Design, synthesis and characterisation of gurmarin and melanocortin analogues of gurmarin

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DESIGN, SYNTHESIS AND CHARACTERISATION OF GURMARIN
AND MELANOCORTIN ANALOGUES OF GURMARIN

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Abstract

In this Ph.D. project the possibility of grafting melanocortin receptor binding sequences into the inhibitor cystine knotted peptide gurmarin was investigated. The goal of designing these analogues was to produce highly stable peptide agonists with potential for obesity treatment.

Inhibitor cystine knotted peptides are a group of peptides found in a variety of plant and animal species. They all contain six cysteines forming three disulphides which determine their tertiary structure and provide them with exceptional proteolytic stability. It has been demonstrated that these peptides can be used as molecular scaffolds into which peptide sequences of pharmaceutical interest can be incorporated, thus providing highly stable drug candidates.

The melanocortin receptors are a family of five G protein-coupled receptors distributed throughout the body. The melanocortin 4 receptor is found in the brain where it regulates appetite and energy expenditure. The melanocortin receptors are activated by peptides containing a His-Phe-Arg-Trp tetrapeptide sequence. Consequently, inhibitor cystine knotted peptides in which this tetrapeptide are grafted could provide novel appetite regulating peptides for the treatment of obesity.

First, a stable method to synthesise gurmarin was developed. The peptide was oxidised by a random oxidation method using a buffer containing a glutathione/cystamine redox pair. Various attempts were made to confirm the disulphide connectivity of the randomly oxidised gurmarin. The native disulphide connectivity of the randomly oxidised gurmarin was ultimately confirmed by a synthetic approach. This approach used a combination of thermolysin cleavage of the randomly oxidised peptide and the development of an orthogonal oxidation method. In this method, the disulphides in gurmarin were oxidised sequentially using three different pairs of cysteine side chain protection groups, which allowed selective disulphide formation.
Secondly, the redox buffer oxidation was used to synthesise six melanocortin analogues of gurmarin. These peptides were designed based on a structural alignment of gurmarin and agouti-related protein, an endogenous antagonist of the melanocortin receptors. The C-terminal part of agouti-related protein contains multiple disulphides similar to the inhibitor cystine knot of gurmarin. In addition, two tetra-disulphide analogues of gurmarin was designed and synthesised based on the alignment with agouti-related protein. The melanocortin analogues were characterised for their binding to the melanocortin receptors. In general, it was proved that it is possible to synthesise gurmarin analogues which bind to the melanocortin receptors. The analogue with highest affinity to the melanocortin 4 receptor had a binding affinity of 500 nM which is around ten-fold lower affinity than the endogenous agonist α-melanocyte stimulating hormone.

Finally, it was demonstrated that it was possible to synthesise melanocortin analogues of the cyclic cystine knotted peptide kalata B1, a plant peptide which two termini are cyclised. These analogues were shown by NMR spectroscopy to adopt similar three-dimensional folds as kalata B1. One analogue was found to bind to the melanocortin 4 receptor with a binding affinity of 29 nM, which is higher affinity than α-melanocortin stimulating hormone. However, the analogue was less potent than α-melanocortin stimulating hormone at activating the melanocortin 4 receptor. In addition, the analogues were shown to be highly resistant to proteolysis compared to α-melanocyte stimulating hormone.

In summary, the possibility of grafting melanocortin binding sequences into cystine knotted peptides was confirmed using two different peptides as molecular scaffolds. The ability of these melanocortin analogues to adopt native folds was confirmed. In addition, it was demonstrated that the synthesised peptides in fact are able to bind to and activate the melanocortin 4 receptor. Moreover, the peptides were resistant to proteolysis. This novel melanocortin grafting approach provides new candidates for the development of drugs treating obesity.
**RESUMÉ**

I dette Pd.D.-projekt blev muligheden for at grafte melanocortin receptor bindende sekvenser i det inhibitor cystine knottepeptid gurmarin undersøgt. Målet med at designe disse analoger var, at producere stabile peptid agonister med potentielle for at behandle fedme.

Inhibitor cystine knottepeptider er en gruppe af peptider, som findes i forskellige plante- og dyrearter. De indeholder alle seks cysteiner, som danner tre disulfider, der bestemmer deres tertiære struktur og giver dem ekseptionel proteolytisk stabilitet. Det er blevet vist, at disse peptider kan blive brugt som molekylære platformer, i hvilke peptidsekvenser af farmaceutisk interesse kan blive indsat, hvormed meget stabile lægemidler kan blive fremstillet.


Endeligt blev det vist, at det var muligt at syntetisere melanocortinanaloger af det cykliske cystine knottede peptid kalata B1, et plantepéptid hvis to termini er cykliserede. Det blev bekræftet med NMR spektroskopi, at disse analoger havde tredimensionelle strukturer lignende kalata B1. Det blev vist at én analog bandt til melanocortin 4 receptoren med en bindingsaffinitet på 29 nM, hvilket er højere affinitet end α-melanocortin stimulating hormone. Analogen var derimod mindre potent end α-melanocortin stimulating hormone til at aktivere melanocortin 4 receptoren. Derudover blev det også vist at analogerne var meget stabile overfor proteolyse sammenlignet med α-melanocyte stimulating hormone.

Alt i alt blev det bekræftet, at det er muligt at grafte melanocortin bindende sekvenser ind i cystine knottede peptider ved brug af to forskellige peptider som molekylær platform. Disse melanocortin analogers evne til at danne native strukturer blev bekræftet. Derudover blev det vist at de syntetiserede peptide kan binde til og aktivere melanocortin 4 receptoren. Yderligere var peptiderne proteolytisk stabile. Denne nye melanocortin grafting-metode har produceret nye kandidater til udviklingen af lægemidler til behandling af fedme.
This Ph.D. thesis consists of four main chapters. The first chapter describes the background of inhibitor cystine knotted peptides and of the melanocortin system. The second chapter concerns different synthesis strategies pursued for the synthesis of the inhibitor cystine knotted peptide gurmarin. In addition it covers structure determination analyses of the synthesised peptides. In the third chapter the design, synthesis and functional characterisation of melanocortin analogues of gurmarin is studied. Finally, the fourth chapter deals with the design, synthesis, functional and structural characterisation of melanocortin analogues of the cyclotide kalata B1.

The work described in this Ph.D. thesis has been performed at Novo Nordisk A/S, Måløv, Denmark, the Department of Micro- and Nanotechnology, Center for Nanomedicine and Theranostics, Technical University of Denmark, Kgs. Lyngby, Denmark and the Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia. The Ph.D. project was co-financed by the Industrial Ph.D. programme, the Danish Agency for Science, Technology and Innovation and the Novo Nordisk R&D Science Talent Attraction and Recruitment (STAR) programme.

Part of the work described in chapter two was summarised in a poster presentation and proceedings paper at the 31st European Peptide Symposium, Copenhagen, 5-9 September 2010. Another part of the work was accepted for publication in Peptides. The work presented in chapter four was submitted for publication.


