected by, subtracting the mean binding values of PYY in the control group from the mean binding values of PYY3-36

and peptide **4** at the different time points in the *in vivo* study. The human PYY ELISA kit (Phoenix Pharmaceuticals) was tested using dilutions of PYY3-36 and peptide **4** respectively, and showed that PYY ELISA kit was very useful for measuring both PYY3-36 as well as peptide **4**. The standard curve showed a linear range between 0.1 to 1 ng/mL for PYY3-36 and 0.1 to 3 ng/mL for peptide **4** (not shown).

Acute mice study.

Animals. The studies were carried out in lean male NMRI mice (Naval Medical Research Institute, Charles River) 8-9 weeks old. Animals were kept on a 12:12-h light-dark cycle (lights on at 03:00 AM) in a temperature-controlled environment (22-24°C). Food for the entire experiment: Standard chow (Altromin standard chow 1324; C. Petersen, Ringsted, Denmark).

Sample preparation. Vehicle for all experiments was 0.9% saline. Compounds were administered via Alzet osmotic minipumps (model 1003; 100µl; 1.0 µl/h, 3 days of delivery). The animals were given either vehicle (0.9% saline), PYY3-36, peptide **3** or **4** in concentrations of 0.25 µmole/kg/day. Pumps were filled in the morning of day 0 and "primed" for 4 hours (pump filled and kept in 0.9% saline at 37 °C until operation, approximately 4-6 hours).

General procedure. The animals (n = 7) were on the day of arrival given water and food for the entire experiment (standard chow; Altromin standard chow 1324; Petersen, Ringsted, Denmark). Upon arrival the animal were transferred to single cages in which they were housed until the start of the experiment (~ 10 days). Body-weight was recorded on the morning of day 0 (7:00-8:00 AM) and the mice were randomized. On day 0, animals were anaesthetized using gas anaesthesia (halothane) and Alzet osmotic pumps (model 1003) implanted subcutaneously in the lower back and surgical between 12:00 and 14:00 PM, wound closed with surgical staples. Povidone iodine solution was applied topically on surgical site. Following the operation, the mice were allowed to recover, and subsequently transferred back to their cages. Pentazocin (5 mg/kg SQ) was administered once as analgesic. Body-weight was recorded in the morning between 8:00 and 10:00 AM from day 0 to day 6.

Chronic DIO mice study.

Animals. Studies were carried out in male C57BL/6J mice. Animals were kept on a 12:12-h light-dark cycle (lights on at 0700) in a temperature-controlled environment (22-24°C) with free access to food and water.

Sample and pump preparation. Vehicle for all experiments was 0.9% saline. Compounds were administered via Alzet osmotic minipumps (model 2002; 200µL; 0.5µL/h, 14 days of delivery). The animals were given either vehicle, PYY3-36 (batch no: IN1009-041) or peptide **2** (batch no: IN1094-071) in concentrations of 0.25 µmole/kg/day or 0.1 µmole/kg/day. Pumps were filled the day before experiment start and "primed" overnight (pump filled and kept in 0.9% saline at 37°C).

General procedure. The animals (n = 9) were aged 6-8 weeks at the time of arrival to the *in vivo* facility. Mice were housed 5 per cage for 20 weeks before experiment start. During this period, mice were given free access to water and a high energy diet containing 60% energy from fat (Diet # D12492; Research Diets, New Jersey, USA). Two weeks prior to pump implantation the animals were transferred to individual cages. Seven days before the experiment start and until end of experiment body-weight (BW) and food intake (FI) were recorded daily at 9.00 AM. The animals were randomized according to body-weight on the day before implantation of the Alzet osmotic mini-pumps. On day 0, animals were anaesthetized using gas anaesthesia (halothane) and Alzet osmotic pumps (model 2002) implanted subcutaneously in the lower back and surgical wound closed with surgical staples. Povidone iodine solution was applied topically on surgical site. Following the operation, mice were allowed to recover, and then transferred back to their cages. Pentazocin (5 mg/kg SQ) was administered once as analgesic. On day 14 of the experiment a morning (9:00 AM) blood sample from ad lib fed mice was obtained for analysis of plasma triglyceride, total cholesterol, free fatty acids, leptin and adiponectin.

Plasma analysis. For measuring plasma triglycerides and total cholesterol and non-esterified free fatty acids (NEFA) blood was sampled from the retroorbital plexus into pre-cooled EDTA coated tubes (K_3-EDTA, 1.6 mg/mL). Samples were immediately centrifuged at 4,800 x G for 15 min and plasma was stored at -80°C until analysis. NEFA was measured in duplicates using a colorimetric enzyme assay kit purchased from FA115, Randox Laboratories, Antrim, United Kingdom). Triglycerides (kit #30364) and total cholesterol (kit #30183) were measured using Labkit from Chemelex S.A. (Barcelona, Spain). For the analysis of plasma leptin and adiponectin blood was sampled into pre-cooled EDTA tubes (K_3-EDTA, 1.6 mg/mL) and plasma stored at -80°C until analysis. Leptin and adiponectin levels were measured using mouse leptin (EZML-82K) and mouse adiponectin (EZMADP-60K) ELISA kit, respectively (Millipore). Fluorescence readout was measured using SpectraMax Gemini Spectrofluorometer (Molecular Devices Corporation, USA).

Conclusions

In conclusion, we report the design of PYY analogs with a different peptide architecture - mainly isoforms, their synthesis, and their pharmacological characterization, which led to the identification of a number of selective and potent Y2 receptor agonists. Particularly, the compounds effectively reduce body-weight in both lean and DIO mice. Interestingly, despite identical stability and PK-profile between peptide **4** and native human PYY3-36, peptide **4** has additional effects on plasma lipids and presumably fat-cell metabolism reflected in increased adiponectin levels. The reported isoforms of PYY hold interesting and unforeseen pharmacological properties, and should be explored further due to their lipid-lowering effects.

Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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SUPPORTING INFORMATION:

Re-design of a peptide hormone: *N*-terminally branched PYY3-36 isoforms give improved lipid and fat cell metabolism in diet-induced obese mice

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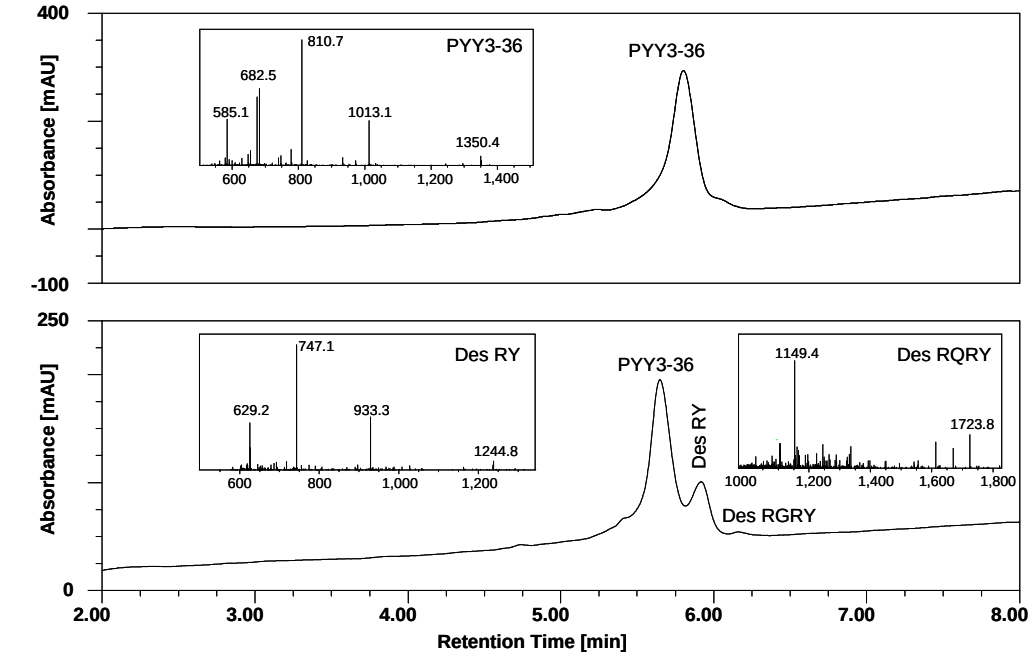

Figure S1. LC-MS. Serum stability of native PYY3-36.

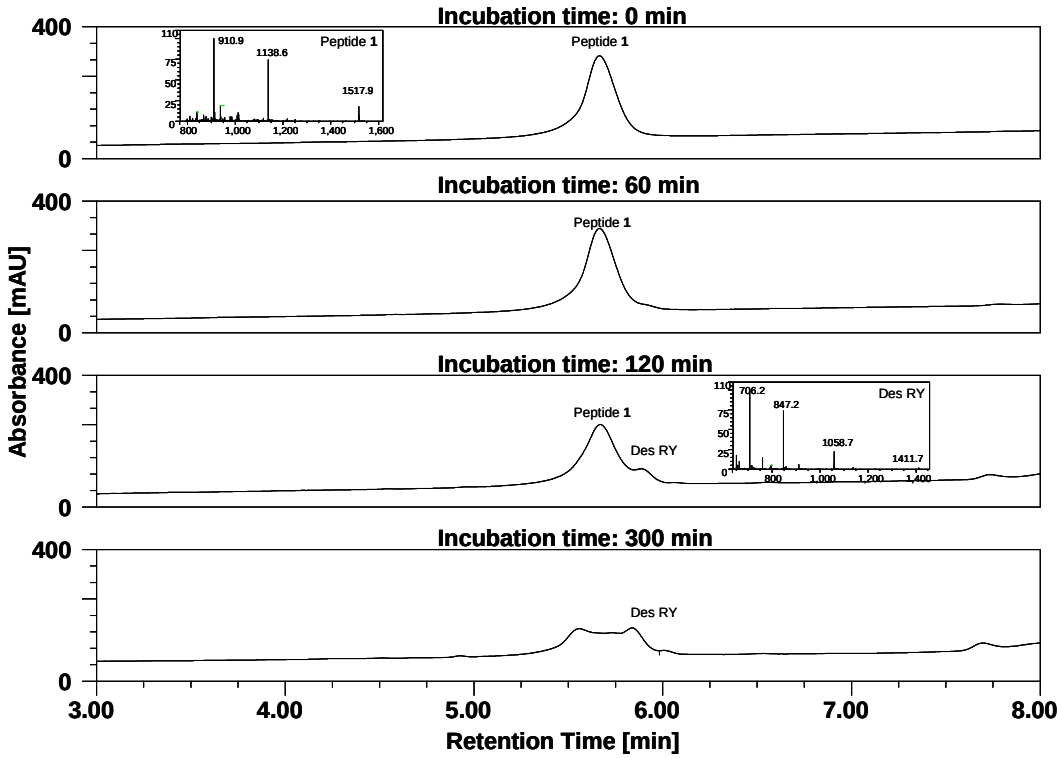

Figure S2. LC-MS. Serum stability of Peptide **1**.

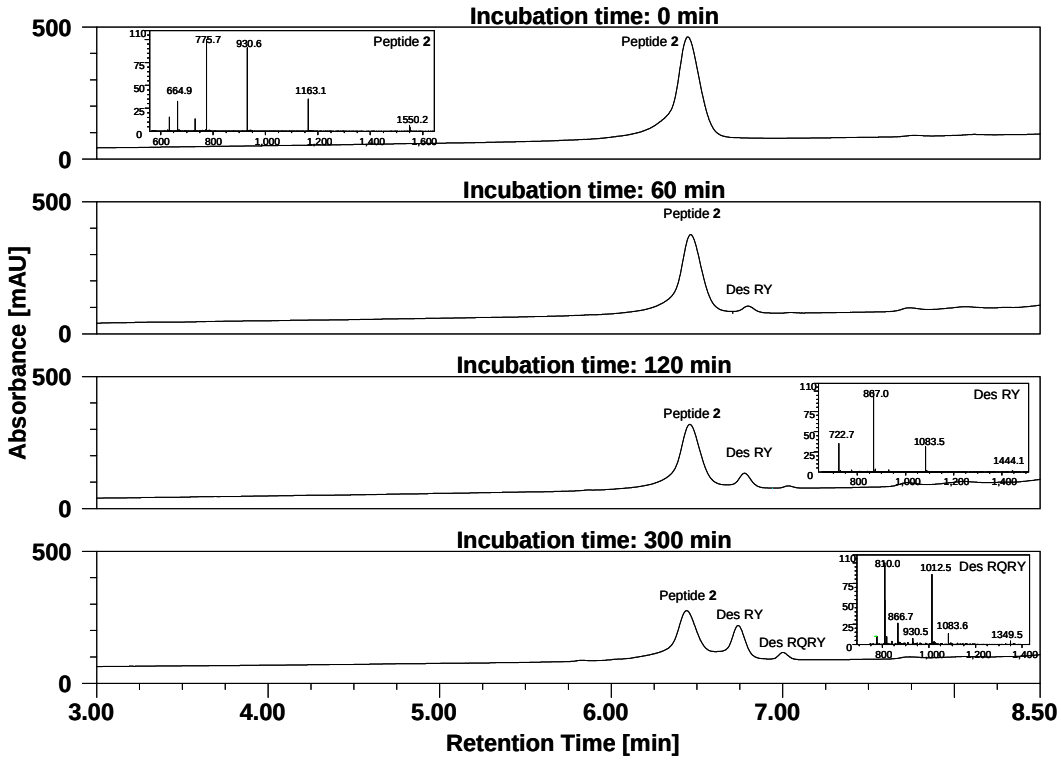

Figure S3. LC-MS. Serum stability of Peptide **2**.

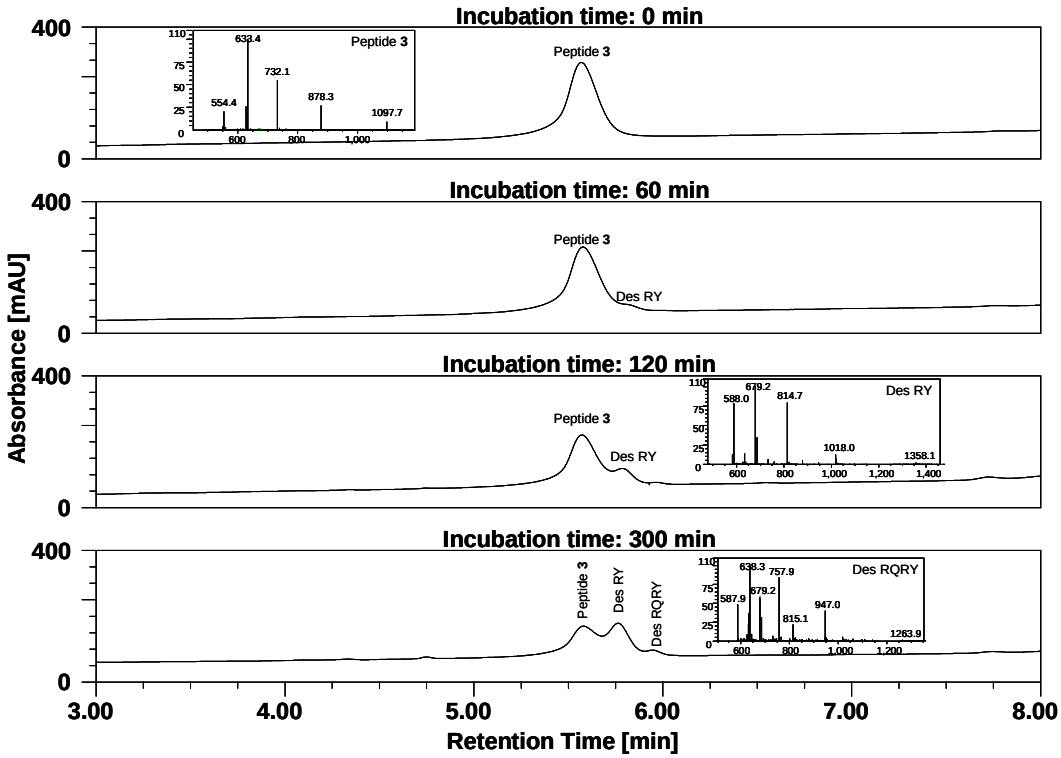

Figure S4. LC-MS. Serum stability of Peptide **3**.