I would like to thank my two supervisors, Principle Scientist at Novo Nordisk A/S Kilian W. Conde-Frieboes and Senior Researcher Thomas L. Andresen at the Department of Micro-and Nanotechnology, Center for Nanomedicine and Theranostics, Technical University of Denmark. They have both shared several valuable thoughts on how to conduct science. In addition, they have allowed me to take most decisions on how to solve the challenges faced in this project and in a high degree determine which direction the focus in the project should be. Finally, they have provided their inputs when they were needed to solve difficult problems.

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Last but not least, I am very grateful to Martina for your support, motivation and inspiration. Thank you.

Rasmus Eliasen

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ABBREVIATIONS

4-MBzl; 4-methylbenzyl
4-PDS; 4,4-dithiodipyridine
Acm; acetamidomethyl
ACTH; adrenocorticotropic hormone
AgRP; agouti-related protein
Boc; tert-butoxycarbonyl
CCK; cyclic cystine knot
CDAP; 1-cyano-4-dimethylaminopyridinium tetrafluoroborate
DCM; dichloromethane
DIC; 1,3-diisopropylcarbodiimide
DIPEA; diisopropylethylamine
DMF; dimethylformamide
DMSO; dimethylsulfoxide
DTT; dithiothreitol
EDTA; ethylenediaminetetraacetic acid
Fmoc; 9-fluorenylmethyloxycarbonyl
GSH; reduced glutathione
GSSG; oxidised glutathione
HBTU; 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HFIP; hexafluoroisopropanol
HOBt; 1-hydroxybenzotriazole
HPLC; high-performance liquid chromatography
ICK; inhibitor cystine knot
MCR; melanocortin receptor
MS; mass spectrometry
MSH; melanocyte stimulating hormone
MT-II; melanotan II
Abbreviations

MW; molecular weight
NDP-α-MSH; [Nle¹,D-Phe⁷]-α-MSH
Nle; norleucine
NMP; N-methyl pyrrolidone
NMR; nuclear magnetic resonance
Pbf; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PEG; polyethylene glycol
POMC; pro-opiomelanocortin
PS; polystyrene
SPPS; solid phase peptide synthesis
S₄Bu; tert-butylthio
tBu; tert-butyl
tCEP; tris-2-carboxyethlylphosphine
TEA; triethylamine
TIPS; triisopropylsilane
TFA; trifluoroacetic acid
Tris; tris(hydroxymethyl)aminomethane
Trt; trityl
UPLC; ultra-performance liquid chromatography
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1. INTRODUCTION

1.1 GURMARIN

Gurmarin is a 35 amino acid peptide (Figure 1.1) isolated from the leaves of the plant Gymnema sylvestre [1], a woody climber found in south India. The leaves of the plant has been used for treatment of type 2 diabetes mellitus in the traditional Ayurvedic medicine [2]. The antidiabetic effect is believed to be caused by a group of glycosides named gymnemic acids, which lower blood glucose and possibly has a regenerative effect on pancreatic beta cells [3,4]. In contrast to gymnemic acids, gurmarin has been found to inhibit sweet taste receptors in rodents [1,5], while only a minimal effect has been observed in humans [1].

*EQCVK KDELCPYDCCPELKVNWWDHKCG
1 6 11 16 21 26 31

**Figure 1.1.** In the top the primary structure of gurmarin is shown. The asterisk indicates an N-terminal pyroglutamic acid. Below is the NMR derived three-dimensional structure of gurmarin. PDB ID: 1c4e.
Inhibitor cystine knotted peptides

The primary structure of gurmarin was first determined by a combination of enzymatic digestions and Edman degradation [6], while a similar approach determined the connectivity of three disulphides [7]. The three disulphides form an inhibitor cysteine knot (ICK) which define the tertiary structure of gurmarin confirmed by two published nuclear magnetic resonance (NMR) derived three-dimensional structures (Figure 1.1) [8,9]. The neighbouring cysteines in position 17 and 18 make a simple determination of the disulphide combination by enzymatic digestion unfeasible due to lack of cysteine-specific proteases [6,7].

1.2 INHIBITOR CYSTINE KNOTTED PEPTIDES

The ICK is defined by six cysteines forming three disulphides connected as Cys(1-4), Cys(2-5) and Cys(3-6), where the numbers refer to the cysteine position from the N-terminal end. Furthermore, the peptide backbone and the Cys(1-4) and Cys(2-5) disulphides form a ring, which is penetrated by the Cys(3-6) disulphide (Figure 1.2) [10].

Figure 1.2. A depiction of the ICK in gurmarin. The six cysteines are numbered 1 to 6 in order of their position in the sequence from the N-terminal end. The ring formed by the backbone and the Cys(1-4) and Cys(2-5) disulphides are highlighted in red. Disulphides are shown in yellow. The N-terminal pyroglutamic acid is marked with an asterisk.

ICK peptides have been found in a variety of species like cone snails [11], spiders [12], squash [13], potato [14] and in other plants [8]. They all share similar functions
as toxins or ion channel inhibitors, which gives rise to the ICK name [10]. A subgroup of
the ICK peptides that has a similar disulphide pattern but also a cyclic backbone are
referred to as cyclotides, and their general structure is referred to as the cyclic cystine knot
(CCK) [15]. These peptides are found in plants where they are believed to function as a
host defence towards insects and other pests [16-19].

Despite their similar function, high sequence diversity besides the six
cysteines is found in ICK peptides [20-22]. This diversity suggests that it is possible to graft
functional peptide sequences into the ICK or cyclotide frameworks. Hence, ICK peptides
would make a suitable scaffold for designing peptide drugs or for agricultural applications
like crop pest protection [20,23,24]. Besides the sequence diversity, a very high enzymatic,
thermal and chemical stability is observed in ICK peptides, especially in cyclotides
[18,19,25]. Since stability often is a concern when developing peptide drugs [26-28], this is a
huge advantage for the ICK scaffold in peptide drug design. Furthermore, injection is often
the delivery route of peptide drugs, but this route can be associated with discomfort and
inconvenience [26,28]. ICK peptides have potential for oral delivery due to their higher
enzymatic stability and their ability to permeate membranes compared to other peptides
[29]. Thereby, they might overcome another obstacle in peptide drug development.