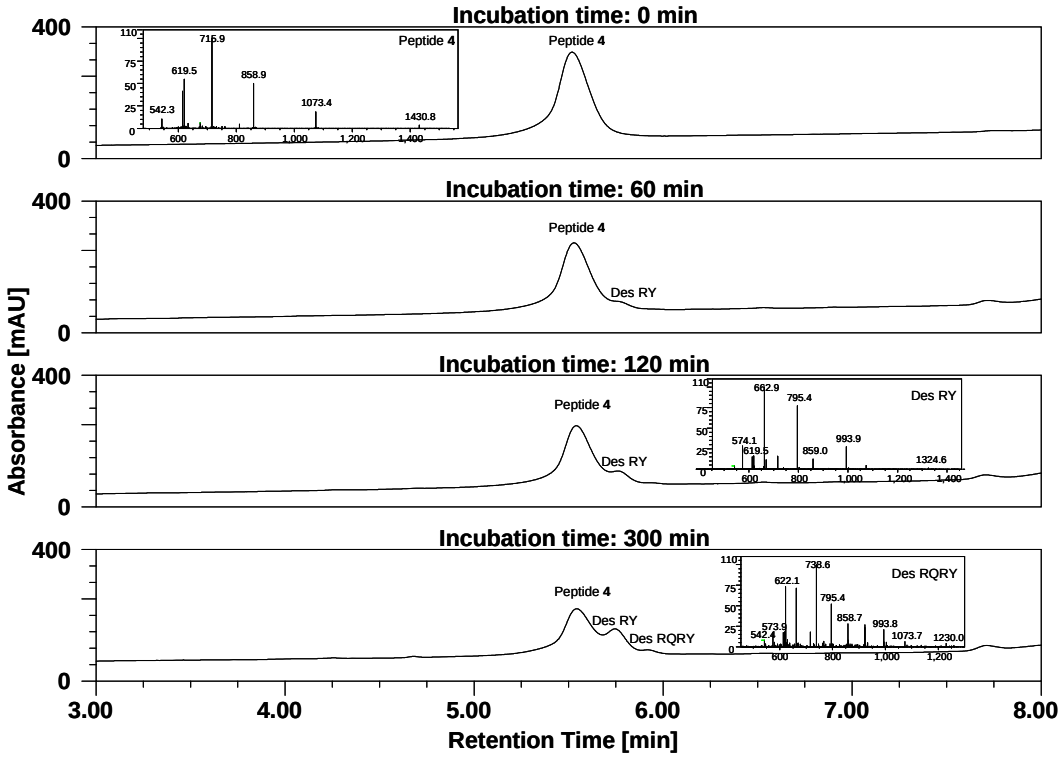

Figure S5. LC-MS. Serum stability of Peptide **4**.

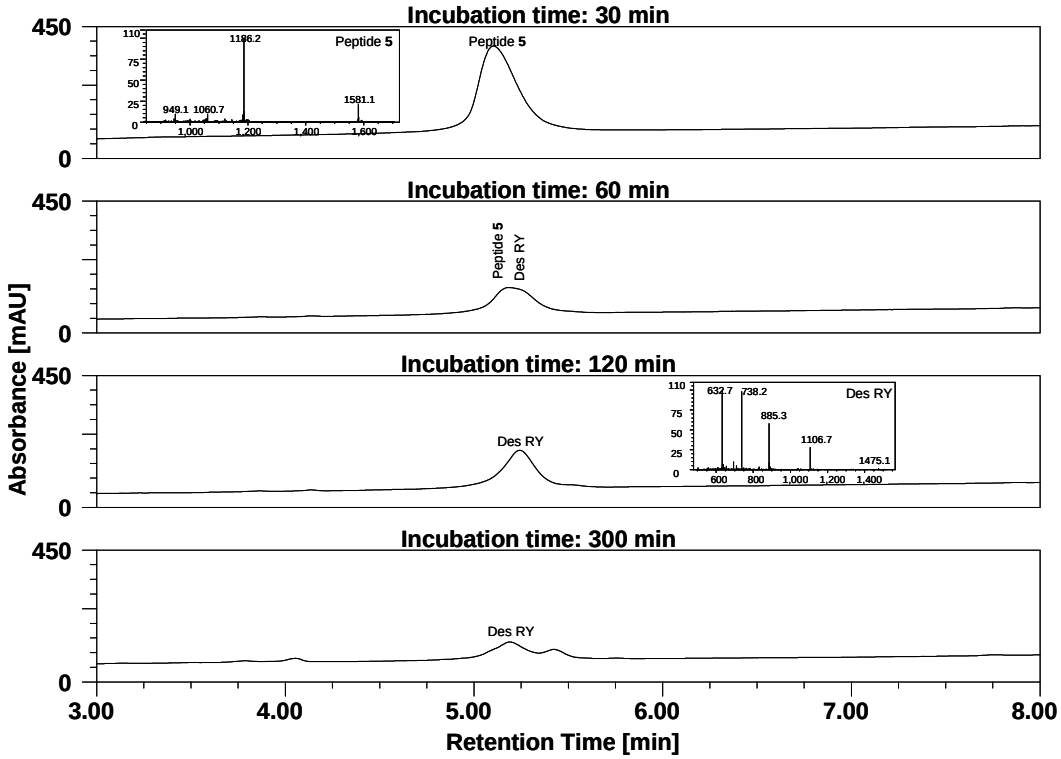

Figure S6. LC-MS. Serum stability of Peptide **5**.

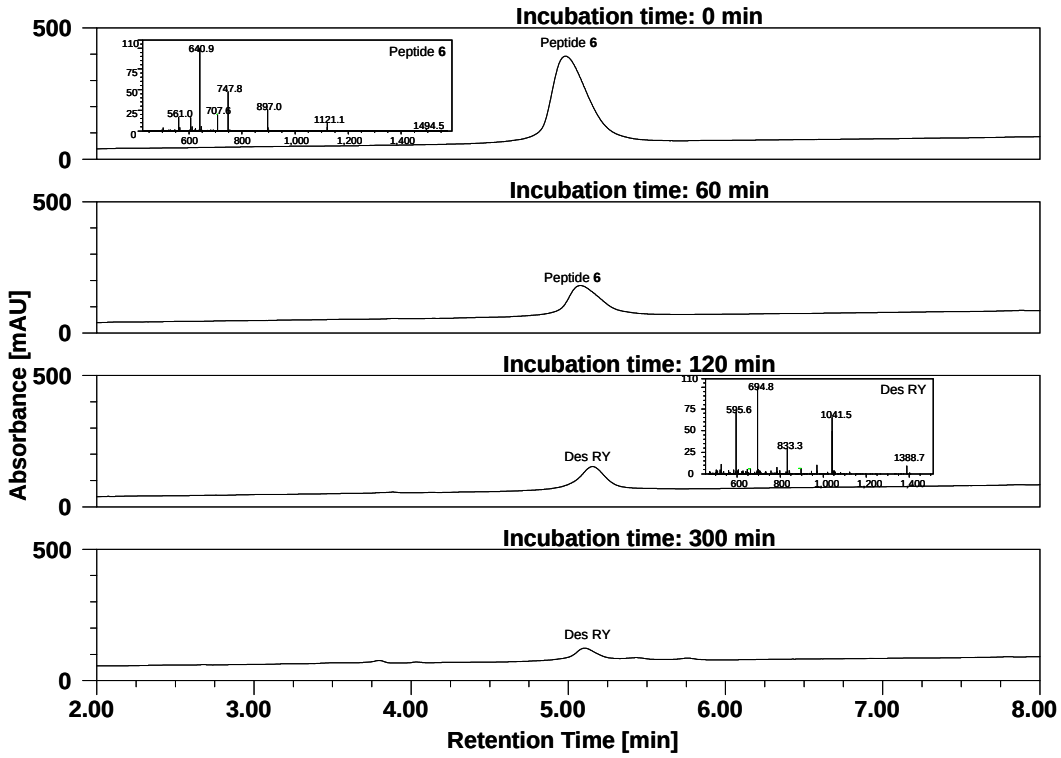

Figure S7. LC-MS. Serum stability of Peptide **6**.

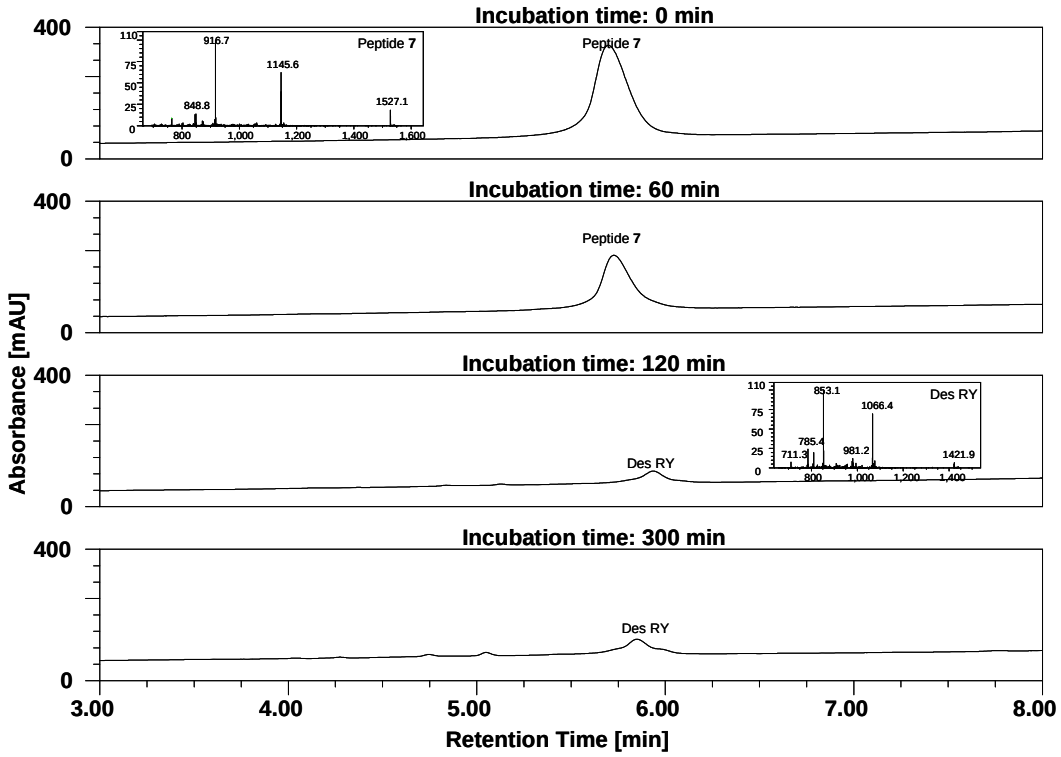

Figure S8. LC-MS. Serum stability of Peptide **7**.

Appendix 4

Paper 4:

Søren L. Pedersen, Pottayil G. Sasikumar, Niels Vrang, and Knud J. Jensen. Peptide-architecture: Adding an α-helix to PYY Lys side-chain maintains binding affinity and demonstrate body-weight lowering effects, *ChemMedChem*, submitted.

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Peptide-architecture: Adding an α-helix to the PYY Lys side-chain provides nanomolar binding and body-weight lowering effects

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The gut hormone PYY3-36 influences food intake and body-weight via interaction with hypothalamic pre-synaptic Y2 receptors. Novel Y2 receptor selective analogs of PYY3-36 are therefore potential drug candidates for the treatment of obesity. It has been hypothesized that PYY3-36 and possibly also the related PP-fold peptides, NPY and PP, bind to the membrane via their amphipathic α-helix prior to receptor interaction. In this view the amphipathic α-helix of PYY3-36 leads to the association of the peptide with the membrane and it is essential for Y receptor potency, as it possibly guides the C-terminal pentapeptide into the correct conformation for receptor activation. Based on this hypothesis, the importance of the amphipathic nature of PYY3-36 as well as the ability of amphipathic α-helices to interact in solution to form di- and tetramers, we re-designed the peptide architecture by addition an amphipathic α-helix via the Lys-4 side-chain of PYY3-36. Two different amphipathic sequences were introduced; first, PYY17-31, the native α-helix of PYY, and secondly, its retro counterpart, PYY31-17, which is also predicted to form an α-helix. Moreover, several different turn motifs between the branching point and the additional α-helix were tested. Several novel peptides with nanomolar Y2 receptor binding affinity as well as increased Y receptor selectivity were synthesized in high purity. CD experiments showed the modifications to be well accepted and an increase in mean ellipticity (ME) per molar peptide at 220 nm was observed, which signifies an increased degree of α-helix per molar peptide. Structure-affinity relationship experiments indicated that the direction of the additional α-helix is less important, in contrast to the turn motifs, which were of high importance for reducing the Y1 receptor binding. The turn motif thus determined the Y1 receptor activity. On the other hand, the structure-activity relationship experiments from acute mice studies showed that the peptide containing the retro-sequences was inactive even though the binding data demonstrated high affinity and selectivity. This demonstrates that radical re-design of peptide architecture can provide nanomolar binding with improved sub-type selectivity and with in vivo efficacy.

Introduction

The worldwide obesity epidemic and the dramatic increases in obesity-associated diseases such as cardiovascular disease and type-II diabetes, calls for not only major emphasis on obesity prevention but also for novel pharmacological approaches to appetite regulation. Over the past 30 years a number of gut peptides with anorectic potential have been described.[1] One of these gut-hormones is PYY3-36 (Table 1), a Y2 receptor agonist, which plays a role in appetite regulation.[2-4] PYY is a member of the PP-fold family of peptides that also consists of pancreatic polypeptide (PP) and neuropeptide Y (NPY) (Table 1).[5] The physiological actions of the PP-fold peptides are expressed through their activation of G-protein coupled receptors (GPCR), the so-called Y receptors (Y1, Y2, Y4 and Y5), which belong to the rhodopsin-like superfamily (class A) of receptors.[6] Both PYY1-36 and NPY display high Y1 and Y5 affinity, while PP is a potent and highly selective Y4 receptor agonist with low affinity to the Y1, Y2, and Y5 receptor subtypes. The endogenous ligand for the Y2 receptor is believed to be PYY3-36. PYY1-36 is co-secreted with GLP-1 postprandially from endocrine L-cells lining the gut[7] and following secretion, PYY1-36 is *N*-terminally cleaved by dipeptidyl peptidase IV (DPP-IV) to produce PYY3-36.[8] The major blood-circulating form of PYY, PYY3-36, has a high affinity towards the Y2 receptor, and to a lesser extent the Y1 receptor.[9] The Y2 receptor is expressed primarily on pre-synaptic terminals in the arcuate nucleus of the hypothalamus, and has been hypothesized to be key mediators of the anorectic

Table 1. Sequences of human NPY, PP, PYY1-36 and PYY3-36. The extra amphipathic α-helix is branching from the side chain amine of Lys-4 in the PYY3-36 sequence.

Peptide	Sequence
PP	H-APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-NH_2
NPY	H-YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-NH_2
PYY1-36	H-YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2
PYY3-36[a]	H-I[4]KPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2

[a] Novel analogs: Additional sequence positioned at the ε-amine of Lys-4 of PYY3-36.

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effects of peripherally administered PYY3-36.[2] Indeed, several studies in both rats and mice using intraperitoneal[4,10] or intravenous[11,12] administration of PYY3-36 have shown acute reductions of food intake as well as chronic effects of PYY3-36 on Body-weight.[4,11] However, since PYY3-36 displays a rather short plasma half-life and also binds to Y1 receptors – potentially counteracting anorectic effects - we aimed to develop more selective (Y2 over Y1) PYY3-36 analogs.

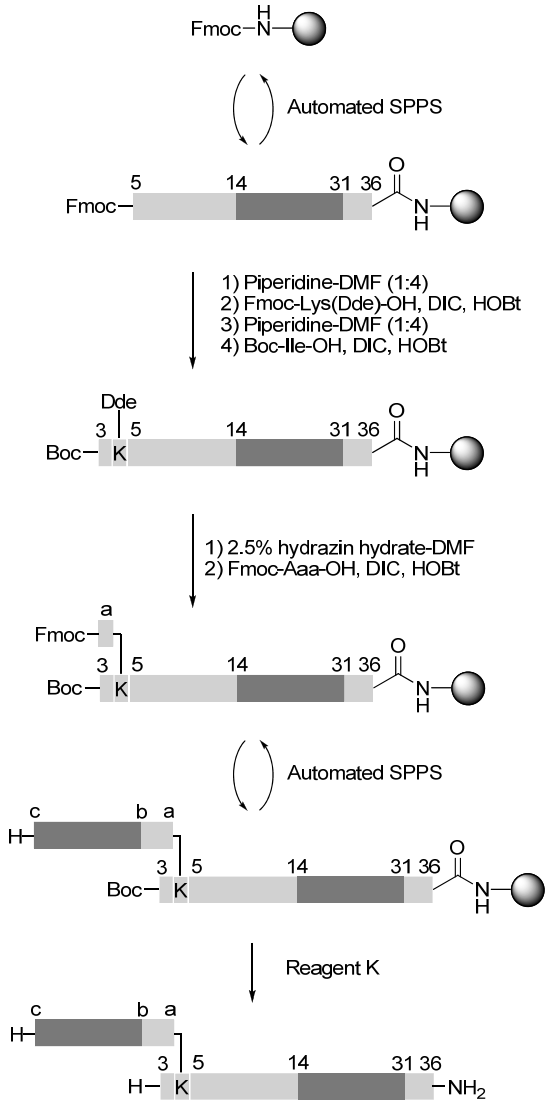
Results and Discussion

Peptide design.

The sequences of the PP-fold family of peptides consist of a *C*-terminal receptor recognizing segment, an amphipathic α-helix and a more flexible *N*-terminus. PYY1-36, as well as PP, are back-folded in solution unlike NPY which is more unstructured.[13] However, when bound to a membrane all the PP-fold peptides show almost identical tertiary structure which led Zerbe and co-workers to the hypothesis that the peptides are not recognized by their receptors directly from solution, but after an association with the membrane, followed by a lateral diffusion along the membrane before binding to the receptor.[13,14] The native amphipathic α-helix of the PP-fold peptides has two functions in the membrane-compartment model[15]: First to direct the peptides to the membrane-water interface and secondly, to guide the *C*-terminal receptor binding segment in the right conformation for activity. The membrane stabilizes both the α-helix and especially the *C*-terminus, which has been shown to be relatively flexible in solution.[13,14] The *N*-terminus (residue 1-12) has been proven very important for the Y receptor selectivity and essential for Y1 and Y4 receptor activity.[16] Recently, it has been suggested that the *N*-terminal segments bind with low affinity to extracellular Y receptor sections thereby acting as an anchor in the transfer from the membrane-bound state into the genuine binding pocket of the receptor for the peptide hormones.[17]

We aimed for a radical re-design of the PYY3-36 architecture based on the original hypotheses regarding peptide hormone interactions with membranes by Kaiser and Kezdy,[18] and Schwyzer,[15] as well as recent results by Zerbe and co-workers regarding PYY3-36.[13,14] The overall aim was to (*i*) improve membrane binding, (*ii*) improve the selectivity for Y2 over Y1, and (*iii*) to test this through binding affinities to Y1, Y2, and Y4 receptors. Rather than trying to obtain this by single amino acid substitutions, we decided for re-designing the overall peptide topology. To achieve this we ornamented PYY3-36 by (*i*) addition of one of two α-helices (PYY17-31 and its retro-sequence) to the ε-amino group in the native Lys-4 (a 'helix branch'), and (*ii*) insertion of one of four different turn motifs between the ε-amine and the new helix, as well as the control without a turn motif (Tables 1 and 2). As previously stated the *N*-terminus of PYY is very important for Y1 receptor affinity[16] and branching from Lys-4 could lead to a decrease in Y1 receptor affinity. We hypothesized that the amphipathic nature of the additional α-helix, and its potentially increased affinity towards the cell membrane, could lead to an accumulation of peptide near the Y receptors. Bringing the peptide from the three-dimensional space in solution on to the two-dimensional plane of the cell membrane should increase the probability of the peptide finding the receptor as well as the ability of activating the receptor multiple times. Secondly, the additional α-helix could in solution potentially back-fold alongside the native α-helix and/or the *N*-terminal segment. Third, as the *N*-terminal end is important for binding to the Y1 receptor, addition of the *N*-terminal branched helix might suppress binding to the Y1 receptor thus providing better Y2 receptor selectivity. Finally, the additional α-helix could possibly support a conformational stabilization of the *C*-terminal pentapeptide leading to an increase in Y receptor selectivity via a decreased Y1 receptor potency.

These analogs of PYY3-36 with α-helical branches gave increased Y receptor selectivity (Y2 over Y1), and body-weight lowering effects in lean male NMRI mice over a period of 3 days. Circular dichroism (CD) indicates that all the novel analogs maintained the native α-helical nature of PYY3-36. Several α-helical branched analogs showed an increased Y receptor selectivity and two of these compounds showed a promising ability to reduce body-weight in a mouse model of obesity.



Scheme 1. Synthetic scheme for solid-phase synthesis of α-helical branched PYY3-36 analogs. The dark grey boxes represent the regions theoretically α-helical, light grey boxes corresponds to the unstructured regions, the *C*-terminal binding segment, turns and the *N*-terminus. Region a-b shows the variable turn motif and b-c illustrates the position of the additional amphipathic α-helix.

Peptide synthesis

All peptides were assembled using the Fmoc/*t*-Bu strategy by automated solid-phase peptide synthesis on Rink Amide TentaGel resin. The N^{α}-Fmoc-protected amino acids were coupled using DIC and HOBt as coupling reagent and additive, in DMF, with coupling times of 2 hours. The Fmoc group was removed using piperidine-DMF (1:4; 3 + 17 min). The sequences of PYY1-36, PYY3-36 and resin-bound PYY5-36 were assembled using standard Fmoc/*t*-Bu chemistry as described above. The sequence of resin-bound PYY5-36 was additionally modified by coupling of the Dde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl) side-chain protected Lys at position 4. After Fmoc removal N^{α}-Boc-protected Ile was coupled at position 3 (Scheme 1). The Dde group was removed by 2.5 % (v/v) hydrazine hydrate in DMF making the side-chain ε-amino group available for assembly of the peptide branch (Scheme 1, a-c), which additionally was performed by standard Fmoc/*t*-Bu chemistry. Except for peptides **1** and **2**, a spacer or turn motif (Scheme 1, a-b) was incorporated between the branch point and the additional α-helix. After completion, the peptides were side-chain deprotected and simultaneously cleaved from the solid support using reagent K. Finally, the peptides were purified by preparative RP-HPLC, quantified by analytical RP-HPLC and characterized by mass spectrometry. The final products were obtained with >95% purity (Table 3).

Table 2. PYY1-36, PYY3-36 and the H-I^{4}**K**PEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2 analogs binding to the Y receptor subtypes Y1, Y2, and Y4, respectively.

	Lys-4 branched sequences			Y1R[a]	Y2R[b]	Y4R[b]
Peptide	α-helix	Turns	Nature of α-helix	IC_{50} [nM]	IC_{50} [nM]	IC_{50} [nM]
PYY1-36				4.0 ± 1.7	3.8 ± 1.6	141 ± 79
PYY3-36				7.8 ± 1.1	2.1 ± 0.2	255 ± 29
1	H-LNRYYASLRHYLNLV	-	PYY17-31	11.7 ± 3.7	6.2 ± 1.1	395 ± 121
2	H-VLNLYHRLSAYYRNL	-	PYY31-17	7.9 ± 0.5	5.4 ± 0.3	496 ± 91
3	H-LNRYYASLRHYLNLV	GPRRP-	PYY17-31	>1000	2.1 ± 0.2	173 ± 60
4	H-VLNLYHRLSAYYRNL	GPRRP-	PYY31-17	>1000	0.4 ± 0.1	101 ± 16
5	H-LNRYYASLRHYLNLV	GG-	PYY17-31	>1000	14.1 ± 4.5	567 ± 40
6	H-VLNLYHRLSAYYRNL	PE-	PYY31-17	17.2 ± 1.8	7.8 ± 2.2	276 ± 5
7	H-LNRYYASLRHYLNLV	SP-	PYY17-31	>1000	7.6 ± 0.4	298 ± 15
8	H-VLNLYHRLSAYYRNL	SP-	PYY31-17	>1000	5.6 ± 1.8	362 ± 127

[a] SK-N-MC cells expressing the Y1 receptor. [b] Transfected Y2 or Y4 receptors in HEK293 Flp-In T-Rex cells.

Structure affinity relationship.

To characterize the influence of adding an amphipathic α-helix and modifying the turn sequence to the side-chain of Lys-4, the binding affinity of the novel PYY3-36 analogs were tested using a radioligand displacement assay (competition binding assay) based on membranes from SK-N-MC and HEK293 Flp-In T-Rex cell lines expressing the human Y1, Y2 and Y4 receptor subtypes, respectively. ^{125}I-[^{31}Leu,^{34}Pro]-pNPY for Y1R, ^{125}I-hPYY3-36 for Y2R and ^{125}I-hPP for Y4R were used as radioligands. Binding affinities (IC_{50} values) for PYY1-36, PYY3-36 and the novel analogs (peptides **1-8**) are summarized in Table 2.

Adding an amphipathic α-helix to the side-chain of Lys-4 of PYY3-36 has only moderate impact on binding affinity towards the Y2 and Y4 receptors. In general, 1-3 fold decreases in affinity towards the Y2 receptor were observed, except for peptide **5**, which gave a 7-fold reduction in Y2 receptor binding affinity. Peptide **4** gave a 5-fold increase in affinity towards the Y2 receptor. The binding data revealed a slight tendency that the peptides containing the retro sequence to be better accepted by the Y2 receptor than the natural sequence, however, the nature of the turn region led to more significant differences in binding affinity towards the Y2 receptor (Table 2 and Figure 1). The GG spacer sequence (helix stop motif) caused poor binding towards all three Y receptor subtypes. The Y2 receptor affinity of analogs without a spacer or turn motif (peptides **1** and **2**) were equipotent to both the PE (peptide **6**) and SP (peptides **7** and **8**) turn motifs, which are relative restricted turns because of the proline residue. The GPRRP turn motif (peptides **3** and **4**) were the best accepted sequence and gave equivalent to enhanced Y2 receptor binding affinities. The GPRRP turn motif was adapted from Hill and

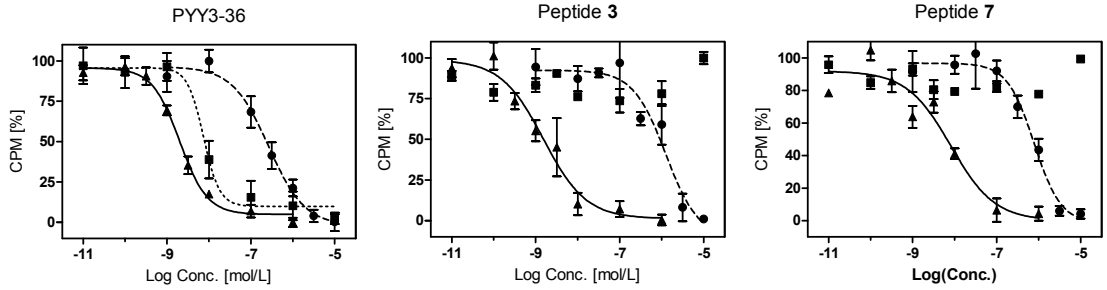


Figure 1. Binding of PYY3-36, peptides **3** and **7** towards the Y1 (■), the Y2 (▲) and Y4 (●) receptors. The curves are given as means of the replicates for each peptide.

Degrado[19] and limited the flexibility of the additional α-helix even more than both the SP and PE turn motifs, which in so doing may guide the *C*-terminal in an optimal conformation for Y2 receptor binding and/or prevents Y1 receptor binding. In addition, the GPRRP motif has a double positive charge. A similar change in binding affinity appears for the Y4 receptor, except the type of the amphipathic α-helix seems to have no consequence for the binding affinity towards the Y4 receptor. Interestingly, the analogs with a GPRRP turn motif (peptides **3** and **4**) were also well tolerated towards the Y4 receptor and led to almost a threefold increase in binding affinity (Table 2 and Figure 1). The PE (peptide **6**) and SP (peptides **7** and **8**) turns were again equipotent to PYY3-36, however, excluding the turn motif (peptides **1** and **2**) proved to be slightly unfavorable. The GG turn (peptide **5**) was equipotent to the analogs without a turn motif and demonstrated about half the potency of PYY3-36 towards the Y4 receptor. The binding affinities towards the Y1 receptor were greatly affected by the addition of an additional amphipathic α-helix. No turn (peptides **1** and **2**) and the PE turn motif (peptide **6**) had little effect on the binding affinity towards the Y1 receptor. The latter gave an almost identical Y receptor binding profile as PYY3-36, which indicates that the additional α-helix has to be positioned in a certain fashion to have influence on the Y receptor binding affinity. Peptides **3**-**5** and **6**-**8** showed a very low Y1 receptor affinity, which consequently leads to improved Y2/Y1 and Y4/Y1 receptor selectivity's compared to PYY3-36 (Table 2 and Figure 1).

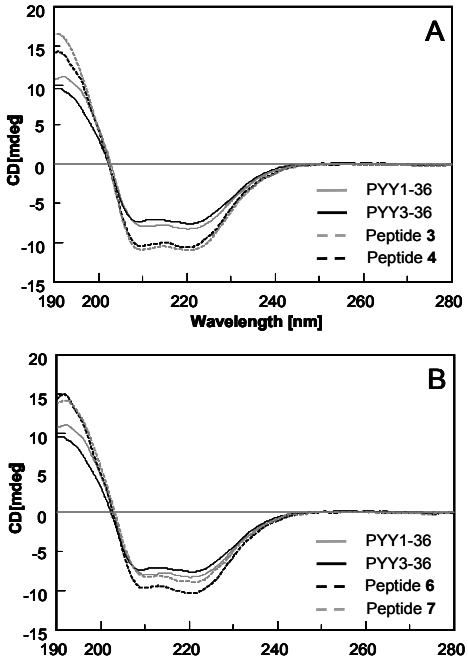


Figure 2. UV CD spectra of PYY1-36, PYY3-36, peptide **3**-**4**, and peptide **6**-**7** in aqueous buffered solution (20 μM peptide in 10 mM phosphate buffer).

Biophysical data

The secondary structures of peptides **1**-**8** in solution were assessed by circular dichroism (CD) in 10 mM phosphate buffer. The spectra obtained are typical for the α-helical structure, showing characteristic minima at 208 nm and 222 nm (Figure 2). The degree of α-helicity of the analyzed peptides were between 20 and 31%, while PYY1-36 and PYY3-36 had an α-helicity of 27 and 26%, respectively. Several branched PYY3-36 analogs showed nearly a doubling in mean molar ellipticity (ME) at 220 nm, *i.e.* per mol peptide, compared to PYY3-36, originating from the increase in the number of α-helical residues (not shown). Peptides **2**, **5**, and **7** show equal ME at 220 nm, compared to PYY3-36, thus for these peptides the addition of a potential α-helix surprisingly did not give a higher ME in solution. For all the novel analogs the introduction of an additional α-helix appears to be well tolerated and do not result in disruption of the secondary structure. Size exclusion chromatography of 40 μM peptide in phosphate buffered saline (PBS) showed the novel analogs to be monomers. The amphipathic nature of the α-helix of the PP-fold peptides is, as previously explained, important for the binding affinity towards the Y receptors.[20] Since the branched peptides are monomeric and several have higher ellipticity per molar (ME) at 220 nm they theoretically have more amphipathic structure exposed for either intra-molecular back-folding of the two α-helices in solution and/or membrane binding.

Structure activity relationship

Peptides **1**-**8** were investigated in an acute *in vivo* model in lean male NMRI mice (Naval Medical Research Institute, Charles River). The vehicle for all experiments was isotonic saline (0.9%). The peptides were administered twice daily subcutaneously in the inguinal region. The mice were given either vehicle (0.9% saline),

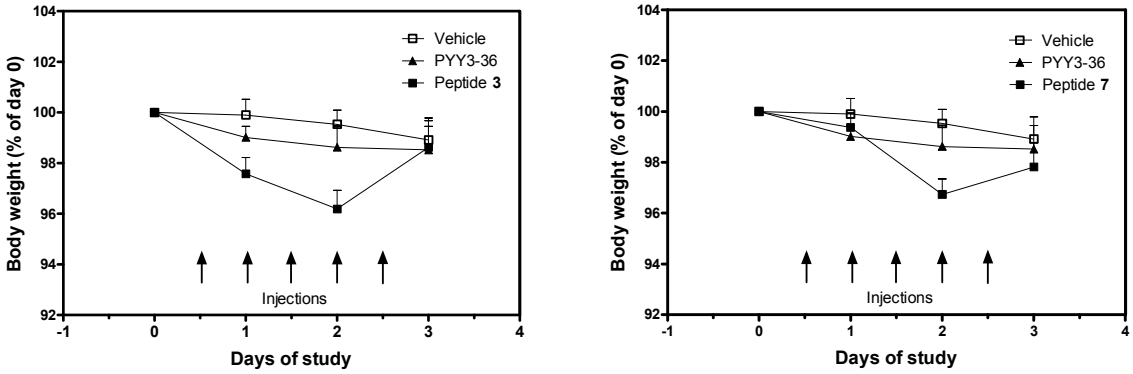


Figure 3. Acute effect of peptides **3** and **7** in lean male NMRI mice. The mice were given either 0.9% saline as vehicle (□), 1.0 μmol/kg/day PYY3-36 (▲), or 1.0 μmol/kg/day branched peptide (■) two times a day for two days, shown by an arrow, with a total of five injections. The body weights were calculated every morning for four days and given as mean body-weight (per cent of day zero). There were seven mice in each group (n=7).

PYY3-36, or peptides **1**-**8** in concentrations of 1.0 µmole/kg/day (volume of 3 mL/kg). The animals were injected a total of five times, starting on day 0.5 and last injection at day 2.5. The body-weights were recorded each morning at day 0 to 4. The animals had free access to food and water throughout the duration of the experiment.

The peptides **1**, **2**, and **6** had poor effect on body-weight compared to PYY3-36, which correlated with the relatively good binding affinity to the Y1 receptor as well as the 3-4 fold reduction in Y2 receptor binding affinity. Also, peptide **5** showed no effect on body-weight, which was probably because of the relatively poor Y2 receptor binding affinity. Binding affinities of peptides **3** and **4** were almost equivalent, except in Y2 receptor affinity of peptide **4** that was superior. However, while peptide **4** did not give any body-weight lowering effect, peptide **3** was efficacious (~4% body-weight reduction) (Figure 3). Almost identical Y receptor affinities were given for the isomeric pair **7** and **8**, nevertheless, peptide **7** reduced body-weight by ~3% (Figure 3) and peptide **8** had no effect, even though peptide **8** has slightly better binding affinity pattern. Peptides **3** and **4** both have the GPRRP turn motif and peptides **7** and **8** both have the SP turn before the additional amphipathic α-helix. The key difference was that the additional α-helix in peptides **4** and **8** was the retro-sequence (PYY31-17), unlike the two compounds showing body-weight reducing efficacy, peptides **3** and **7** (Figure 3), which had the forward sequences (PYY17-31). Thus, in contrast to the binding experiments the orientation of the additional amphipathic α-helix, *i.e.* forward vs. retro sequence, was important for *in vivo* efficacy. Moreover, the *in vivo* experiments generally confirm the results from the affinity study that the turn or spacer motif was of central importance.