1.2.1 Grafting examples in inhibitor cystine knotted peptides

The grafting principle in ICK peptides has been demonstrated by designing and
synthesising a chimeric protease inhibiting peptide inhibiting two different proteases [30],
and by altering the specificity of protease inhibitors [31]. In addition, inhibitors of
fibrinogen binding and platelet aggregation have been synthesised by grafting RGD
containing sequences into ICK scaffolds [32]. It has been shown that one of the most
studied cyclotides, kalata B1 is able to obtain the native CCK fold after substitution of
amino acids responsible for haemolytic effect of the peptide [33]. Further grafting work
with kalata B1 has produced analogues that are promising for treatment of angiogenesis
[34] and inflammatory pain [35], the latter having oral bioavailability. Another cyclotide,
MCoTI-II has been engineered to produce a foot-and mouth disease virus inhibitor [36].
Inhibitor cystine knotted peptides

Figure 1.3. Illustration of two oxidation strategies for ICK peptides. a) In a random redox buffer oxidation, the reduced cysteines are oxidised to the ICK peptide via multiple scrambled disulphide isomers. The native product is expected to be the most thermodynamically favoured product and therefore it is also expected to be the most abundant product. b) In an orthogonal oxidation, three pairs of different side chain protection groups are selectively removed one at a time and the corresponding disulphides can thus be formed selectively one at a time. This ensures that only the native ICK conformation is obtained.
1.2.2 Synthesis of inhibitor cystine knotted peptides

The synthesis of ICK peptides is complicated by the fact that the six cysteines can be connected by 15 different disulphide combinations. A common procedure used to fold ICK peptides builds on a method using a redox buffer mixture of reduced glutathione (GSH) and oxidised glutathione (GSSG) or other low molecular weight (MW) thiol-disulphide pairs [37]. This procedure is able to re-oxidise reduced proteins into their native conformation by disulphide scrambling of non-native disulphides into native, based on the hypothesis that the native conformation is the one with the lowest Gibbs free energy [38]. The oxidation procedure has been used for the synthesis of a variety of ICK peptides [39-42] and is illustrated in Figure 1.3a. Another oxidation procedure uses the enzyme protein disulphide isomerase that analogously to the redox pair is able to catalyse the scrambling of non-native disulphides into native [43,44]. This enzyme has been used to catalyse ICK peptides [45], however it does not appear to be widely used in ICK peptide oxidation.

An enzymatic approach has also been used in the backbone cyclisation of cyclotides [46] where immobilised trypsin was used for cyclisation and affinity purification. However, the most commonly used principle for backbone cyclisation uses a C-terminal thioester to cyclise the backbone via native chemical ligation [47,48]. The C-terminal thioester is easily formed using tert-butoxycarbonyl (Boc)-solid phase peptide synthesis (SPPS), and the cyclisation and disulphide formation can be done concurrently in a redox buffer as described above [49]. The use of HF to cleave peptides from the resin after Boc-SPPS is a limitation to this strategy. Unfortunately, the C-terminal thioester is not as easily incorporated using 9-fluorenylmethyloxycarbonyl (Fmoc)-SPPS, due to low stability towards the deprotection conditions used in Fmoc-SPPS. Consequently, Fmoc-compatible methods have been developed where the thioester is formed on-resin after the linear peptide synthesis [50] or where the side chain protected peptide is cleaved from the resin and the thioester formed in solution [51]. The latter method has been adapted for the synthesis of cyclotides [52].

It has been shown that mature ICK peptide sequences provide sufficient information to obtain native structures and disulphide connections [42,45]. However, since the formation of native peptide is reduced when urea is added during the oxidation, non-
Inhibitor cystine knotted peptides

covalent interactions and not only the amino acid sequence are affecting the folding into native structures [40,42]. In the case of a double chain cysteine rich peptide like insulin, the native structure was not obtained when protein disulphide isomerase was used for the oxidation [53]. This indicates that insulin is oxidised as linear pro-insulin, which is subsequently cleaved into mature insulin. This illustrates that it is not always the case that mature peptide sequences contain sufficient information to obtain the native structure.

Another method to form the disulphides in ICK peptides involves orthogonal side chain protection of the involved cysteines [54]. These allow selective deprotection and oxidation of the three disulphides as illustrated in Figure 1.3b. Even though various orthogonal synthesis pathways have been suggested [55], only few examples are reported of such syntheses [56-58].

The orthogonal oxidation method ensures that only the desired disulphide connectivity is produced. Partly orthogonal synthesis methods have been used to confirm that native conformations are obtained when using random oxidation of ICK peptides [59,60]. NMR spectroscopy has been widely used to determine the three-dimensional structure of ICK peptides [24]. However, the NMR determination of the disulphide connectivity can be difficult. This was illustrated by a suggested revision of the initial structure determination of kalata B1 [61]. The revision was contradicted by a confirmation of the initial and generally accepted disulphide connectivity though [62]. Nonetheless, these findings suggest that other ways of determining the disulphide connectivity should also be pursued. A chemical determination of disulphide connectivity has been described in which a partial disulphide reduction is followed by cyanylation of reduced cysteines [63]. This produces a mixture of peptides that are first separated by high-performance liquid chromatography (HPLC), then selectively cleaved at cyanylated cysteine residues and finally analysed by mass spectrometry (MS). In another method inspired by this procedure, peptides are partially reduced and reduced cysteines N-ethyl maleimide alkylated, followed by a full reduction and reaction with 2-bromoethylamine. The ethylamine alkylation produces lysine-like modified cysteines that are amenable to tryptic digestion and thus the disulphide connectivity can be determined [64]. These chemical methods to confirm the disulphide connectivity provides a supplement to NMR based determination.
1.3 **The central melanocortin system**

The central melanocortin system is defined as brainstem and hypothalamic neurons expressing neuropeptide Y, agouti-related protein (AgRP) or pro-opiomelanocortin (POMC) along with their downstream targets expressing melanocortin receptors (MCR) 3 and 4 [65]. The central melanocortin system is involved in energy homeostasis and appetite regulation and consequently it is a target for the development of drugs for treatment of obesity [65-67].

1.3.1 **The melanocortin receptors**

The MCRs is a family of five G protein-coupled receptors named in the order of their discovery [68-72]. The MC1R is mainly found in melanocyte cells [68,69]. The MC2R is found in the adrenal cortex and regulates steroidogenesis [68]. Of the endogenous agonists only adrenocorticotropic hormone (ACTH) is able to bind to the MC2R [73]. The MC3R is mainly found in the arcuate nucleus of the hypothalamus [70,74] where it is suggested to function as an autoinhibitory receptor that downregulates the POMC neuron signalling [75,76]. In addition it has been shown that stimulation of the MC3R increases food intake in mice [77]. The MC4R is found throughout the brain [71,78] and is known to be involved in weight regulation, since obesity is observed both in MC4R deficient humans and mice [79,80]. Furthermore, it has been shown that stimulation of the MC4R increases energy expenditure, decreases appetite and as a result thereof reduces weight in mice. This was illustrated by the lack of response in MC4R knockout mice to stimulation by the MCR agonist melanotan II (MT-II) (described below) compared to the response in wild type mice [81,82]. The MC5R is distributed throughout the body with high levels found in skeletal muscle, brain and exocrine glands and consequently it exerts a variety of functions [72,83,84].
1.3.2 Melanocortin receptor ligands

The endogenous ligands for the melanocortin receptors consists of four agonists, α-melanocyte stimulating hormone (MSH), β-MSH, γ-MSH and ATCH all derived from the precursor POMC (see Figure 1.4a) [85,86] and two antagonists agouti protein [87] and AgRP [88].

![Diagram of POMC and peptide hormones](image)

**Figure 1.4.** a) Illustration of POMC and the peptide hormones that are produced from its processing. The melanocortin agonists ACTH, α-MSH, β-MSH and γ-MSH are shaded in grey. The other peptides derived from POMC are β-lipotropin, corticotropin-like intermediate peptide (CLIP), γ-lipotropin and β-endorphin. These peptides will not be discussed in this work, since they are not active on the MCRs. b) The primary structures of the four endogenous MCR agonists. The His-Phe-Arg-Trp pharmacophore is highlighted in bold.
The four agonists share the His-Phe-Arg-Trp pharmacophore (see Figure 1.4b) and it has been shown that this tetrapeptide alone is able to activate the MCRs (except the MC2R) [89]. Engineering of the endogenous MSH peptides has led to more potent and protease stable analogues. Substitution of Met$^4$ to norleucine (Nle) and Phe$^7$ to D-Phe in α-MSH produced the highly potent nonselective agonist [Nle$^4$,D-Phe$^7$]-α-MSH (NDP-α-MSH) [90], which is often used as a standard in MCR binding and activity assays. In another engineering work of α-MSH, Met$^4$ and Gly$^{10}$ were replaced with cysteines, which were oxidised to form a disulphide cyclisation of the pharmacophore [91]. This analogue had both increased potency and prolonged activity compared to α-MSH. These two analogues were proposed to have stabilised an active conformation of the pharmacophore and thereby increased the potency [90,91]. By combining these two approaches MT-II was designed. This peptide is a truncated version of NDP-α-MSH (residues 4-10) in which an inserted Asp residue in position 5 and a Lys residue in position 10 forms a stabilising lactam bridge [92]. As mentioned above, MT-II has been used in a number of studies to illustrate the functions of the MCRs.

The C-terminal residues 87-132 of the MCR antagonist AgRP contain five disulphides in a similar conformation as the ICK [93]. Engineering of this C-terminal part produced a 34 residue ICK peptide AgRP(87-120, C105A) with four disulphides (see Figure 1.5). Both peptides show high antagonist activity at the MCRs [94]. The fourth non-ICK disulphide surrounds the loop in which the antagonist binding motif Arg-Phe-Phe [95] is present (see Figure 1.6a). Similarly to the engineered cyclic melanocortin agonists described above, the disulphide may induce a favourable conformation of the active loop for the MCRs. Further engineering of the four disulphide ICK peptide AgRP(87-120, C105A) reversed the antagonist activity. This was done by replacing the Arg-Phe-Phe motif with the His-D-Phe-Arg-Trp pharmacophore and a consequently a nanomolar affinity agonist at the MCRs was obtained [96].
The central melanocortin system

Figure 1.5. Structural alignment of AgRP(87-120, C105A) (PDB ID: 1mr0) coloured in magenta with gurmarin (PDB ID: 1c4e) coloured in green. The active loop in AgRP(87-120, C105A) is in the bottom and stabilised by the fourth non-ICK disulphide, which is not present in gurmarin. The aligned sequences of AgRP(87-120; C105A) and gurmarin are shown at the bottom with disulphides indicated as lines. The asterisk indicates an N-terminal pyroglutamic acid.

1.3.3 Development of melanocortin peptide pharmaceuticals

Some inconclusive studies on body fat reduction have been reported when ACTH(4-10) was administered intranasally [97,98]. In normal weight humans, fat mass reduction and weight loss was observed, while no significant effect was observed in overweight humans. The lack of response could be explained by changes in the MC4R reported in some obese
people [80]. Another explanation is a possible MSH resistance observed in obese people [99], thus suggesting that higher doses or more potent and selective compounds are necessary.

**Figure 1.6.** An illustration of the grafting principle in this work. a) The active loop in AgRP(87-120, C105A) (PDB ID: 1mr0) coloured in magenta aligned with the similar loop in gurmarin (PDB ID: 1c4e) coloured in green. The side chains of the binding residues Arg-Phe-Phe in AgRP(87-120, C105A) are shown. b) The side chains of residues 26-31 in the gurmarin loop are shown. c) A model of the His-Phe-Arg-Trp pharmacophore mutation in the gurmarin loop at residues 27-30. The four residues are coloured in grey.

Despite many years of research, no melanocortin drugs are in advanced human trials for obesity [67,100]. It has been proposed that this could be due to the high similarity between the MCRs, and consequently side effects due to lack of selectivity for one receptor [100], or due to the often observed low half-life of peptides *in vivo* [101,102]. It has also been suggested that a fine tuning of the selectivity in order to achieve an optimal signalling e.g. of the MC3R and MC4R could improve the outcome of clinical studies with melanocortin drugs [67]. However, the lack of knowledge of the interplay between the MC3R and MC4R in regulating appetite and energy homeostasis is a limitation to the pursuit of this hypothesis.
The non-selective MCR agonists MT-II and its C-terminal acid analogue [103] have been evaluated in phase 1 clinical trials for MCR mediated penile erection and sexual desire. The compounds were effective in these trials, and the most common side effects observed were flushing, nausea, stretching/yawning, headache, hypertension, taste disturbance and feeling hot [104-108]. Since the agonists were not selective, it is not known which MCR is responsible for mediating the observed effects. In addition, it has previously been suggested that hypertension effects of γ-MSH are exerted through a non-MCR mediated pathway [109]. The side-effects in the trials were observed at a mild level and might be acceptable when administering a drug occasionally for treatment of sexual disorders. However, when stimulating the MC4R chronically for treatment of obesity, these side effects along with the potential increase in penile erection and sexual desire may not be deemed acceptable. Consequently, there is a need for more stable and selective MC4R ligands if this pathway should be used for treatment of obesity.

1.4 Project aims

It is possible that gurmarin based melanocortin analogues may overcome some of the limitations of current analogues described in this chapter. Due to the ICK framework, the peptides will most likely be more stable towards enzymatic degradation than native MSH-peptides. In addition, they could possibly also be more selective due to a more stabilised conformation within the ICK framework. Furthermore, it is possible that their non-melanocortin nature could reduce some of the side-effects that have been observed, if these are exerted on other receptors than the MCRs and not by the His-Phe-Arg-Trp pharmacophore.

Due to the ICK nature of the engineered AgRP(87-120, C105A) a structural alignment with gurmarin was made. This indicated that an inter-cysteine loop was present in gurmarin with high similarity to the active loop in AgRP(87-120, C105A) (see Figure 1.5).

Consequently, the aim of the project was to pursue the grafting principle by synthesising melanocortin analogues of gurmarin. This was done by engineering the His-
Phe-Arg-Trp pharmacophore into gurmarin (illustrated in Figure 1.6) in a similar fashion as was described for AgRP(87-120; C105A) [96]. The synthesised analogues were evaluated for their binding to the MCRs. Before synthesising melanocortin analogues of gurmarin a stable gurmarin synthesis route was established by comparing orthogonal disulphide formation with random redox oxidation of the disulphides in gurmarin.
2. SYNTHESIS OF GURMARIN

2.1 INTRODUCTION

This chapter will present the work done on optimising the synthesis of gurmarin and confirmation of the disulphide connectivity. All peptide structures synthesised are given a number and a complete list of the structures is found in Appendix A. The work in this chapter has led to one poster presentation (Appendix B) and proceedings paper (Appendix C) along with one paper accepted for publication (Appendix D). An experimental section is found at the end of this chapter.