Conclusion

In conclusion, based on the hypothesis that PYY3-36 interacts with the membrane prior to receptor binding, we have designed PYY3-36 analogs with a novel peptide architecture. An additional amphipathic α-helix was introduced on the Lys-4 side-chain in the PYY3-36 sequence. This increased the selectivity for the Y2 receptor subtype over the Y1 receptor through a decrease in binding to the latter, possibly by guiding the *C*-terminal pentapeptide into a beneficial binding mode. In addition, previous studies had shown that *N*-terminal truncation of PYY3-36 had dramatically decreased its Y1 binding[16]; thus placing an additional helix at the *N*-terminus might decrease Y1 binding. The turn motif showed to be a switch for Y1 receptor potency without affecting the Y2 receptor potency. Incorporating the restricted GPRRP and SP turns gave high potency both *in vitro* and *in vivo*. In contrast, the GG and PE turns, as well as the absence of a turn in the sequence, gave either poor Y2 receptor affinity or relative high Y1 receptor affinity *in vitro* and showed no activity *in vivo*. Introduction of an additional α-helix also provides more possibilities for self-assembly of these novel PYY3-36 analogs. The addition of an amphipathic α-helix generally leads to an increased degree of mean ellipticity (ME). CD indicates non or little disturbance of the native α-helical structure of the peptides and SEC indicates the peptides are monomers in PBS. Peptides with the retro-sequence were inactive in the *in vivo* experiments, in contrast to the structure-affinity relationship experiments which shown good selectivity and affinity. An increased Y receptor selectivity, compared to PYY3-36, led to a 3-4% reduction in body-weight for the peptides **3** and **7** in lean male NMRI mice. This demonstrates that radical re-design of the PYY3-36 architecture can provide new agonists with nanomolar affinities and improved sub-type selectivity as well as *in vivo* efficacy.

Experimental Section

Materials. The organic solvents and reagents for peptide synthesis were all of analytical reagent grade and were obtained from Fluka, Advanced Chemtech, Sigma Aldrich, Chemimpex, Chemlabs or Spectrochem. TentaGel S Rink Amide resin was obtained from Fluka. Human Embryonic Kidney 293 cells (HEK293 Flp-In T-Rex), and pcDNA3.1 vector were purchased from Invitrogen. Dulbecco's Modified Eagle's Medium (D-MEM), fetal calf serum, penicillin-streptomycin solution, phosphate buffered saline (PBS), sucrose, and 99% glycerol were all obtained from Sigma-Aldrich. FuGENE 6 transfection reagent, complete protease inhibitor cocktail tablets and bovine serum albumin (BSA) were purchased from Roche. 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS), $MgCl_2 \cdot 6H_2O$, $CaCl_2$, and NaCl were obtained from AppliChem GmbH. (*2S,3S*)-1,4-bis-sulfanylbutane-2,3-diol (DTT) was obtained from GE Healthcare and radioactive labeled agonists were purchased from Phoenix pharmaceuticals. 96-Well filtration MultiScreen HTS, DV plates were obtained from Millipore.

Peptide synthesis. The peptides were prepared by automated peptide synthesis on a Symphony parallel synthesizer (PTI) by standard solid-phase peptide synthesis (SPPS) on TentaGel S Rink Amide resin (0.24 mmol/g loading) with 9-fluorenylmethyloxycarbonyl (Fmoc) for protection of N^{α}-amino groups except for the *N*-terminal Ile of the main-chain, where Boc (*tert*-butoxycarbonyl) was used. Side-chain protecting groups were *tert*-butyl (Ser, Thr, Tyr, Asp, Glu), *tert*-butoxycarbonyl (Lys), 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde, for branching at Lys), 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf, for Arg), and trityl (Trt, for Asn, Gln, His). The peptides syntheses were conducted in 0.1 mmol scale. N^{α}-Fmoc amino acids (6.0 equiv) were coupled using diisopropylcarbodiimide (DIC) (6.0 equiv) as coupling agent and 1-hydroxybenzotriazole (HOBt) (6.0 equiv) as additive in DMF for 120 min. N^{α}-Fmoc deprotection was performed using piperidine-DMF (1:4) for 3 min, followed by piperidine-DMF (1:4) for 17 min. The Lys(Dde) residue was side-chain deprotected with 2.5% (v/v) hydrazine hydrate in DMF at room temperature for 15 min. The peptide amides were

Table 3. Quantification and characterization data of PYY1-36, PYY3-36 and peptide **1-8**.

	ESI-MS (m/z)[a]		Purity[b]
Peptide	Calcd. MS	Found, $[M+4H]^{4+}$	[%]
PYY1-36	4309.8	1078.1	98
PYY3-36	4049.5	1012.8	98
1	5926.3	1482.4	97
2	5926.3	1482.5	96
3	6490.4	1623.3	96
4	6490.4	1623.3	96
5	6042.0	1510.6	95
6	6111.0	1528.5	95
7	6111.0	1528.1	96
8	6153.0	1538.7	95

[a] Identified by ESI-MS on a Agilent Technologies LC/MSD VL.

[b] Quantified by RP-HPLC at 215 nm. Eluent A: 0.1% TFA in H_2O. Eluent B: 0.1% TFA in AcN.

released from the solid support by treatment with reagent K (trifluoroacetic acid (TFA)-phenol-thioanisol-1,2-ethanedithiol (EDT)-H_2O (82.5:5:5:2.5)) for 2.5 hours. The TFA solutions were concentrated by nitrogen flow and the compounds were precipitated with diethylether to yield the crude materials as white powders.

Purification was accomplished by RP-HPLC (Agilent 1200 series) on a preparative column (Zorbax-Eclipse XDB-C18, 7 *μ*m particles, 21.2×250 mm) using the following solvent system: solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA. B gradient elution (0-2 min 5%, 2-5 min 5-25%, 5-50 min 25-60%) was applied at a flow rate of 20 mL min^{-1} and column effluent was monitored by UV absorbance at 220 nm. Identification and quantification were carried out by LC-MS (Agilent Technologies LC/MSD VL) using the eluent system A-B (solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA). The eluent system A-B was applied on a C18 analytical column (Zorbax Eclipse XDB, 80 Å 5 *μ*m 4.6×150 mm) where the B gradient elution (0-25 min: 5-50%) was applied at a flow rate of 1.0 mL min^{-1}. Quantification and characterization data are given in Table 3.

Size exclusion chromatography. SEC was performed on an ÄKTA purifier equipped with a Superdex peptide HR10/30 column (GE healthcare) with an internal diameter of 10 mm. Packing height was 30 cm and with an approximated column volume of 24 mL. The following standards dissolved in PBS (1.0 mg mL^{-1}) were used; Aprotinin (6500 Da), ribonuclease A (13700 Da), carbonic anhydrase (29000 Da) and ovalbumin (43000 Da). As mobile phase phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer) at pH 7.4, was used. The samples were dissolved in 10 mM phosphate buffer at pH 7.4 to a final peptide concentration of 40 μM. Inject volume was 50 μL. The column effluent was monitored at 215, 254 and 280 nm.

Circular dichroism spectroscopy. UV CD spectra were recorded on a JASCO J-810 circular dichroism system using rectangular Hellma quartz cells with a light path of 1 mm. The peptide solutions were approximately 20 μM in 10 mM phosphate buffer, pH 7.4. The absolute concentration was determined spectroscopically (tyrosine absorption at 274 nm, using ε = 1420 $cm^{-1}M^{-1}$). The mean residue ellipticity (MRE) calculated according to Yang *et al.* in which the number *n* refers to the number of residues.[21] The α-helicity was calculated according to a formula by Yang and co-workers.[21]

Radioligand displacement assay. Cell culture and receptor expression: The SK-N-MC cells were cultured in a 1:1 mix of HAM F12 and Dulbecco's Modified Eagle's Medium (D-MEM 1885), containing 15% (v/v) fetal calf serum (FCS), 1% non essential amino acids and 1% (v/v) penicillin-streptomycin in humidified atmosphere of 5% carbon dioxide and 95% air, at 37°C. The human embryonic kidney (HEK293 Flp-In T-Rex) derived cell line was cultured in Dulbecco's Modified Eagle's Medium (D-MEM), containing 10% (v/v) fetal calf serum (FCS) and 1% (v/v) penicillin-streptomycin. Cells were grown as monolayers in humidified atmosphere of 5% carbon dioxide and 95% air, for 48 hours at 37°C. Using serum-free D-MEM and FuGENE 6 transfection reagent HEK293 Flp-In T-Rex cells were transiently transfected by pcDNA3.1 vectors which encode either the human Y2 or Y4 receptor (FuGENE 6/pcDNA3.1, 3:1). The transfected HEK293 Flp-In T-Rex cells were set to express the receptors in humidified atmosphere of 5% carbon dioxide and 95% air, for another 48 hours at 37°C.

Preparation of membrane fractions. The SK-N-MC and the transiently transfected HEK293 Flp-In T-Rex cells were washed with phosphate buffered saline (PBS) and homogenized in cold homogenization buffer (50 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.0 mM DTT, complete protease inhibitor cocktail (one tablet per 50 mL buffer)). An equal amount of 0.6 M sucrose was added to the cell mixture. The homogenate was centrifuged (10,000 g, 10 min at 4°C). Cell pellets were washed in washing buffer (SK-N-MC cells: 50 mM TRIS (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.2% (w/w) BSA fraction V. HEK293-Flp-In-T-Rex: 50 mM TRIS (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 mL buffer), 1.0% (w/w) BSA fraction V), and subsequently the suspensions were centrifuged (10,000 g, 10 min at 4°C). The pellets were re-suspended in glycerol containing binding buffer (50 mM TRIS (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 mL buffer), 1.0% (w/w) BSA fraction V, 20% (v/v) glycerol) and concentration was adjusted to an OD600 of 1.6.

Binding affinity. All binding experiments were performed in 96-well filtration MultiScreen HTS, DV plates (Millipore) and every concentration point was performed as triplicates. The absolute stock concentration of the unlabeled peptides were determined spectroscopically (tyrosine absorption at 274 nm, using ε = 1420 $cm^{-1}M^{-1}$). The unlabeled peptide (25 μL) at concentrations between 10 pM and 10 μM, cell membrane suspension (3.5 μL), binding buffer (61.5 μL, 50 mM TRIS, pH 7.5, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 mL buffer), 1.0% (w/w) BSA fraction V)), radioligand solution (10 μL; Y1R: ^{125}I-[^{31}Leu,^{34}Pro]-pNPY; Y2R: ^{125}I-PYY3-36; and Y4R: ^{125}I-PP; specific activity of 800-1000 Ci $mmole^{-1}$). After 1-2 hours of incubation the assay was terminated by filtration. Finally, the membrane-receptor-ligand complexes were washed twice in cold TRIS buffer (50 mM TRIS, pH 7.5, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$) containing 5.0% BSA fraction V. Bound radioactivity was determined as counts per minute (Wallac 1470 Wizard™ Automated Gamma Counter). Binding data were analyzed with GraphPad Prism 5.0 (GraphPad Software, Inc. San Diego, CA, USA). The displacement experiments were replicated until the standard error of mean (S.E.M.) of –log IC_{50} was below 0.1 (n = 2-5).

***In vivo* experiments.** All experiments were conducted in accordance with internationally accepted principles for the care and use of laboratory animals and were approved by the Danish Committee for Animal Research.

Animals. The studies were carried out in lean male NMRI mice (Naval Medical Research Institute, Charles River) 8-9 weeks old. Studies were performed in the In Vivo Pharmacology Department of Rheoscience A/S. Animals were kept on a 12:12-h light-dark cycle (lights on at 03:00 AM) in a temperature-controlled environment (22–24°C).

Sample preparation. Vehicle for all experiments was isotonic saline. Compounds were administered twice daily subcutaneously in the inguinal region (inject volume: 3 mL/kg; for a 30 gram mouse 0.09 mL). The animals were given either vehicle (0.9% saline), PYY3-36 or peptides **1**-**8** in concentrations of 1.0 μmole/kg/day.

General procedure. The animals were on the day of arrival given water and food for the entire experiment (standard chow; Altromin standard chow 1324; Petersen, Ringsted, Denmark) and transferred to single cages upon arrival. After 5 days body-weight was recorded on the morning of day 0 (7:00 AM) and the mice were randomized. In the afternoon of day 0 (14:30 PM) the mice are given the first injection and the following 1.5 days mice are injected daily (06:30 AM and 14:30 PM) with a total of 5 injections. Body-weight was recorded daily in the morning immediately after injection. Last injection was at 14:30 PM on day 2. Body-weight was also recorded on the morning of day 3 and 4.

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Keywords: PYY3-36 · Y2 receptor agonist · Amphipathic α-helix · Obesity · Gut hormone

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Appendix 5

Paper 5:

Søren L. Pedersen, Birgitte Holst, Maria H. Pedersen, Niels Vrang and Knud J. Jensen. Unique design concept of novel PYY3-36 analogs: Introducing an amphipathic *de novo* designed α-helix leads to increased Y2 receptor potency and selectivity, *in manuscript*.

Unique design concept of novel PYY3-36 analogs: Introducing an amphipathic *de novo* designed α-helix leads to increased Y2 receptor potency and selectivity

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Keywords

PYY, PYY3-36, Y2 receptor agonist, Gut hormone, Obesity

Abstract

Food intake and energy expenditure are homeostaticly regulated by the hypothalamus and brainstem by central and peripheral hunger and satiety signals, such as PYY3-36. PYY3-36 expresses its actions by binding to an appetite and body-weight controlling G-protein coupled receptor family, the so-called Y receptors. It has been hypothesized that PYY3-36 accumulates on the surface of the membrane prior to receptor activation. Based on this phenomenon we have designed a collection of PYY analogs, which could possess a higher affinity towards the membrane and consequently, an increased peptide concentration in the vicinity of the Y receptor. The *N*-terminus of PYY was exchanged with different *de novo* designed heptad repeat α-helices. In doing so, the hydrophobicity and the amphipathic nature of the ligands were increased. The results show increased Y2 receptor affinity and potency for a number of our novel Y2 receptor agonists. Additionally, the affinity and potency towards the Y1 and Y4 receptors were either unaffected or decreased, most likely as a result of the removal of the flexible *N*-terminal segment. These novel ligands further support the hypothesis that PYY3-36 associates with the membrane prior to binding to the receptor.

Introduction

Food intake and energy expenditure are homeostaticly regulated by the hypothalamus and brainstem. Central and peripheral hunger and satiety signals, such as neural and hormonal messengers from the gut and adipose tissue, supply key brain regions with information of the current state of energy.[1] However, the regulatory system is generally unable to cope with large amounts of high-energy foods in conjunction with an inactive lifestyle, resulting in a gain in body-weight.[2] Even though the system, in some cases, has proven unable to control food intake, elements of the regulatory system might be the key to combat obesity.

The only effective treatment of morbid obesity is a gastric bypass operation, which often leads to 30-40% sustained weight-loss.[3] In addition to mechanical factors, gastric bypass also leads to changes in the release of gut hormones, such as increased secretion of peptide YY (PYY3-36) and GLP-1.[4] Full-length PYY1-36 is postprandially secreted by L-cells in the distal gut.[5] Subsequently, the *N*-terminal dipeptide, Tyr-Pro, is cleaved off by the ubiquitously expressed enzyme, dipeptidyl peptidase IV (DPP-IV),[6] which produces the major circulating form of the hormone in both the fed and fasted state, PYY3-36.[7] Furthermore, it has recently been shown that PYY3-36 play a role in energy homeostasis and has additionally been proposed as a novel class of peptide hormones for the treatment of obesity.[8] Moreover, peripheral administration of PYY3-36 reduces body-weight in several animal models of obesity further demonstrating that Y2 agonists are pharmacological relevant.[8,9]

PYY is a member of the PP-fold family of peptides, that also consists of pancreatic polypeptide (PP) and neuropeptides Y (NPY),[10] which expresses their actions

by binding to an appetite and body-weight controlling G-protein coupled receptor family, the so-called Y receptors (Y1, Y2, Y4 and Y5).[11] PYY1-36 is an agonist in the nanomolar range stimulating both the Y1 and Y2 receptors sub-types,[12,13] leading to numerous actions especially on the gastrointestinal function.[14] PYY1-36 acts through the Y1 receptor in the CNS to stimulate appetite and promote weight gain. Conversely, the major blood-circulating form of PYY, the *N*-terminally truncated PYY3-36, binds in a lesser extent to the Y1 receptor, but with a high affinity to the Y2 receptor.[12] Given the possibility that PYY3-36 analogs, when given in pharmacological doses that affects appetite and food intake,[15] may also engage central orexigenic Y1 receptors we aimed to develop more selective (Y2 over Y1) PYY3-36 analogs.