2.2 FIRST SYNTHESIS STRATEGY

The initial strategy involved acetamidomethyl (Acm) protection of all six cysteines (Table 2.1). Acm-protected cysteines can be oxidised to disulphides using iodine, which forms an iodide activated cysteine that is able to react with another Acm protected or iodide activated cysteine [110,111]. The advantage of this method is a fast oxidation, while the lack of control over which disulphides are formed is a drawback.

Table 2.1. Positions of cysteine side chain protection groups in the first strategy and the partially orthogonal gurmarin oxidation strategy. The lower case letter refers to the different synthesis procedures pursued for the synthesis of gurmarin (1).

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Cys3-18</th>
<th>Cys10-23</th>
<th>Cys17-33</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Acm</td>
<td>Acm</td>
<td>Acm</td>
</tr>
<tr>
<td>1b</td>
<td>Acm</td>
<td>Acm</td>
<td>Trt</td>
</tr>
<tr>
<td>1c</td>
<td>Acm</td>
<td>Trt</td>
<td>Acm</td>
</tr>
<tr>
<td>1d</td>
<td>Trt</td>
<td>Acm</td>
<td>Acm</td>
</tr>
</tbody>
</table>

Linear gurmarin (1a) was synthesised on an automated peptide synthesiser using standard Fmoc-SPPS chemistry with and without use of microwaves for coupling
and deprotection. The MW of the main product was 18 Da lower than expected. This was suggested to be due to aspartimide formation of the Asp16-Cys(Acm)17 bond (Figure 1.1), since it has been reported that this sequence is prone to aspartimide formation [112]. Another explanation could be aspartimide formation of Asp7 or Asp30, however the -18 Da MW appeared after coupling of Asp16. To confirm the aspartimide hypothesis, a gurmarin analogue with Asp16 substituted with Ala was synthesised. In this synthesis only the expected MW was observed confirming the aspartimide formation of Asp16.

Figure 2.1. Treatment of the aspartimide peptide with NaOH. In the top left are the aspartimide and non-aspartimide peptide UPLC (ultra-performance liquid chromatography) chromatogram and mass spectra of the triple charged peptides (non-aspartimide exp. MW: 1390.64). Below are the peptides after treatment with NaOH. The four peaks observed are due to the four isomers produced from hydrolysis of the aspartimide as depicted to the right. R indicates surrounding amino acids. The gradient used on UPLC are 20-60% of 70:30 acetonitrile:water against 10% acetonitrile in 0.2 M Na₂SO₄, 0.04 M H₃PO₄, pH 3.5 over 8 min.
The aspartimide was stable when dissolving the peptide and during purification. Treatment of a mixture of aspartimide peptide and non-aspartimide peptide with NaOH gave four products all with the same mass as the non-aspartimide peptide. This corresponds to D- and L- stereoisomers of alpha and beta branched peptide as illustrated in Figure 2.1 that shows the NaOH treatment of reduced gurmarin with Asn27 substituted for Ala [113].

To reduce the aspartimide formation, the protection groups of Cys17 and Cys33 were changed to trityl (Trt), since the Asp-Cys(Trt) bond should be less prone to aspartimide formation [112]. In addition, the introduction of a second pair of cysteine protection groups made selective formation of the first disulphide possible. It has previously been shown that a partially orthogonal oxidation of three disulphides in a non-ICK peptide, where two disulphides are formed first and the third disulphide is formed selectively afterwards produces the expected product [114]. It was suggested that the formation of the correct disulphide combination was induced by reducing the possible disulphide combinations from fifteen to three. In addition, positioning of the orthogonal protection group on cysteines that would normally form non-native disulphides based on experience was proposed to increase the yield. Consequently, it is possible that the selective formation of the first disulphide in gurmarin induces the correct formation of the final two disulphides.

Linear peptide 1b (Table 2.1) was synthesised on an automated microwave peptide synthesiser using standard Fmoc-SPPS chemistry. As expected, the aspartimide formation was reduced but not eliminated by introducing Cys(Trt) as residue 17. The peptide was cleaved from the resin using trifluoroacetic acid (TFA) and scavengers, which removed Trt protection groups on Cys17 and Cys33 concurrently and did not affect the Acm-protected cysteines. The first disulphide was formed by air oxidation in an aqueous tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 7.8 with 2 vol% dimethylsulfoxide (DMSO). The final two disulphides were formed by iodine oxidation using 20 eq. of iodine in 1:4 water:methanol. Only one product with the expected MW was observed and isolated in yields from 0.25-0.5% based on the loading of the resin.
To investigate the disulphide connectivity of 1b, a chymotrypsin cleavage of the peptide was done. As illustrated in Figure 2.2, a chymotrypsin cleavage of gurmarin should not produce multiple fragments, since the fragments would be connected by the disulphides. However, a mass spectrum of the chymotrypsin cleavage of 1b showed two products corresponding to residues 1-13 and 14-35 as illustrated in Figure 2.2. Since the Cys17-Cys33 disulphide is formed selectively, the depicted disulphide connectivity with two non-native disulphides is the only possible combination.

Figure 2.2. The cleavage sites for chymotrypsin (red) and thermolysin (green) in gurmarin is highlighted on top. Chymotrypsin cleaves the C-terminal peptide bond of aromatic amino acids. Thermolysin cleaves the N-terminal peptide bond of hydrophobic acids. The asterisk indicates an N-terminal pyroglutamic acid residue. In the middle are two non-native gurmarin fragments observed in a chymotrypsin cleavage of peptide 1b. The bottom shows the observed mass spectra of the fragments from the chymotrypsin cleavage. The calculated mass of the mono-disulphide fragment is 1546.70 and the calculated mass of the bis-disulphide fragment is 2677.20 and 2677.22 calculated from the triple and double charged peptide respectively.

Consequently, the synthesis strategy 1b did not produce gurmarin (1) but a disulphide isomer of gurmarin (2). The pH during the chymotrypsin cleavage was 7.8 and consequently scrambling of the disulphides was a possibility. However, only the mass peaks depicted in Figure 2.2 were observed, which indicate that scrambling did not occur.
First synthesis strategy

Similar synthesis strategies were used for peptide 1c and 1d, which only differed in the position of the Trt protection groups as shown in Table 2.1. Since Cys17 was Acm-protected in these peptides aspartimide was observed in both syntheses and the overall yields of the syntheses were less than 0.1%.