PYY1-36 consists of a *C*-terminal receptor-recognizing pentapeptide, subsequent to an amphipathic α-helix and followed by a flexible *N*-terminal polyproline helix. Both PYY1-36 and PP are back-folded in solution, which is not the case for NPY, however, when bound to the membrane NPY and PYY1-36 show almost identical secondary structures, which are in alignment with the virtually matching binding profiles towards the Y receptor sub-types, as well as their high degree of sequence homology.[16] In contrast, PP is pharmacologically different from the other members of PP-fold family of peptides as it selectively activates the Y4 receptor. It has a different secondary structure in the membrane-bound state - especially in the *C*-terminal region.[16-18] Zerbe and co-workers have hypothesized that the PP-fold peptides bind to the Y receptors by a mechanism referred to as the membrane compartment model,[16-18] which initially was proposed by Kaiser and Kezdy,[19] and further developed by Schwyzer *et al*.[20] Prior to binding to the receptor, the amphipathic peptide ligands associate with the membrane

surface and accumulate in the vicinity of the receptor. The bioactive conformations are induced, which leads to a reduced entropic loss upon receptor binding and activation. It is hypothesized that the amphipathic α-helix of the PP-fold peptides associates with the membrane and guide the *C*-terminal receptor-recognizing segment into the optimal conformation for receptor activation, thus the receptor-membrane associated state could be close to or in the bioactive conformation.[17]

We have taken advantage of the hypothesized membrane-ligand binding mechanism in the design of an array of new PYY analogs. The *N*-terminus, which is less important for Y2 receptor activity,[11,21] was exchanged by an amphipathic α-helix to increase the affinity of the agonist towards the cell membrane, thus an increased concentration of ligand should accumulate in the vicinity of the Y receptors. The accumulated membrane-bound peptide is assumed to be in the receptor activating conformation. Additionally, we believe that an additional amphipathic α-helix can induce the inherent PYY α-helix in the conformation profitable for Y2 receptor activation. Given, that the *N*-terminus of PYY is not part of the construct of the novel analogs, we presume that the Y1 receptor will be less activated compared to the natural agonist. The *de novo* α-helical segments are sequences derived from heptad repeat peptides initially designed by Koksch and co-workers,[22] Lazo and Downing,[23] Mutter and co-workers,[24] or Causton and Sherman[25]. These heptad repeat sequences were not originally designed for membrane binding, but for the study of self-assembly or coiled-coil systems in different environments.[22-25] The majority of the introduced amphipathic *de novo* α-helix fragments have a neutral net charge, however, positive charged segments has also been exploited.

Heptad repeat (seven residues) motifs are known to be able to assemble specific α-helices into larger structures such as coiled coils. Adjacent heptad repeat motifs have been recognized in a diverse range of fibrous and globular proteins, and have the form $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_m$ where about 70-75% of the *a* and *d* positions are occupied by apolar residues.[26] Position *e* and *g* are often charged residues and position *b*, *c*, and *f* are preferably polar residues. This form of heptad repeat pattern results in α-helices which have 3.6 residues per turn and are amphipathic.[27]

Materials and methods

Materials. The organic solvents and reagents for peptide synthesis were all of analytical reagent grade and were obtained from Iris Biotech GmbH (Germany). TentaGel S Rink Amide resin was obtained from Rapp Polymere GmbH. Milli-Q (Millipore) water was used for RP-HPLC analyses and purifications. Human Embryonic Kidney 293 cells (HEK293 Flp-In T-Rex), and pcDNA3.1 vector were purchased from Invitrogen. Dulbecco's Modified Eagle's Medium (D-MEM), fetal calf serum, penicillin-streptomycin solution, phosphate buffered saline (PBS), sucrose, and 99% glycerol were all obtained from Sigma-Aldrich. FuGENE 6 transfection reagent, complete protease inhibitor cocktail tablets and bovine serum albumin (BSA) were purchased from Roche. 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS), $MgCl_2 \cdot 6H_2O$, $CaCl_2$, and NaCl were obtained from AppliChem GmbH. (*2S,3S*)-1,4-bis-sulfanylbutane-2,3-diol (DTT) was obtained from GE Healthcare and radioactive labeled agonists were purchased from Phoenix pharmaceuticals. 96-Well filtration MultiScreen HTS, DV plates were obtained from Millipore. [^{3}H]-*myo*-inositol (PT6-271) was purchased from Amersham.

Peptide synthesis. The peptides were prepared by automated peptide synthesis on a Syro II peptide synthesizer (MultiSynTech) by standard solid-phase peptide synthesis (SPPS) on TentaGel S Rink Amide resin with 9-fluorenylmethyloxycarbonyl (Fmoc) for protection of N^{α}-amino groups. Side-chain protecting groups were *tert*-butyl (Ser, Thr, Tyr), 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf, for Arg), and trityl (Trt, for Asn, Gln, His). N^{α}-Fmoc amino acids (4.0 equiv) were coupled using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide HBTU (3.8 equiv), 1-hydroxybenzotriazole (HOBt) (3.6 equiv), 1-hydroxy-7-azabenzotriazole (HOAt) (0.4 equiv) and *N*,*N*-diisopropylethylamine (DIEA) (7.8 equiv) as coupling reagents in DMF for 2 hours. N^{α}-Fmoc deprotection was performed using piperidine-DMF (2:3) for 3 min, followed by piperidine-DMF (1:4) for 12 min. The peptides were released from the solid support by treatment of trifluoroacetic acid (TFA)-triethylsilane (TES)-H_2O (95:2:3) for 2 × 2.5 hours. The TFA solutions were concentrated by nitrogen flow and the compounds were precipitated with diethylether to yield the crude materials as white powders. Purification was accomplished by preparative RP-HPLC (Dionex Ultimate 3000 system) on a preparative column (FeF Chemicals, 300 Å 5 μm C4 particles, 2.1×200 mm) followed by a second purification on a preparative C18 column (FeF Chemicals, 200 Å 10 μm C18 particles, 2.1×200 mm) using the following solvent system: solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA. B gradient elution (0-50 min: 10% to 60%) was applied at a flow rate of 10 mL min^{-1} and column effluent was monitored by UV absorbance at 215 and 254 nm simultaneously. Identification was carried out by ESI-MS (MSQ Plus Mass Spectrometer, Thermo). The peptides were quantified by analytical HPLC (Dionex

Ultimate 3000 system equipped with a PDA UV detector) using the eluent system A-B (solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA). Eluent system A-B was applied on a C4 analytical column (phenomenex, Jupiter, 300 Å 5 μm C4 particles, 3.9×150 mm) where a B gradient elution (0-14 min: 5% to 100%) was applied at a flow rate of 1.0 mL min^{-1}. Quantification and characterization data are given in Table 1.

Radioligand displacement assay. Cell culture and receptor expression: The SK-N-MC cells were cultured in a 1:1 mix of HAM F12 and Dulbecco's Modified Eagle's Medium (D-MEM 1885), containing 15% (v/v) fetal calf serum (FCS), 1% non essential amino acids and 1% (v/v) penicillin-streptomycin in humidified atmosphere of 5% carbon dioxide and 95% air, at 37°C. The human embryonic kidney (HEK293 Flp-In T-Rex) derived cell line was cultured in Dulbecco's Modified Eagle's Medium (D-MEM), containing 10% (v/v) fetal calf serum (FCS) and 1% (v/v) penicillin-streptomycin. Cells were grown as monolayers in humidified atmosphere of 5% carbon dioxide and 95% air, for 48 hours at 37°C. Using serum-free D-MEM and FuGENE6 transfection reagent HEK293 Flp-In T-Rex cells were transiently transfected by pcDNA3.1 vectors which encode either the human Y2 or Y4 receptor (FuGENE6-pcDNA3.1, 3:1). The transfected HEK293 Flp-In T-Rex cells were set to express the receptors in humidified atmosphere of 5% carbon dioxide and 95% air, for another 48 hours at 37°C.

Preparation of membrane fractions: The SK-N-MC and the transiently transfected HEK293 Flp-In T-Rex cells were washed with phosphate buffered saline (PBS) and homogenized in cold homogenization buffer (50 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.0 mM DTT, complete protease inhibitor cocktail (one tablet per 50 mL

buffer)). An equal amount of 0.6 M sucrose was added to the cell mixture. The homogenate was centrifuged (10,000 g, 10 min at 4°C). Cell pellets were washed in washing buffer (50 mM TRIS (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 mL buffer), 1.0% (w/w) BSA fraction V), and subsequently the suspensions were centrifuged (10,000 g, 10 min at 4°C). The pellets were re-suspended in glycerol containing binding buffer (50 mM TRIS (pH 7.5), 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 mL buffer), 1.0% (w/w) BSA fraction V, 20% (v/v) glycerol) and concentration was adjusted to an OD600 of 1.6.

Binding affinity: All binding experiments were performed in 96-well filtration MultiScreen HTS, DV plates and every concentration point was performed as triplicates. The unlabeled peptide (25 µL) at concentrations between 10 pM and 10 µM, cell membrane suspension (3.5 µL), binding buffer (61.5 µL, 50 mM TRIS, pH 7.5, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, complete protease inhibitor cocktail (one tablet per 50 mL buffer), 1.0 % (w/w) BSA fraction V)), radioligand solution (10 µL; Y1R: ^{125}I-[^{31}Leu,^{34}Pro]-pNPY; Y2R: ^{125}I-PYY3-36; and Y4R: ^{125}I-PP; specific activity of 800-1000 Ci/mmole) were added. After 1-2 hours of incubation the assay was terminated by filtration. Finally, the membrane-receptor-ligand complexes were washed twice in cold TRIS buffer (50 mM TRIS, pH 7.5, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$). Bound radioactivity was determined as counts per minute (Wallac 1470 Wizard^{TM} Automated Gamma Counter). Binding data were analyzed with GraphPad Prism 5.0 (GraphPad Software, Inc. San Diego, CA, USA). The displacement experiments were replicated until the

standard error of mean (S.E.M.) of –log IC_{50} was below 0.1 (n = 2-5), except when different is stated.

Functional assay. Transfections and tissue culture: COS-7 cells were grown in Dulbecco's modified Eagle's medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine and 0.01 mg/mL gentamicin. Cells were transfected with 10 μg cDNA of wild type Y1 or Y2 receptors and 10 μg cDNA of a GαΔ6qi4myr[28] using the calcium phosphate precipitation method with chloroquine addition. The chimeric G-proteins allow the Gαi coupled receptors to signal through the signal transduction pathways known for the Gαq coupled receptors. Receptors from the PP fold family of peptides - the Y1 and Y2 receptors - were cloned from a human cDNA library and expressed in a pcDNA3.1 vector.

Phosphatidylinositol turnover: One day after transfection, COS-7 cells were incubated for 24 hours with 5 μCi of [^{3}H]-*myo*-inositol in 1 mL medium, washed twice in buffer, 20 mM HEPES, pH 7.4, and were subsequently incubated in 0.5 ml buffer supplemented with 10 mM LiCl at 37˚C for 30 min. After stimulation with various concentrations of PYY3-36 or the synthetic analogs for 45 min at 37˚C, cells were extracted with 10% ice-cold formic acid followed by incubation on ice for 30 min. The generated [^{3}H]-inositol phosphate was purified on Bio-Rad AG 1-X8 anion-exchange resins. Determinations of each measuring point were made in duplicates. The functional assays were replicated at least 3 times.

Size exclusion chromatography. SEC was performed on an ÄKTA purifier equipped with a Superdex Peptide HR10/30 column (GE healthcare) with an internal diameter of 10 mm. Packing height was 30 cm and with an approximated column volume

of 24 mL. The following standards dissolved in PBS (1.0 mg mL^{-1}) were used; Aprotinin (6500 Da), ribonuclease A (13700 Da), Carbonic anhydrase (29000 Da) and ovalbumin (43000 Da). The mobile phase was phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer) at pH 7.4. The samples were dissolved in 10 mM phosphate buffer at pH 7.4 to a final peptide concentration of 40 μM. Inject volume was 50 μL. The column effluent was monitored at 215, 254 and 280 nm.

Circular dichroism spectroscopy. UV CD spectra were recorded on a JASCO J-810 circular dichroism system using rectangular Hellma quartz cells with a light path of 1 mm. The peptide solutions were approximately 20 μM in 10 mM phosphate buffer, pH 7.4. The absolute concentration was determined spectroscopically (tyrosine absorption at 274 nm, using ε = 1420 $cm^{-1}M^{-1}$). The mean residue ellipticity (MRE) and degree α-helicity were calculated according to Yang *et al*.[29] The number *n* refers to the number of residues.

Results and Discussion

Peptide synthesis. The peptides were synthesized on Rink Amide TentaGel resin using the Fmoc/*t*-Bu strategy by automated solid-phase peptide synthesis. The N^{α}-Fmoc-protected amino acids were coupled using HBTU as coupling reagent, DIEA as base, and HOBt and HOAt as additive, in DMF. Coupling times were generally 2 × 2.5 hours. The peptides were side-chain deprotected and at the same time cleaved from the solid support by a TFA cocktail containing TES and H_2O as scavengers. Finally, the peptides were purified by RP-HPLC and characterized by analytical HPLC and mass spectrometry. The final products were obtained with > 95% purity (Table 1).

Structure evaluation. UV CD of PYY3-36 as well as the novel Y2 agonists (20 μM) were obtained in 10 mM phosphate buffer (pH 7.4) using a JASCO J-810 circular dichroism system. PYY3-36 gave a degree of α-helicity of 26%, which is in agreement with literature.[30] The spectra acquired for peptides **1**-**4**, **7**-**9**, **11**-**14**, **17**, **20**, and **21** (α-helicity between 25-52%) are typical for the α-helical structure, showing characteristic minima at 208 nm and 222 nm (Figure 1). The spectra obtained of peptides **1**, **5**, **10**, **15**, and **16** (α-helicity between 8-16%) also showed typical α-helical structures, however, to a considerable lower extent. Peptides **6**, **18,** and **19** were not investigated.

UV CD also show that substituting Leu with Trp (peptide **2** (α-helicity: 45%) and **12** (α-helicity: 50%), respectively) slightly stabilizes the *de novo* α-helix, however, introducing Tyr instead of Lys (peptide **15** (α-helicity: 8%) and **2** (α-helicity: 45%), respectively) resulted in a nearly abolishment of α-helical structure (not shown). By exchanging the positively charged, hydrophilic Lys with the neutral, hydrophobic Tyr residue the ionic interactions between positive and negative charged residues of the *de novo* α-helix is reduced which consequently could lead to a destabilization of the α-helix. Generally, circular dichroism reveals that large reduction in α-helicity results in poor binding towards all the Y receptor subtypes, which is in alignment with the membrane compartment model for the PP-fold peptides.[16-18]

Despite, the amphipathic nature and the high degree of α-helicity of the novel PYY3-36 analogs, size exclusion chromatography in PBS showed no self-assembly of the tested analogs, which could indicate a back-folding of the additional heptad repeat sequence onto the native amphipathic PYY α-helix resulting in a hydrophobic core between the two α-helix segments. Another possibility could be that the peptides are

highly aggregated thus unable to elute from the SEC-column due to size and interaction with the column-material.

Affinity and activity evaluation. To characterize the influence of substituting the flexible hydrophilic *N*-terminus with amphipathic *de novo* designed α-helices, the binding affinity of the novel PYY3-36 analogs were tested using radioligand displacement assay (competition binding assay) based on membranes from SK-N-MC and HEK293 Flp-In T-Rex cell lines expressing the human Y1, Y2 and Y4 receptor sub-types, respectively. ^{125}I-[^{31}Leu,^{34}Pro]-pNPY for Y1 receptor, ^{125}I-hPYY3-36 for Y2 receptor and ^{125}I-hPP for Y4 receptor were used as radioligands. The analogs with the highest affinity as well as selectivity towards the Y2 receptor were additionally analyzed for their ability to activate the Y1 and Y2 receptor sub-types. The signal transduction assay was based on COS-7 cells expressing the chimeric G-protein GαΔ6qi4myr,[28] and either the Y1 or Y2 receptor sub-type, which allows the Gαi coupled receptors to signal through the signal transduction pathways known for the Gαq coupled receptors. After incubation the generated [^{3}H]-inositol phosphate was purified and measured. Binding affinities (IC_{50} values) and activation potencies (EC_{50} values) for the PYY3-36 analogs as well as native PYY3-36 are summarized in Table 2.

Structure affinity and activity relationship. The binding and signal transduction studies of native human PYY3-36 confirms the high potency towards the Y2 receptor subtype.[31] PYY3-36 is, however, only moderately selective because of the additional engagement to the Y1 receptor (Table 2).[31] Though a *N*-terminal truncation version of PYY3-36, e.g. PYY13-36 (peptide **1**), leads to a high degree of Y2 receptor selectivity, the sub-nanomolar affinity towards the anorectic Y2 receptor also drops, which confirms

previous reports.[13,32] Introducing an amphipathic heptad repeat sequence to PYY13-36 retains part of the affinity towards the Y2 receptor first shown by peptide **2** - the high Y2 receptor selectivity shown by PYY13-36 (peptide **1**) was nevertheless, preserved. Modifying peptide **2** at the *N*-terminus by acetylation or lipidation did not result in altered Y receptor affinity or selectivity (peptides **3** and **4**). Conversely, adding PYY3-9 in the *N*-terminus of peptide **2**, giving peptide **5**, resulted in an undesirable Y1 receptor affinity gain and a fivefold decrease in Y2 receptor affinity compared to peptide **2**. Exchanging the *N*-terminal Tyr of peptide **2** with a hydrophobic Ala (peptide **6**), slightly improved the Y2 receptor affinity compared to peptide **2** but, the signal transduction assay showed a 6-fold decrease in Y2 receptor activity compared to inherent PYY3-36 (Table 2). Altering the stereogenic center of the amino acid residues of the turn motif by incorporating D-amino acids into the loop of the peptide **2** sequence (peptides **7**-**10**) resulted in only minor changes in the affinity towards the Y receptors compared to peptide **2**. This indicates that changing the geometry of this type of turn has less impact on the binding affinity towards the Y receptors.

Substituting the hydrophobic Leu of the heptad repeat segment with the less hydrophobic Phe (peptide **11**) or Trp (peptide **12**) resulted in equipotency towards the Y2 receptor subtype compared to peptide **2**. Interestingly the Phe containing analog demonstrated a major increase in Y1 receptor affinity (eleven-fold) compared to peptide **2**. Moreover, selected Glu or Lys residues of peptide **2** were substituted with Ser, peptides **13** and **14**, to change the isoelectric point and the net charge of the heptad repeat segment, only resulted in minor increases in Y2 receptor affinity towards the Y receptors compared to peptide **2**. However, substituting the Ser of peptide **14** for Tyr (peptide **15**)

gave poor binding affinities towards all Y receptor subtypes, which may originate from a destabilization of the α-helices (α-helicity: 8%). The side-chain of Ser residues can make specific hydrogen bonds to the carbonyl oxygen of the third or fourth residue earlier in the sequence, which may stabilize the α-helix.[33] Furthermore, peptide **15** is more hydrophobic than peptide 1**4**, which could alter the binding mode towards the membrane, thus also the binding conformation towards the Y receptors. Moreover, moving the *de novo* segment further away from the native PYY α-helix (peptide **16**) also destabilized the secondary structure of the novel Y2 receptor agonist (α-helicity: 16%) as well as demonstrating a poor binding towards the Y receptors.

The affinity towards the Y2 receptor was significantly improved when the *N*-terminal α-helix of peptide **2** was exchanged by different heptad repeat sequences[22-24] (peptides **17**-**20**). Peptides **17** and **18** differ at position *c* and *g* of the heptad repeat segment which are swap over - heptad repeat sequences of peptides **17** (LKELERK) and **18** (LKKLERE) (Table 2). The binding assays show that peptide **17** and PYY3-36 have equivalent binding affinities towards the Y1 and Y2 receptor sub-types. Contrary, peptide **18** resulted in a slight and significant decrease in binding affinity towards the Y2 and Y1 receptors, respectively, consequently leading to a highly selective analog. The major differences in binding affinity towards the Y1 receptor were not shown by the signal transduction assay of peptides **17** and **18** (Table 2 and Figure 2), which, however, resulted in a two- to threefold increase in potency towards the Y2 receptor and only minor decreases towards the Y1 receptor. Interestingly, peptide **19** resulted in an exceptional high affinity and potency towards the Y2 receptor (fivefold increase compared to PYY3-36), in addition to a slight reduction in Y1 receptor potency. The high

affinity may originate from the high Arg and Lys content of the amphipathic heptad repeat α-helix sequence. The positively charged residues may have a high affinity towards the negatively charged phospholipid bilayer of the membrane that additionally pre-orientates the inherent PYY α-helix in a conformation profitable for activation of the Y2 receptor subtype. Moreover, binding experiments of peptide **20** showed high affinity towards the Y2 receptor even though the *de novo* designed sequence has a neutral net charge, however, all the positively charged Lys are clustered in the middle of the heptad repeat sequence, which may again lead to a local net charge that are positive. Finally, peptide **21** showed poor Y2 receptor affinity compared to PYY3-36 that could originate from an unfavorable conformation of the *C*-terminal helix for Y receptor binding. Peptide **21** additionally, as the only compound of this series of PYY analogs, shows a better affinity towards the Y4 receptor compared to PYY3-36.

To summarize; to our knowledge no previously reported analogs of PYY3-36 has shown a fivefold increase in Y2 receptor potency in addition to a fourfold increase in Y receptor selectivity (Y2 over Y1) compared to PYY3-36. A neutral net charge of the heptad repeat sequences (peptide **2**-**12**, **14**-**16**, and **21**) generally showed low to poor Y2 receptor affinity and significantly decreased Y1 and Y4 receptor affinity, even though the sequences were designed to be amphipathic of nature. The amphipathic sequences with a positive net charge (peptides **13**, and **17**-**20**) showed up to a fivefold increase in Y2 receptor potency without affecting the Y1 receptor potency, consequently leading to an increase in Y receptor selectivity (Y2 over Y1) (Table 2).

Conclusion

We have taken advantage of the phenomenon that the PP-fold peptides associate with the membrane prior the receptor activation, in the design of an array of novel Y2 receptor ligands. We believe that this rational design concept for the development of novel PYY analogs is very unique and innovative. Our novel Y receptor ligands increase both Y2 receptor affinity and selectivity, as well as support the hypothesis that members of PP-fold family of peptides associates with the membrane prior to receptor activation. These preliminary results point towards that increasing the affinity towards the membrane enhances the potency towards the Y2 receptor subtype. Our novel PYY analogs give a superior Y2 affinity over Y1, which potentially could lead to an enhanced pharmacological effect. The Y2 receptor agonists with the highest potency have a positive net charge in their heptad repeat segments, which could associate with the phospholipid bilayer, and thereby may lead to an increased ligand accumulation in the vicinity of the membrane, and in this manner also the Y receptors. Finally, we established by circular dichroism that high degree of MRE at 220 nm is important for achieving potent *de novo* designed PYY analogs. We believe this knowledge can contribute to the development of more efficacious therapeutics against obesity in the future.

TABLES:

Table 1. Quantification and characterization data of PYY3-36 and R^1-NRYYASLRHYLNLVTRQRY-NH_2 (R^1-PYY18-36) analogs.

Peptide	*N*-terminal, R^1	ESI-MS (*m/z*)[a]					Purity[b]
		Calcd. MS	Found	Assigned	Found	Assigned	(%)
PYY3-36	H-IKPEAPGEDASPEEL-	4049.46	1012.8	$[M+4H]^{4+}$	810.2	$[M+5H]^{5+}$	98
1	H-SPEEL-	3040.6	1041.6	$[M+3H]^{3+}$	761.4	$[M+4H]^{4+}$	98
2	H-YEELLKKLEELLKKASPEEL-	4870.6	1219.5	$[M+4H]^{4+}$	975.8	$[M+5H]^{5+}$	99
3	**$CH_3(CH_2)_4CO$**-YEELLKKLEELLKKASPEEL-	4968.8	1243.0	$[M+4H]^{4+}$	994.5	$[M+5H]^{5+}$	99
4	**Ac**-YEELLKKLEELLKKASPEEL-	4912.6	1228.4	$[M+4H]^{4+}$	983.0	$[M+5H]^{5+}$	99
5	H-**IKPEAPG**YEELLKKLEELLKKASPEEL-	5563.4	1391.7	$[M+4H]^{4+}$	1113.6	$[M+5H]^{5+}$	99
6	H-**A**EELLKKLEELLKKASPEEL-	4778.5	1195.3	$[M+4H]^{4+}$	956.2	$[M+5H]^{5+}$	99
7	H-YEELLKKLEELLKKAS**p**EEL-	4870.6	812.5	$[M+6H]^{6+}$	696.5	$[M+7H]^{7+}$	99
8	H-YEELLKKLEELLKKASP**e**EL-	4870.6	812.3	$[M+6H]^{6+}$	696.7	$[M+7H]^{7+}$	95
9	H-YEELLKKLEELLKKASPE**e**L-	4870.6	812.2	$[M+6H]^{6+}$	696.2	$[M+7H]^{7+}$	97
10	H-YEELLKKLEELLKKAS**pe**EL-	4870.6	812.5	$[M+6H]^{6+}$	696.4	$[M+7H]^{7+}$	99
11	H-YEE**FF**KKLEEL**F**KKASPEEL-	4972.6	1243.9	$[M+4H]^{4+}$	995.4	$[M+5H]^{5+}$	99
12	H-YEE**WW**KKLEEL**W**KKASPEEL-	5089.7	1273.2	$[M+4H]^{4+}$	1018.8	$[M+5H]^{5+}$	99
13	H-YE**S**LLKKL**S**ELLKKASPEEL-	4786.5	1197.4	$[M+4H]^{4+}$	958.0	$[M+5H]^{5+}$	99
14	H-YEELLK**S**LEELLK**S**ASPEEL-	4788.4	1197.7	$[M+4H]^{4+}$	958.0	$[M+5H]^{5+}$	99
15	H-YEELLK**Y**LEELLK**Y**ASPEEL-	4940.6	1235.8	$[M+4H]^{4+}$	989.0	$[M+5H]^{5+}$	99
16	H-YEELLKKLEELLKKA**GEDA**SPEEL-	5242.9	1311.1	$[M+4H]^{4+}$	1049.1	$[M+5H]^{5+}$	99
17	H-YLERKLKELERKLKELSPEEL-	5111.9	1278.6	$[M+4H]^{4+}$	1023.2	$[M+5H]^{5+}$	99
18	H-YLER**E**LK**K**LER**E**LK**K**LSPEEL-	5111.9	1279.2	$[M+4H]^{4+}$	1023.3	$[M+5H]^{5+}$	99
19	H-YLKALKEALKALKEALKSPEEL-	4953.7	1239.2	$[M+4H]^{4+}$	991.6	$[M+5H]^{5+}$	99
20	H-NLEELKKKLEELKGSPEEL-	4694.3	1174.3	$[M+4H]^{4+}$	939.8	$[M+5H]^{5+}$	99
21	H-YLKALEEKLKALEEKLKALEEKGSPEEL-	5697.5	1425.0	$[M+4H]^{4+}$	1140.4	$[M+5H]^{5+}$	98

[a] Identified by ESI-MS on a MSQ Plus Mass Spectrometer (Dionex). [b] Quantified by RP-HPLC at 215 nm. Eluent system A-B: Eluent A: 0.1% TFA in H_2O. Eluent B: 0.1% TFA in AcN.

Table 2. PYY3-36[a] and R^1-NRYYASLRHYLNLVTRQRY-NH_2 (R^1-PYY18-36) binding to Y receptor subtypes Y1, Y2, and Y4, in addition to functional Y1 and Y2 receptor evaluation of the most potent Y2R agonists.

		Binding affinity assay IC_{50} [nM]			Signal transduction assay EC_{50} [nM]	
Peptide	*N*-terminal, R^1	Y1R[b]	Y2R[c]	Y4R[c]	Y1R[d]	Y2R[d]
PYY3-36	H-IKPEAPGEDASPEEL-	7.8 ± 1.1	0.50 ± 0.09	255 ± 29	90 ± 12	5.6 ± 1.1
1	H-SPEEL-	>1000	36.2 ± 7.2	>1000	-	-
2	H-YEELLKKLEELLKKASPEEL-	518 ± 350	10.7 ± 2.5	>1000	-	-
3	**$CH_3(CH_2)_4CO$**-YEELLKKLEELLKKASPEEL-	>1000	9.1 ± 6.7	>1000	-	-
4	**Ac**-YEELLKKLEELLKKASPEEL-	794 ± 291	8.6 ± 1.8	>1000	-	-
5	H-**IKPEAPG**YEELLKKLEELLKKASPEEL-	230 ± 59	54.0 ± 21.4	>1000	-	-
6	H-**A**EELLKKLEELLKKASPEEL-	424 ± 2	6.1 ± 1.8	>1000	141 ± 11	31.6 ± 12.5
7	H-YEELLKKLEELLKKAS**p**EEL-	141 ± 8	5.5 ± 1.4	>1000	-	-
8	H-YEELLKKLEELLKKASP**e**EL-	>1000	29.1 ± 10.9	>1000	-	-
9	H-YEELLKKLEELLKKASPE**e**L-	>1000	11.8 ± 3.3	>1000	-	-
10	H-YEELLKKLEELLKKAS**pe**EL-	774 ± 104	12.0 ± 3.5	>1000	-	-
11	H-YEE**FF**KKLEEL**F**KKASPEEL-	48 ± 4	8.0 ± 3.5	>1000	-	-
12	H-YEE**WW**KKLEEL**W**KKASPEEL-	>1000	9.1 ± 6.7	>1000	-	-
13	H-YE**S**LLKKL**S**ELLKKASPEEL-	239 ± 124	4.9 ± 2.7	>1000	94 ± 33	3.9 ± 2.8
14	H-YEELLK**S**LEELLK**S**ASPEEL-	805 ± 43	4.6 ± 1.1	>1000	-	-
15	H-YEELLK**Y**LEELLK**Y**ASPEEL-	>1000	14 ± 9[e]	>1000	-	-
16	H-YEELLKKLEELLKKA**GEDA**SPEEL-	340 ± 107	14.1 ± 3.4	>1000	-	-
17	H-YLERKLKELERKLKELSPEEL-	11 ± 1.4	0.9 ± 0.4	>1000	113 ± 21	2.3 ± 1.2
18	H-YLER**E**LK**K**LER**E**LK**K**LSPEEL-	>1000	3.2 ± 2.6	>1000	97 ± 31	3.1 ± 1.8
19	H-YLKALKEALKALKEALKSPEEL-	>1000	4.3 ± 0.5	664 ± 276	63 ± 27	1.1 ± 1.0
20	H-NLEELKKKLEELKGSPEEL-	27 ± 0.4	2.1 ± 0.5	532 ± 354	-	-
21	H-YLKALEEKLKALEEKLKALEEKGSPEEL-	141 ± 8	36.8 ± 19.0	174 ± 40	-	-

[a] Native human sequences. [b] SK-N-MC cells expressing the Y1R. [c] Y2R or Y4R transfected HEK293 Flp-In T-Rex cells.
[d] Y1R or Y2R and GαΔ6qi4myr transfected COS-7 cells. [e] log S.E.M > 0.1

FIGURES:

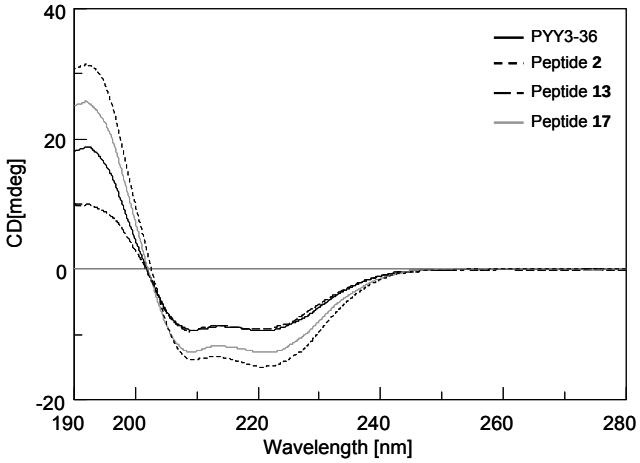

Figure 1. UV CD spectra of PYY3-36, peptide **2**, **13**, and **17** in aqueous buffered solution (20 μM peptide in 10 mM phosphate buffer).

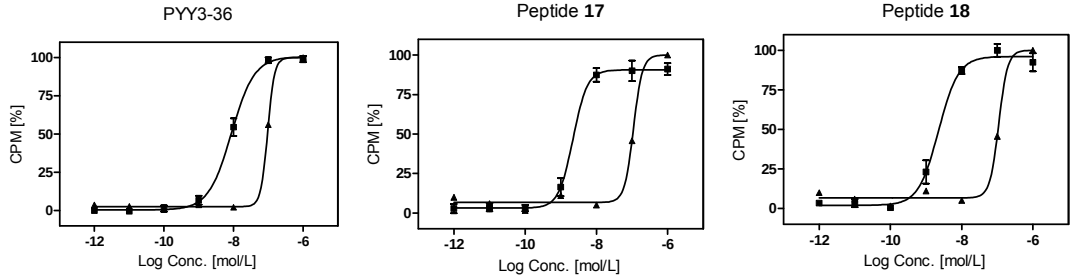

Figure 2. Functional assay. The activity of PYY3-36, peptide **17** and **18** towards the Y1 (▲) and Y2 (■) receptor subtypes.

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Appendix 6

Paper 6:

Søren L. Pedersen, Kasper K. Sørensen and Knud J. Jensen. Semi-automated microwave-assisted SPPS: Optimization of protocols and synthesis of difficult sequences, *Biopolymers*, **2009**, accepted.

Semi-automated microwave-assisted SPPS: Optimization of protocols and synthesis of difficult sequences

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Abstract

Precise microwave heating has emerged as a valuable addition to solid-phase peptide synthesis. New methods and protocols are required to utilize this potential and make it a reliable technique. Here we describe a new semi-automated instrument for solid-phase peptide synthesis with microwave heating and report protocols for its reliable use. The instrument features a flow-through reaction vessel that is placed in the microwave instrument throughout the synthesis. Bubbling with N_2 gas during the microwave irradiation proved important for temperature control. Washing and Fmoc removal steps were automated, while activated Fmoc-amino acids were added manually. Several linkers and resins were evaluated and protocols for synthesis of difficult sequences were developed. These included on-resin reductive amination of BAL handles under microwave heating. We believe that the instrument, as well as the protocols for its use, will be useful tools for peptide chemists.

Keywords

Microwave-assisted solid-phase peptide synthesis, SPPS, difficult sequences, PYY, LysM domain, *ortho* backbone amide linker

Introduction

Solid-phase peptide synthesis (SPPS) is still often faced with difficulties in the assembly of long and so-called difficult sequences due to incomplete reactions, possibly caused by aggregation and sterical hindrance. These problems have only partly been solved by new coupling reagents and solid supports. Precise microwave heating has emerged as one new, additional parameter for SPPS.[1-6] Combining SPPS with precise microwave heating has the potential to enable syntheses of long and/or difficult sequences and to improve purity.