Table 2.2. A list of the fragments observed by thermolysin cleavage of peptides 1b-d. The asterisk indicates an N-terminal pyroglutamic acid residue.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Fragment</th>
<th>Theoretical mass</th>
<th>Observed mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b</td>
<td>*EQC</td>
<td>803.34*</td>
<td>803.35</td>
</tr>
<tr>
<td></td>
<td>LC IP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L DCCEP</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>LECKK</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WD HKC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1c</td>
<td>*EQC</td>
<td>523.69*</td>
<td>523.69</td>
</tr>
<tr>
<td></td>
<td>L DCCEP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WD HKC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LECKK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>*EQC</td>
<td>861.80*</td>
<td>861.81†</td>
</tr>
<tr>
<td></td>
<td>L DCCEP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WD HKC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LECKK</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LC IP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gurmarin</td>
<td>*EQCVK</td>
<td>531.78*</td>
<td>531.79†</td>
</tr>
<tr>
<td></td>
<td>KDELIC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IPYLYL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DCCEP</td>
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<tr>
<td></td>
<td>LECKK</td>
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<tr>
<td></td>
<td>VNWWD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HKDGI</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td></td>
<td>31</td>
</tr>
</tbody>
</table>

† (M+1H+), ‡ (M+2H+), † the peptide fragment contains only native disulphides

Peptides 1b-d were cleaved with thermolysin to investigate the disulphide connectivity. The possible cleavage sites for thermolysin in gurmarin are shown in Figure 2.2. The observed masses of the fragments from the cleavage are listed in Table 2.2. For all three peptides, the major fragments were non-native. In peptide 1c, a fragment containing the selectively formed Cys10-Cys23 disulphide was found though. In peptide 1d, two fragments containing only native disulphides were observed: the Cys10-Cys23 fragment also observed in peptide 1c, and a fragment containing the final two disulphides. Since the
Cys3-Cys18 disulphide was formed selectively in peptide 1d, the final disulphide must be the native Cys17-Cys33 and consequently native gurmarin (1) was produced in the synthesis of peptide 1d. However, the amounts of the native fragments were lower than the amounts of the non-native fragments also observed. Consequently, native gurmarin was a by-product of this synthesis. The peptide obtained from the 1c procedure was another disulphide isomer of gurmarin (3), while the major product obtained from the 1d procedure was a third disulphide isomer of gurmarin (4).

This work with the partially orthogonal synthesis of gurmarin and thermolysin cleavage of the peptides was presented as a poster shown in Appendix B and published as a proceedings paper, which is found in appendix C.

### 2.3 Redox Buffer Oxidation Strategy

Since the first synthesis strategy only produced gurmarin in very small amounts not separable from the non-native disulphide isomers of gurmarin also produced, a new random redox oxidation strategy was attempted. This strategy uses a low MW thiol-disulphide pair to induce scrambling of the disulphides in gurmarin until the most thermodynamically favourable conformation is obtained as described in the introduction. The redox buffer oxidised gurmarin is called peptide 1e.

Linear peptide 1e was synthesised on an automated microwave peptide synthesiser using standard Fmoc-SPPS chemistry with all cysteines Trt protected. The aspartimide formation was not eliminated by the introduction of Cys(Trt) in position 17. Consequently, 0.05 M 1-hydroxybenzotriazole (HOBt) was added to the piperidine solution used for Fmoc-deprotection, since this has been shown to suppress aspartimide formation [112,115]. As depicted in Figure 2.3, the addition of HOBt almost eliminated the aspartimide formation.

Following preparative HPLC purification, the peptide was oxidised in a 0.1 M Tris-HCl (pH 7.8), 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM GSH, 2 mM cystamine, 35 vol% DMSO, 6 vol% 2-dodecyloxyethanol buffer that had previously been optimised for the synthesis of the cyclotide cycloviolacin O2 [52]. The peptide was oxidised
Redox buffer oxidation strategy

for 48 hours in 0.01 mM concentration. Subsequently, analysis by UPLC and MS indicated that one product was formed with the expected MW. However, the preparative HPLC purification was complicated by the viscous detergent 2-dodecyloxyethanol.

![Figure 2.3](image)

**Figure 2.3.** Aspartimide formation with and without HOBt addition during Fmoc-deprotection. The mass spectra correspond to triple charged gurmarin (exp. MW: 1404.97).

To improve the oxidation and to facilitate the final purification, the redox buffer was optimised. Nine different conditions were tested (Table 2.3). The conditions were chosen based on a review on disulphide bond formation in peptides [54] and the buffer used initially. The detergent 2-dodecyloxyethanol was not included in the oxidations. It was added to favour solubility of folded peptides with solvent exposed hydrophobic residues [52]. However, since other reported redox oxidations of ICK peptides did not include detergents, it was anticipated that gurmarin would be able to fold with no detergent added. This would ultimately make the purification less complicated.

Not surprisingly, the yields were higher in the Tris buffer because of the higher pH and no intermolecular disulphide bonds were observed in either concentration tested. The addition of EDTA suppressed the oxidation. In the initial report on GSH-GSSG oxidation, EDTA was added in order to reduce variations in the oxidation, which was proposed to be caused by copper ions. When EDTA was present without GSH and GSSG, the oxidation was suppressed similarly to what was observed in this experiment [37]. DMSO increased the oxidation yield, however only minor differences were observed
between 20 vol% and 10 vol% after 72 h. The 1:1 ratio of GSH:GSSG was superior to the 1:10 ratio and cystamine was superior to GSSG. This is consistent with the report that cystamine is a more powerful oxidation agent than GSSG [116].

Table 2.3. The conditions used in the buffer optimisation. The peptides were oxidised for 72 h.

<table>
<thead>
<tr>
<th>Peptide concentration</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 mM</td>
<td>0.1 M ammonium acetate (pH 6.5)</td>
</tr>
<tr>
<td>0.01 mM</td>
<td>0.1 M Tris-HCl (pH 7.8)</td>
</tr>
<tr>
<td>0.01 mM</td>
<td>0.1 M ammonium acetate (pH 6.5), 1 mM EDTA</td>
</tr>
<tr>
<td>0.01 mM</td>
<td>0.1 M ammonium acetate (pH 6.5), 20 vol% DMSO</td>
</tr>
<tr>
<td>0.01 mM</td>
<td>0.1 M ammonium acetate (pH 6.5), 10 vol% DMSO</td>
</tr>
<tr>
<td>0.01 mM</td>
<td>0.1 M ammonium acetate (pH 6.5), 1 mM GSH, 0.1 mM GSSG</td>
</tr>
<tr>
<td>0.01 mM</td>
<td>0.1 M ammonium acetate (pH 6.5), 1 mM GSH, 1 mM GSSG</td>
</tr>
<tr>
<td>0.01 mM</td>
<td>0.1 M ammonium acetate (pH 6.5), 1 mM GSH, 0.1 mM cystamine</td>
</tr>
<tr>
<td>0.02 mM</td>
<td>0.1 M ammonium acetate (pH 6.5)</td>
</tr>
</tbody>
</table>

Since the oxidation buffer containing cystamine gave the highest amount of fully oxidised peptide, peptide 1e was oxidised using 0.1 M Tris-HCl (pH 7.8), 1 mM GSH, 1 mM cystamine as redox buffer. DMSO was not included in the buffer, since the buffer optimisation indicated that the DMSO oxidation mechanism was inferior to the GSH:cystamine facilitated oxidation, from which fully oxidised peptide was obtained.