During the synthesis of difficult sequences the peptide chain most likely becomes partially inaccessible typically due to the formation of secondary structures, especially of β-sheets.[7] Methods to suppress intra-molecular aggregation have been described and include pseudo-prolines,[7] solvent composition,[8] and chaotropic salts,[9,10] however, the utility is limited and the efficiency is variable. Inter-molecular aggregation could lead to a poor solvation of the peptidyl-polymer, but it is less pronounced with low-loading resins. Heating is likely to reduce both the inter- and intra-molecular derived aggregation and thereby to decrease the reaction time and improve the coupling efficiency of bulky and β-branched amino acids, as well as N^{α}-deprotection of sterically hindered peptidyl-polymers. Kappe *et al.* have recently probed microwave-assisted SPPS by comparing it

with oil bath-heating SPPS and concluded that the effect was of thermal nature.[11] Non-thermal effects were absent, which contradicts some previous suggestions.[12,13] Microwave irradiation is an advantageous tool in peptide chemistry because of the rapid and precise elevation of the temperature, and the efficient temperature control during the synthesis.

In this paper a new, flexible semi-automated instrument for the application of precise microwave heating in solid-phase peptide synthesis is presented. It combines a slightly modified Biotage Initiator microwave instrument, with a custom-modified semi-automated peptide synthesizer from MultiSynTech (Figure S1, Supporting Information). A specially made flow-through reaction vessel is placed in the microwave oven for the duration of the synthesis (Figure S2, Supporting Information). Mixing is achieved by nitrogen bubbling and is necessary to reach a homogenous heat distribution in the reactor vessel,[4] as magnetic stirring is problematic during solid-phase synthesis. The washing steps are automated, though the activated amino acid derivatives have to be added manually.

Protocols were optimized for both N^{α}-deprotection and for peptide coupling reactions. The external conditions, temperature and time, for Fmoc removal were first studied on Fmoc-Rink polystyrene resin (PS) (**1**) and the optimal conditions were further investigated on a resin-bound Fmoc protected 37-mer, Fmoc-VATTLYENLTNWNIVQASNPGVNPYLLPERVKVVFPL-Rink-TG (**2**), derived from the Nod-factor receptor 5 (Nfr5), LysM2 domain (*Lotus japonicus* sequence).[14]

Coupling conditions, temperature and time, were optimized using decapeptide **3** (Table 1), which Carpino and co-workers have reported to be a very difficult sequence,[15]

and originates from the *C*-terminus of the MuLV CTL epitope.[16] The optimized protocol has been utilized to synthesize the following peptides: Peptide hormone PYY1-36[17] (Table 1, peptide **4**), different regions of the Nfr5, LysM2 domain (*Lotus japonicus* sequence) (Table 1, peptides **5** and **6**),[14] and finally a glycopeptide aldehyde (Table 1, peptide **7**).

Results and discussion

Microwave-assisted Fmoc removal. As a model system for Fmoc removal the high-loading (0.71 mmol/g) Fmoc-Rink PS (**1**) was chosen, as it in our hands required long reaction times at room temperature to be fully deprotected. The Rink Amide PS was deprotected by piperidine-DMF (1:4) using variable temperatures (RT, 60°C, 80°C) obtained by microwave irradiation and reaction times (2-15 min). After initial Fmoc removal the residual loading was determined by a loading test. Residual loading after N^{α}-deprotection at RT for 2 min was 0.99 μmol per gram resin, however residual loading after N^{α}-deprotection at 60°C for 2 min was merely 0.13 μmol per gram resin (Table 2). The difference is noteworthy when synthesizing long and difficult sequences because the N^{α}-group becomes more hindered as the peptidyl-resin chain grows. The results were integrated in an optimized N^{α}-deprotection protocol: piperidine-DMF (1:4) for 1 min at RT followed by piperidine-DMF (1:4) for 2 min at 60°C. The protocol was verified on the N^{α}-deprotection of peptidyl-resin **2**, which is the *C*-terminal 37 residue segment of the LysM2 domain. After N^{α}-deprotection the residual loading was analyzed by a loading test and was determined to be < 0.0005 mmol/g (Figure 1.B). The protocol has been used in all the following microwave-assisted experiments including the coupling optimization,

except the N^{α}-deprotection of aspartic acid, which was deprotected without the use of microwave irradiation to avoid any risk of epimerization.

Microwave-assisted amino acid coupling optimization. The decapeptide sequence **3** derived from the MuLV CTL epitope (26 amino acids) was first reported as a difficult sequence by Jung and Redemann, who showed major deletion peptides within the first nine amino acid coupling steps.[16] As a difficult sequence, peptide **3** was chosen to determine the optimal coupling conditions (temperature and time) for the semi-automated microwave-assisted peptide synthesizer (SAMPS) using HBTU, HOBt, and DIPEA as coupling reagents. The coupling temperature was RT, 60°C or 80°C and the coupling time 2 min, 5 min or 10 min. On a fully automated synthesizer (Syro II, MultiSynTech) two control syntheses were additionally performed with coupling times of 45 min and 2×150 min at RT. All the coupling conditions were analyzed by LC-MS and the efficiency was measured by the percentage of peak area of peptide **3**. Coupling at RT for 2 min gave rise to a poor purity of 6% and the major deletion peptides were des-Phe and des-(Phe Thr) (**10**), des-(Trp Thr) (**12**) and des-(Trp Phe) (**13**). Peptide couplings performed at 60°C for 2 min improved the purity of peptide **3** considerably to 20%, however, elevating the temperature to 80°C for 2 min only increased the efficiency by an additional few per cent to 24% (Figure 2). Prolonging the reaction time from 2 min to 10 min at 60°C improved the purity of the final, crude peptide from 20% to 30% and the latter is compatible with conventional peptide synthesis at RT for 45 min (32%). Interestingly, a twofold increase in purity was observed when the reaction time was extended from 2 min to 10 min at 80°C, from 24% to 44%, and approximately equaled conventional peptide synthesis for 2×150 min at RT.

Based on comparison with reported literature data on epimers[15] our LC-MS analysis did not show any epimer formation of peptide **3**. Carpino *et al.* did however observe the D-Ser epimer of peptide **3** as byproduct during their synthesis, even at RT, which probably can be explained by the absence of additives in the coupling mixture.[15]

To summarize, these experiments show that microwave-assisted peptide coupling at 80°C for 10 min performs equal to double couplings for 150 min at RT. No epimerization was observed in any of the used protocols, as detected by the absence of additional peaks with identical masses. Peptide **3** is indeed a very difficult sequence and the optimal performance describe in literature, to our knowledge, provides a purity of 49%, which was achieved using N^{α}-Bsmoc protection, HATU and DIPEA system with 7 min preactivation, coupling times of 30 min and 15 min deblocking.[15] We believe that less aggregating and sterically unhindered peptides can easily be synthesized using couplings at 80°C for only 2 min. This reduces the synthesis times significantly and provides a higher peptide turnover.

Application to other sequences

Peptide YY. PYY1-36, **4**, is a 36 amino acid long peptide hormone involved in the regulation of food intake. It consists of a *C*-terminal α-helix and a *N*-terminal poly-proline helix.[17] PYY1-36 was synthesized on the SAMPS using the optimized protocol (10 min at 80°C). LC-MS showed a crude purity of 41% (data not shown) which demonstrates that microwave-assisted synthesis can potentially decrease the overall synthesis time of PYY1-36 considerably.

Nfr5 receptor peptides. In an ongoing study of Nfr5, difficulties during synthesis of a variety of analogs of the LysM2 domain were observed. Especially the synthesis of the *C*-terminal and the *N*-terminal regions were problematic, presumably due to formation of *β*-sheet like aggregates. LysM domains consist of two α-helices which are situated alongside a two-stranded anti-parallel *β*-sheet (*βαaβ* structure) and has been identified in Nfr5 by sequence alignment of the crystal structure with the LysM domain of *Bacillus subtilis* ykuD.[18]

The 11-mer **5** derives from the *C*-terminus of the LysM2 domain and consists of several *β*-branched and bulky amino acid residues resulting in sterical hindrance during peptide chain assembly. Nevertheless peptide **5**, which is a very difficult sequence to synthesize, was prepared in an acceptable purity (48%) using the SAMPS (Figure 3).

Furthermore, it is also difficult to synthesize the *N*-terminal 11-mer region of the LysM2 domain (residue 42-52) hence the SAMPS was used to synthesize this region. The first 41 amino acid were coupled using automated SPPS with coupling times of 2×150 min at RT (the underlined part of entry **6**, Table 2) and residue 42-52 were coupled using the optimized protocol for the SAMPS (80°C for 10 min). The microwave heating was a prerequisite for obtaining this protein segment after HPLC purification. However, it was difficult to estimate the crude purity due to close-lying peaks.

Glyco-peptide aldehyde. The *α*-GalNAc moiety is a Tn cancer antigen which is recognized by a variety of lectins e.g. macrophage galactose lectin (MCL),[19] consequently cancer-associated glycopeptides and proteins represent a potential target for immunodiagnostics and therapeutics.[20] We have previously reported a method for peptide coupling with microwave heating in a sealed container. This also worked particularly

well in the reductive amination of the *ortho* backbone amide linker (*o*-BAL) on TentaGel polymer.[5] For the synthesis of glycopeptide aldehyde **7** by SAMPS in an open vessel, methanol was substituted for DMF in the reductive amination. The subsequent acylation of the secondary amine was performed in DMF-DCE (1:9) at 60°C, as the latter has a b.p. of 83°C (Scheme 1).

Glycopeptide aldehyde **7** was subsequently synthesized using the optimized SAMPS protocol (coupling at 80°C for 2 min). After cleavage from the *o*-BAL functionalized TG resin four major peaks appeared in the analytical RP-HPLC chromatogram, however, by LC-MS it is conclusive that all the major peaks were glycopeptide **7** derivatives. Peak **18** is the fully acetylated version of peptide **7**, peak **17** and **19** are versions where one acetyl group has been lost, and peak **16** is a version where two acetyl groups has been lost (Figure 4.A). The partial *O*-deacetylation could possibly be due to the activation of DIPEA at elevated temperature, however Matsushita *et al.* does not report any problems during microwave-assisted synthesis of glycopeptides at 50°C.[2] These findings indicate that coupling glyco-amino acids at 80°C, can cause partial deacetylation of the glycan, but this may not be a major problem. Treating the peptide mixture with 0.1 M methoxide (NaOMe, pH 10) completely deprotected the glycan hydroxyl groups to yield the *O*-linked *N*-acetylgalactoseamine peptide aldehyde **7**. The overall purity of glycopeptide **7** was 92% (Figure 4.B).

Conclusion

The semi-automated synthesizer (SAMPS) provides a flexible platform for peptide synthesis and different types of on-resin chemistry such as anchoring of *o*-BAL.

Several types of resins, with different functionalities were tested successfully. A systematic study of temperature and time showed that the reaction time for Fmoc removal can in most cases be reduced to 2 min at 60°C and the coupling time could be reduced to 2-10 min at 80°C. Using the optimized protocol for the semi-automated microwave assisted peptide synthesizer numerous peptides was synthesized. The peptides were completed faster than conventionally and in addition some were made in a significantly higher purity.

Materials and methods

General experimental. Materials were obtained from commercial suppliers (Sigma-Aldrich, Iris Biotech GmbH, Novabiochem, Sussex Research, Varian (Polymer laboratory). Milli-Q (Millipore) water was used for LC-MS analysis. The peptides were synthesized on either TentaGel S Rink Amide resin (loading 0.24 mmol/g), polystyrene Rink Amide resin (loading 0.71 mmol/g) or TentaGel S resin (loading 0.26 mmol/g), with 9-fluorenylmethyloxycarbonyl (Fmoc) for protection of N^{α}-amino groups. Side-chain protecting groups were *tert*-butyl (Ser, Thr, Tyr), 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf, for Arg), and trityl (Trt, for Asn, Gln, His). Peptides prepared on the semi-automated solid-phase microwave-assisted peptide synthesizer were prepared using N^{α}-Fmoc amino acids (4.0 equiv, 0.20 mol/L) which were coupled using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) (3.8 equiv, 0.19 mol/L), 1-hydroxybenzotriazole (HOBt) (4.0 equiv, 0.20 mol/L) and *N*,*N*-diisopropylethylamine (DIEA) (7.8 equiv, 0.39 mol/L) as coupling agents in DMF, except His which was coupled in NMP. N^{α}-Fmoc

deprotection was performed using piperidine-DMF (1:4). Control peptides were prepared conventionally on a fully automated Syro II peptide synthesizer (MultiSynTech) using a similar protocol except that a 9:1 mixture of HOBt-HOAt (HOAt; 1-hydroxy-7-azabenzotriazole) was used as additive (4.0 equiv). N^{α}-Fmoc deprotection was performed using piperidine-DMF (2:3) for 3 min, followed by piperidine-DMF (1:4) for 12 min. All peptides were cleaved from the solid support by treatment with trifluoroacetic acid (TFA)-triethylsilane (TES)-H_2O (95:2:3) for 2 hours, except the glycopeptide aldehyde which was released with by TFA-H_2O (95:5). The TFA mixtures were concentrated by nitrogen flow and the compounds were precipitated with diethylether to yield the crude materials as white powders. Quantification was performed by LC-MS (Dionex Ultimate 3000 system equipped with PDA) on an analytical C4 column (phenomenex, Jupiter, 300 Å 5 μm particles, 3.9×150 mm) or on a C18 column (phenomenex, Germini, 110 Å 3 μm particles, 4.6×50 mm). The following solvent system was used: solvent A, water containing 0.1% formic acid; solvent B, acetonitrile containing 0.1% formic acid. Identification was carried out by ESI-MS (MSQ Plus Mass Spectrometer, Thermo).

The reaction-time for all microwave-assisted reactions was for technical reasons extended by 1 min (+1 min) beyond the time of heating (Supporting Information). Thus, 2 min at 60°C means 2 min at 60°C plus 1 min with no irradiation giving a total of 3 min reaction time, however, only two minutes of heating.

Amine deprotection, Fmoc-Rink Amide PS. Fmoc-Rink Amide PS resin with an initial loading of 0.71 mmol/g was deprotected by treatment with piperidine-DMF (1:4) using various reaction times (2 min, 5 min, 10 min, 15 min) and temperatures (RT,

60°C, 80°C). The resin was washed 3 times with DMF and the residual loading was determined by a loading test on 35-45 mg of resin (Table 3).[21]

Amine deprotection, LysM2 domain (37 residues). Peptide **2** was synthesized by conventional SPPS at RT on a fully automated peptide synthesizer. The peptide was synthesized on TentaGel Rink amide resin (loading 0.18 mmol/g) with coupling times of 2×150 min and N^{α}-deprotection with piperidine-DMF (2:3) for 3 min followed by piperidine-DMF (1:4) for 12 min, and finally 16 min with piperidine-DMF (1:4). Released peptide **2** was analyzed by LC-MS (C18 column) both before (Figure 1.A) and after (Figure 1.B) the final N^{α}-deprotection via B gradient elution (0-8 min: 5% to 38%, 8-10 min: 100%, 10-11 min: 100%) with an applied flow rate of 1.0 mL min^{-1}. The loading of peptide **2** was determined by loading test: 0.044 mmol/g, and the residual loading after N^{α}-deprotection of peptidyl-resin **2** was determined by an additional loading test: < 0.0005 mmol/g. ESI-MS of released Fmoc protected peptide **2**, calculated average isotopic composition for $C_{209}H_{313}N_{49}O_{55}$, 4391.3 Da. Found. m/z 1464.9 $[M+3H]^{3+}$, 1099.0 $[M+4H]^{4+}$. ESI-MS of Fmoc deprotected peptide **2**, calculated average isotopic composition for $C_{194}H_{303}N_{49}O_{53}$, 4169.4 Da. Found: m/z 1390.9 $[M+3H]^{3+}$, 1043.4 $[M+4H]^{4+}$.

Coupling optimization, peptide 3. Microwave-assisted amino acid coupling optimization was conducted on Rink amide PS resin (loading 0.71 mmol/g) using various amino acid coupling temperatures (RT, 60°C, 80°C) and times (2 min, 5 min, 10 min). Fmoc removal was carried out by treatment with piperidine-DMF (1:4) at RT followed by 2 min at 60°C. After cleavage, the crude reaction mixtures were analyzed by LC-MS (C18 column) via B gradient elution (0-5 min: 5% to 37%, 5-8.5 min: 37%, 8.5-9.5 min:

37%-100%, 9.5-10 min: 100%) with an applied flow rate of 1.0 mL min^{-1}. Purity: 6% (RT, coupling time 2 min), 20% (60°C, 2 min), 30% (60°C, 10 min), 24% (80°C, 2 min), 33% (80°C, 5 min), 44% (80°C, 2 min). ESI-MS (80°C, 2 min), calculated monoisotopic composition for $C_{58}H_{90}N_{12}O_{14}S$, 1210.6 Da. Found: m/z 1211.9 $[M+H]^+$.

Control peptides were synthesized conventionally at RT on Rink amide PS resin (loading 0.71 mmol/g) using the general protocol for automated peptide synthesis with amino acid coupling times of 45 min and 2×150 min. The crude mixture was analyzed by LC-MS (C18 column) via B gradient elution (0-5 min: 5% to 37%, 5-8.5 min: 37%, 8.5-9.5 min: 37%-100%, 9.5-10 min: 100%) with an applied flow rate of 1.0 mL min^{-1}. Purity: 32% (RT, coupling time 45 min), 43% (RT, 2×150 min). ESI-MS, calculated monoisotopic composition for $C_{58}H_{90}N_{12}O_{14}S$, 1210.6 Da. Found: m/z 1211.6 $[M+H]^+$.