Figure 2.4 shows UPLC chromatograms illustrating the progress of the oxidation. The purified yield of reduced peptide was 24.7%. The yield of the oxidation and purification was 57.1% and consequently, the overall yield of peptide 1e was 14.1%.

### 2.4 Structure Confirmation

Since the redox buffer oxidation does not provide control over the disulphide connectivity, 15 possible combinations can be obtained when the peptide contains six cysteines. Consequently, a chemical determination of the disulphide connectivity mentioned in the introduction was pursued [63].
2.4.1 Partial reduction and cyanylation

The principle of the method is outlined in Figure 2.5. The advantage of the method is that it makes it possible to distinguish between neighbouring cysteines. This is not possible when using traditional proteolytic approaches due to lack of cysteine specific proteases. In addition, the reduction and cyanylation is done at pH 3, which reduce the possibility of disulphide scrambling of the peptide.
Figure 2.5. An example of the chemical determination of the disulphide connectivity. A) The peptide is partially reduced using tris-2-carboxyethylphosphine (TCEP) producing a mixture of peptides that are fully oxidised, has two disulphides, one disulphide or are fully reduced. In this example the Cys10-23 disulphide is reduced. B) The reduced disulphides are cyanlated using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP). C) After HPLC separation, the peptides are cleaved on the N-terminal bond of cyanlated cysteines using methylamine. D) The peptides are fully reduced using TCEP and analysed by MS.
The reduction and cyanylation of gurmarin was optimised by varying the reaction times and the amounts of TCEP in order to achieve the highest amount of mono and double reduced and cyanylated gurmarin species. However, the changes mostly affected the amounts of fully reduced and fully oxidised peptides. Subsequently, the separation of the peptides was optimised using different HPLC systems and gradients. Finally, the cleavage at the cyanylated cysteines was optimised by using methylamine instead of ammonia and by adding guanidinium chloride to the cleavage to denature the peptide.

Peptide 1e was reduced for 30 minutes using 30 eq. of TCEP. The reduced cysteines were cyanylated using 1200 eq. of CDAP. The partially reduced and cyanylated peptides were purified using 20-30% acetonitrile against 0.1% TFA in water over 30 min on an analytical HPLC and manual fractionation (see Figure 2.6).

In general, the fractions were not very pure and the amount of peptide was low, which complicated the subsequent MS analysis. The two peaks containing double reduced and cyanylated gurmarin (Figure 2.6) were the most pure, and consequently these
were cleaved with aqueous methylamine containing guanidinium chloride. The sequence and mass spectra of the fragments observed are shown in Figure 2.7. The fragments found in the two peaks were shown to contain the Cys10-23 disulphide and the Cys17-33 disulphide. From these results, it can be deduced that the third disulphide must be the Cys3-Cys18 confirming the native disulphide connectivity.

Figure 2.7. Sequence and mass spectra of the observed fragments found after methylamine cleavage of double reduced and cyanylated gurmarin. Cys10-23 and Cys17-33 are native disulphides while Cys23-33 is a non-native disulphide.
Low purity and concentration of the purified peptide fragments, which is responsible for low resolution mass spectra, gives rise to some uncertainty of the accuracy of the disulphide determination though. In addition, when the analysis was repeated a non-native Cys23-33 fragment was found as shown in Figure 2.7. This either means that multiple disulphide combinations are present in peptide 1e or that scrambling occurs in the assay despite the low pH that should prevent this.

### 2.4.2 Thermolysin cleavage

Due to the complicated and laborious separation of the cyanylated fragments described above, a different attempt was made to investigate the disulphide connectivity of peptide 1e. As shown in Figure 2.2, a thermolysin cleavage of gurmarin would produce a fragment containing the Cys10-23 disulphide (8) and a fragment containing the other two disulphides (6 or 7).

The MS analysis of a thermolysin cleavage of peptide 1e is shown in Figure 2.8. Only two peaks with disulphide containing fragments were observed; one with the Cys10-23 disulphide (8) and another containing the bis-disulphide fragment (6 or 7). However, it is not possible to distinguish the connectivities of Cys17 and Cys18. Consequently, peptide 1e either has the native disulphide connectivity or a non-native Cys3-17, Cys18-33 connectivity. This possible non-native disulphide combination is a fourth disulphide isomer of gurmarin (9).

Since the thermolysin cleavage was done at pH 6.5, disulphide scrambling is a possibility as it was in the chymotrypsin cleavage of peptide 1b. But since only two disulphide-containing fragments were found, it is not likely that scrambling had occurred during the cleavage.
2.4.3  Fragment synthesis

To investigate the disulphide connectivity of the bis-disulphide fragment (6 or 7) in Figure 2.8, the two bis-disulphide fragments were synthesised as a linear peptides with the two cysteine pairs Trt and Acm protected (10 and 11). This allowed for selective disulphide formation of both the native fragment 6 and non-native fragment 7 disulphide connectivity showed in Figure 2.8. The peptides were oxidised using the same conditions as employed.
in the first synthesis strategy described in section 2.2. It was expected that a thermolysin cleavage of peptides 10 and 11 would produce fragments 6 and 7 respectively, however 10 and 11 were not cleaved by thermolysin. This was suggested to be due to these short fragments containing two disulphides. As a consequence, they are most likely locked in a conformation that does not favour the interaction of thermolysin with the cleavage sites.

To overcome the thermolysin cleavage problem, two gurmarin analogues were synthesised with Cys10 and Cys23 substituted with alanine using the same synthesis strategy as described above. The first analogue (12) had two native disulphides, while the second analogue (13) had two non-native disulphides as in fragment 7. These two analogues were cleaved with thermolysin to produce fragment 6 and 7. The thermolysin cleaved peptides 12 and 13 were co-injected with thermolysin cleaved peptide 1e on both UPLC and HPLC-MS, but it was not possible to distinguish the native fragment 6 from the non-native fragment 7 using these methods since the fragments co-eluted.

### 2.5 **Orthogonal synthesis strategy**

To overcome the uncertainty of the disulphide connectivity an orthogonal synthesis strategy as outlined in (Figure 1.3b) was pursued. Besides the Trt and Acm protection groups already mentioned, tert-butylthio (S\textsubscript{t}Bu) which can be removed using reduction agents as mercaptoethanol, dithiothreitol (DTT) or TCEP \[117,118\] and monomethoxytrityl (Mmt), which can be removed by dilute TFA and scavengers \[119\] was used as cysteine protection groups.