PYY1-36, peptide 4. Microwave-assisted synthesis was performed on TentaGel Rink amide resin (loading 0.24 mmol/g) using the optimized SAMPS protocol: coupling times of 10 min at 80°C and Fmoc removal by treatment with piperidine-DMF (1:4) for 1 min, without heating, followed by 2 min at 60°C. The crude product was analyzed by LC-MS (C4 column) via B gradient elution (0-14 min: 5% to 100%) with an applied flow rate of 1.0 mL min^{-1}. Purity 41%, ESI-MS, calculated average isotopic composition for $C_{194}H_{295}N_{55}O_{57}$, 4309.2 Da. Found: m/z 1437.9 $[M+3H]^{3+}$, 1078.3 $[M+4H]^{4+}$, 863.0 $[M+5H]^{5+}$, 719.3 $[M+6H]^{6+}$, 616.7 $[M+7H]^{7+}$.

***C*-terminal LysM2 domain, peptide 5.** Synthesized on TentaGel Rink amide resin (loading 0.24 mmol/g) using the optimized SAMPS protocol: coupling times of 10 min at 80°C and Fmoc elimination by treatment with piperidine-DMF (1:4) for 1 min, without heating, followed by 2 min at 60°C. The crude product was analyzed by LC-MS

(C18 column) via B gradient elution (0-8 min: 5% to 38%, 8-10 min: 38% to 100%) with an applied flow rate of 1.0 mL min^{-1}. Purity 48%, ESI-MS, calculated average isotopic composition for $C_{63}H_{105}N_{15}O_{14}$, 1294.8 Da Found: m/z 1295.8 $[M+H]^+$, 648.3 $[M+2H]^{2+}$, 432.8 $[M+3H]^{3+}$.

***N*-terminal LysM2 domain, peptide 6.** Synthesized on TentaGel Rink amide resin (loading 0.24 mmol/g). The first 41 amino acid couplings were conducted conventionally using the general protocol for automated peptide synthesis with coupling times of 2×150 min, however, the N^{α}-deprotection was performed with piperidine-DMF (2:3) for 3 min followed by piperidine-DMF (1:4) for 12 min, and finally 16 min with piperidine-DMF (1:4). The coupling of the final 11 *C*-terminal amino acids were completed using the optimized protocol for the SAMPS: coupling times of 10 min at 80°C and Fmoc elimination by treatment of piperidine-DMF (1:4) for 1 min, no heat, followed by 2 min at 60°C. The product was analyzed by LC-MS (C18 column) via B gradient elution (0-3 min: 5% to 37%, 3-8 min: 37% to 38%, 8-11 min: 38% to 100%) with an applied flow rate of 1.0 mL min^{-1}. ESI-MS, calculated average isotopic composition for $C_{272}H_{411}N_{67}O_{81}$, 5916.0 Da. Found: m/z 1479.6 $[M+4H]^{4+}$, 1183.8 $[M+5H]^{5+}$.

***Ortho* backbone amide linker (*o*-BAL).** *Anchoring* (double coupling): The TentaGel S resin (loading 0.26 mmol/g) was washed with NMP. 5-(2-Formyl-3,5-dimethoxyphenoxy)pentanoic acid (4.0 equiv), HBTU (3.8 equiv), HOBt (4.0 equiv), and DIEA (7.8 equiv) in DMF were added and reacted by microwave heating at 60°C for 10 min. The resin was washed (3×DMF). *Reductive amination* (performed twice): Aminoacetaldehyde-dimethylacetal 99% (10 equiv) and $NaBH_3CN$ (10 equiv) were

suspended in AcOH–DMF (1:99), added to the *o*-BAL-resin, and heated by microwave irradiation at 60°C for 10 min. The resin was washed (3×DMF). *Symmetrical anhydride* (performed twice): Fmoc-Gly-OH (10 equiv) and DIC (5 equiv) were suspended in 1,2-dichlorothane (DCE) in DMF (10:1) and pre-activated for 10 min. The activated amino acid residue was added to the resin and heated by microwave irradiation at 60°C for 10 min. The resin was washed (3×DMF).

Glycopeptide aldehyde 7. Synthesized on the previously prepared *o*-BAL TentaGel resin using the optimized SAMPS protocol: coupling times of 10 min at 80°C and Fmoc removal by treatment with piperidine-DMF (1:4) for 1 min, without heating, followed by 2 min at 60°C. The glycan hydroxyl groups were deacetylated on-resin using 0.1 M methoxide (NaOMe, pH 10) for 2×30 min to yield the *O*-linked *N*-acetylgalactoseamine peptide aldehyde **7**. The product was analyzed by LC-MS (C18 column) via B gradient elution (0-8 min: 5% to 38%, 8-10 min: 38% to 100%) with an applied flow rate of 1.0 mL min^{-1}. Purity: 92%. ESI-MS, calculated monoisotopic composition for $C_{35}H_{43}N_5O_{14}$, 757.3 Da. Found: m/z 391.1 $[M+Na+H]^{2+}$, 261.1 $[M+Na+2H]^{3+}$.

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Table 1. Peptide sequences synthesized on the SAMPS.	
No	Sequence[a]
3	H-WFTTLISTIM-NH_2
4	H-YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2
5	H-LPERVKVVFPL-NH_2
6	Ac-NATSYQIQLGDSYDFVATTLYENLTNWNIVQASNPGVNPYLLPERVKVVFPL-NH_2
7	Fmoc-S(GalNAc)EGG-H
[a] Underlined sequences were synthesized conventionally.	

Table 2. Residual loading [μmole/g] after deprotection of Fmoc-Rink-PS resin at various reaction conditions.[a]

Reaction time	Temperature [°C][b]		
	RT	60	80
2 min	0.99	0.13	0.08
5 min	0.08	0.20	0.13
10 min	0.05	0.07	0.12
15 min	0.03	0.08	ND

[a] Loading determined by UV at 301 nm.[21] [b] Obtained by microwave irradiation.

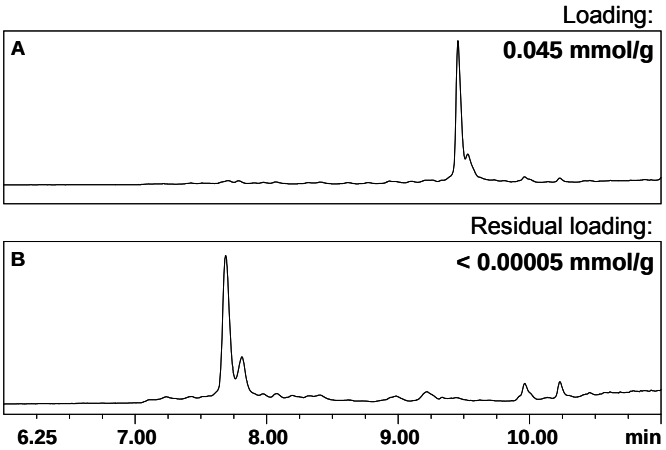


Figure 1. The N^{α}-deprotection of peptidyl-resin **2** using microwave-assisted heating was evaluated by HPLC. Before (A, Fmoc LysM2 37-mer resin) and after (B, LysM2 37-mer resin) Fmoc removal, the peptides were released from the solid support and subsequently analyzed by LC-MS. Additionally, loading tests[21] were carried out both before and after the N^{α}-deprotection to determine the performance of the microwave assisted Fmoc removal. The side peaks at 9.6 min (A) and 7.8 min (B) results from a deletion peptide.

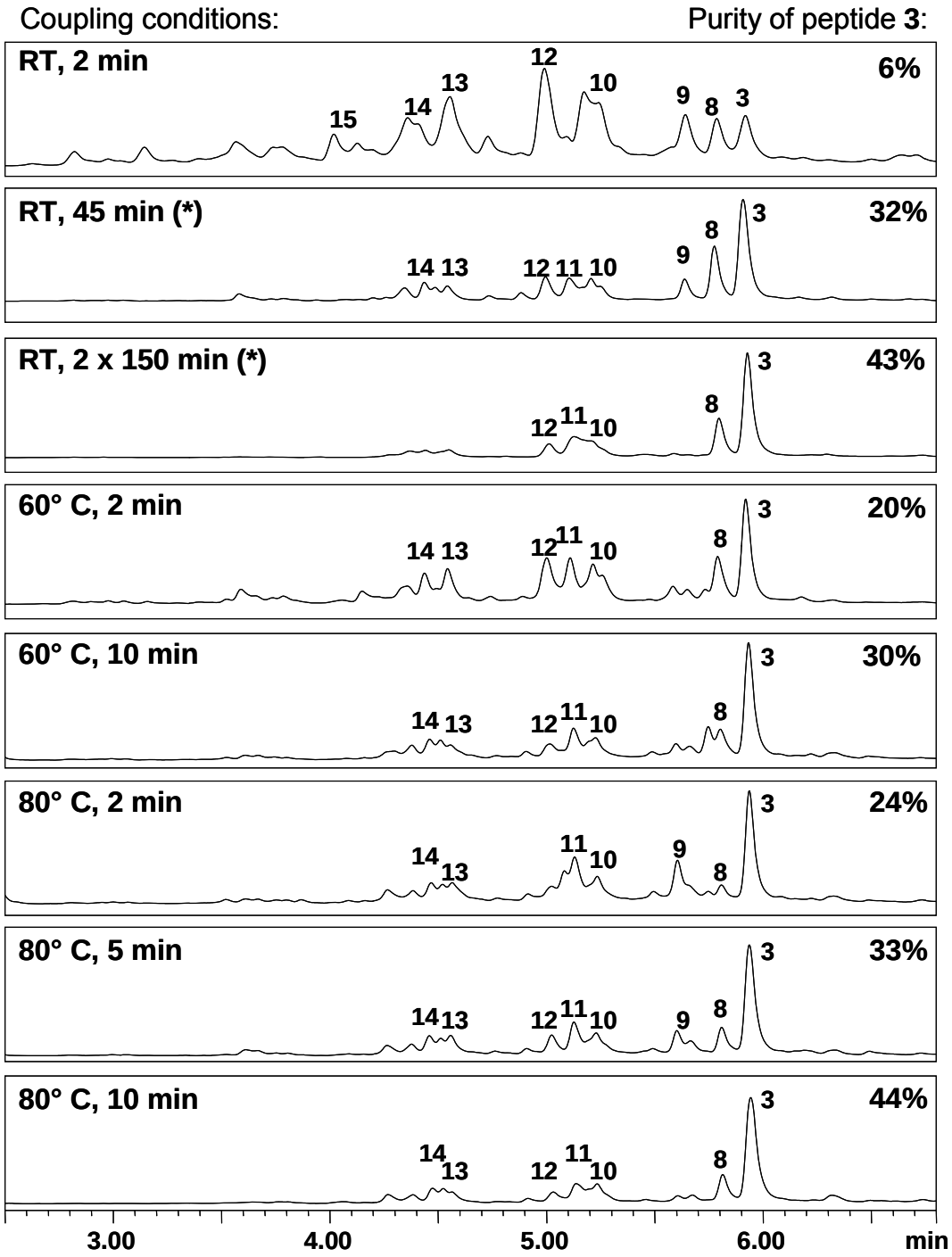


Figure 2. HPLC chromatograms of coupling optimization experiments. The coupling conditions are shown at left and the purity of peptide **3** is given in the right side of each chromatogram. **3** (H-WFTTLISTIM-NH_2), des-(Thr) (**8**), des-(Thr Thr) (**9**), des-(Phe) and des-(Phe Thr) (**10**), des-(Trp) (**11**), des-(Trp Thr) (**12**), des-(Trp Phe) (**13**), des-(Trp Phe Thr) (**14**), and des-(Trp Ile) (**15**). Conventional peptide synthesis (*).

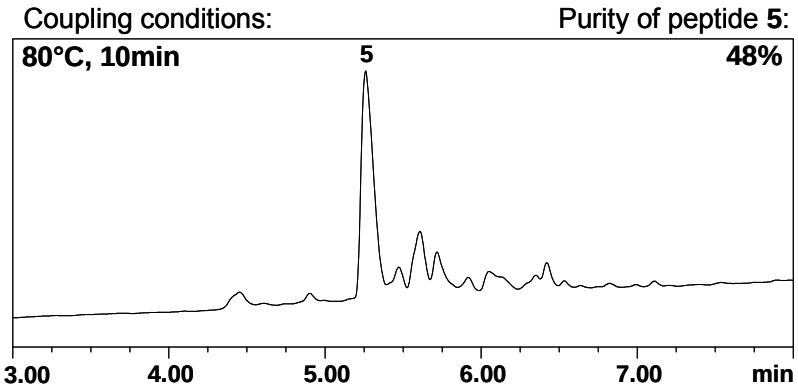


Figure 3. Analytical RP-HPLC chromatogram of the *C*-terminal 11-mer (**5**) of the LysM2 domain synthesized on the SAMPS.

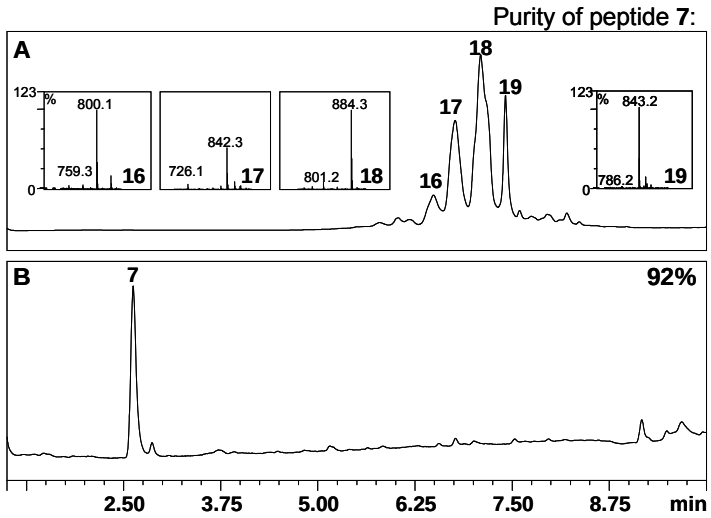
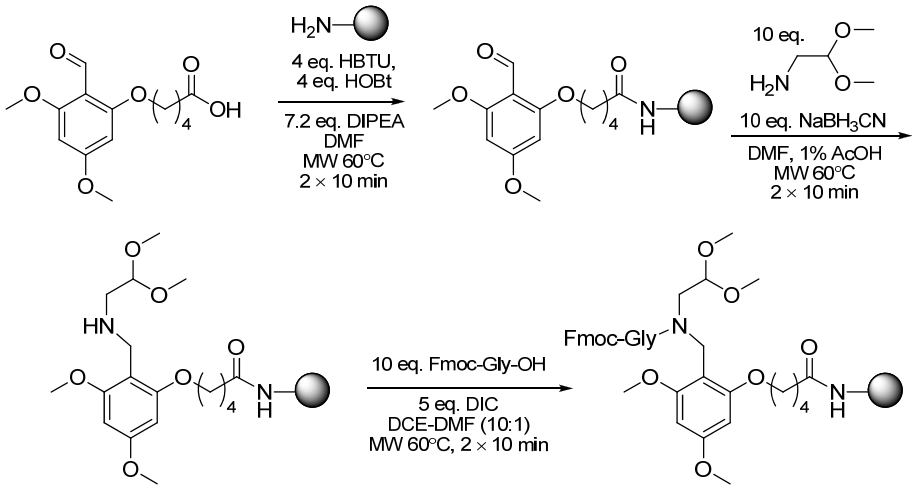


Figure 4. Analytical RP-HPLC chromatogram of the glycopeptide **7**, before (A) and after (B) deacetylation. Glycopeptide **7** fully acetylated (**18**), loss of one acetyl group (**17** and **19**) and loss of two acetyl groups (**16**).



Scheme 1. Modified SAMPS protocol for preparing *o*-BAL TG.

Supporting Information:

Semi-automated microwave-assisted SPPS: Optimization of protocols and synthesis of difficult sequences

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Figure S1. Semi-automated microwave-assisted peptide synthesizer (SAMPS). The two parts of the SAMPS do not communicate with each other, thus the microwave heating program on the Biotage Initiator has to be started manually each time heating is needed. The Initiator was slightly customized: the `blast-box´ underneath the reaction chamber was removed to allow suction in the bottom of the reaction vial, and the Initiator was operated in the 'service mode' (programmed to keep the lid open during heating). The amino acids is added manually thus the SAMPS program starts with the coupling. The washing and N^{α}-deprotetion is fully automated by the modified MultiSynTech SAP. The SAP module was modified by removing the original reaction flask and substituting magnetic stirring with nitrogen bubbling for mixing. A cycle consits of (1) coupling (microwave irradiation started manually), (2) drain, (3) wash, (4) N^{α}-deprotection (microwave irradiation started manually), (5) drain, and (6) wash. To avoid microwave irradiation on non-

solvated resin after coupling and N^{α}-deprotection steps (the microwave irradiation was started manually while the solvents were added automatic which may give a time gap between the two parallel running programs) the SAP unit was programmed to react one more minute than the Initiator heated (+1 min).

Figure S2. The custom-made, flow reactor vessels (10–20 mL and 2-5 mL) for the (SAMPS).