A number of different conditions were tested for deprotection and oxidation of the cysteines as outlined in Table 2.4 and Table 2.5. In addition, different resins were used to investigate their effect on deprotection and on-resin oxidation. Finally, a number of different combinations of the protection groups were synthesised as outlined in Table 2.6.
Table 2.4. Deprotection methods used in the orthogonal oxidation strategy.

<table>
<thead>
<tr>
<th>Method</th>
<th>Protection group</th>
<th>Deprotection conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>S/Bu</td>
<td>10.1 ml 80:20:1 NMP:mercaptoethanol:DIPEA, 3 x &gt;5 h</td>
</tr>
<tr>
<td>II</td>
<td>S/Bu</td>
<td>50 eq. DTT in 10 ml dioxane, 2 x &gt;5 h</td>
</tr>
<tr>
<td>III</td>
<td>S/Bu</td>
<td>20.2 ml 80:20:1 DCM:mercaptoethanol:DIPEA, 3 x &gt;5 h</td>
</tr>
<tr>
<td>IV</td>
<td>S/Bu</td>
<td>80 eq. DTT, 5 eq. DIPEA in 20 ml water, 5 x 24 h</td>
</tr>
<tr>
<td>V</td>
<td>Mmt</td>
<td>10 ml 70:27:3 HFIP:DCM:TIPS, 2 x 15 min</td>
</tr>
<tr>
<td>VI</td>
<td>Mmt</td>
<td>10 ml 94:4:2 DCM:TIPS:TFA, 5 x 4 x 5 min</td>
</tr>
<tr>
<td>VII</td>
<td>Mmt</td>
<td>10 ml 95:4:1 DCM:TIPS:TFA, 3 x 4 x 5 min</td>
</tr>
<tr>
<td>VIII</td>
<td>Mmt</td>
<td>10 ml 95:4:1 DCM:TIPS:TFA, 10 x 5 min</td>
</tr>
<tr>
<td>IX</td>
<td>S/Bu</td>
<td>10.1 ml 80:20:1 NMP:mercaptoethanol:DIPEA, 3 x 60 min, 60°C</td>
</tr>
<tr>
<td>X</td>
<td>S/Bu</td>
<td>10.1 ml 80:20:1 NMP:mercaptoethanol:DIPEA, 3 x 24 h</td>
</tr>
<tr>
<td>XI</td>
<td>Mmt</td>
<td>10 ml 95:4:1 DCM:TIPS:TFA, 10 x 2 min</td>
</tr>
</tbody>
</table>

NMP;  N-methyl pyrrolidone. DIPEA; diisopropylethylamine. DCM; dichloromethane. HFIP; hexafluoroisopropanol. TIPS; triisopropylsilane. Eq. are compared to resin loading. Unless otherwise stated, the deprotection was done at room temperature.

Table 2.5. Oxidation methods used in the orthogonal oxidation strategy.

<table>
<thead>
<tr>
<th>Method</th>
<th>Oxidation conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>XII</td>
<td>10 eq. TEA in 20 ml NMP, 48 h</td>
</tr>
<tr>
<td>XIII</td>
<td>10 eq. 4-PDS in HPLC eluents, 0.05 mM peptide, 48 h</td>
</tr>
<tr>
<td>XIV</td>
<td>100 eq. I₂ in 4:1 acetic acid:HPLC eluents, 0.5 μM peptide, 2 h</td>
</tr>
<tr>
<td>XV</td>
<td>10 eq. 4-PDS in 20 ml NMP, &gt;5 h</td>
</tr>
<tr>
<td>XVI</td>
<td>1 eq. 4-PDS in 20 ml NMP, &gt;5 h</td>
</tr>
<tr>
<td>XVII</td>
<td>0.5 eq. 4-PDS in 20 ml NMP, &gt;5 h</td>
</tr>
<tr>
<td>XVIII</td>
<td>20 ml 4:1 NMP:DMSO, 4 h</td>
</tr>
<tr>
<td>XIX</td>
<td>10 eq. 4-PDS in HPLC eluents diluted with water, 0.05 mM peptide, 2 h</td>
</tr>
<tr>
<td>XX</td>
<td>100 eq. I₂ in 4:1 acetic acid:HPLC eluents, 0.05 mM peptide, 2 h</td>
</tr>
</tbody>
</table>

TEA; triethylamine. 4-PDS; 4,4-dithiodipyridine. HPLC eluents were approximately 69.9:30:0.1 water:acetonitrile:TFA.
Orthogonal synthesis strategy

Table 2.6. Overview and nomenclature of the conditions used in the orthogonal syntheses. Only the protection groups that were deprotected in the respective procedures are mentioned.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Linker and resin</th>
<th>Protection group</th>
<th>Position</th>
<th>Deprotection method</th>
<th>Oxidation method</th>
</tr>
</thead>
<tbody>
<tr>
<td>If</td>
<td>Wang-PS</td>
<td>S\text{Bu}</td>
<td>C3-18</td>
<td>I, II</td>
<td>-</td>
</tr>
<tr>
<td>Ig</td>
<td>NovaSynTGT</td>
<td>S\text{Bu}</td>
<td>C3-18</td>
<td>I, II</td>
<td>-</td>
</tr>
<tr>
<td>Ih</td>
<td>Wang-TentaGel</td>
<td>S\text{Bu}</td>
<td>C3-18</td>
<td>I, II</td>
<td>-</td>
</tr>
<tr>
<td>Ii</td>
<td>2-CITrtp-PS</td>
<td>S\text{Bu}</td>
<td>C3-18</td>
<td>I, II</td>
<td>-</td>
</tr>
<tr>
<td>Ij</td>
<td>Wang-PS</td>
<td>S\text{Bu}</td>
<td>C10-23</td>
<td>I, II</td>
<td>XII</td>
</tr>
<tr>
<td>Ik</td>
<td>Wang-PS</td>
<td>S\text{Bu}</td>
<td>C17-33</td>
<td>I, II</td>
<td>XII</td>
</tr>
<tr>
<td>Il</td>
<td>HMPB-Chemmatrix</td>
<td>S\text{Bu}</td>
<td>C10-23</td>
<td>III, IV</td>
<td>XII</td>
</tr>
<tr>
<td>Im</td>
<td>HMPB-Chemmatrix</td>
<td>Mmt</td>
<td>C10-23</td>
<td>V</td>
<td>XII</td>
</tr>
<tr>
<td>In</td>
<td>Wang-Chemmatrix</td>
<td>Mmt</td>
<td>C10-23</td>
<td>VI</td>
<td>XII</td>
</tr>
<tr>
<td>Io</td>
<td>Wang-Chemmatrix</td>
<td>Mmt, Trt, Acm</td>
<td>C10-23, C17-33, C3-18</td>
<td>V, VII</td>
<td>XII, XIII, XIV</td>
</tr>
<tr>
<td>Ip</td>
<td>Wang-Chemmatrix</td>
<td>Mmt</td>
<td>C10-23</td>
<td>VII, VIII</td>
<td>XII</td>
</tr>
<tr>
<td>Iq</td>
<td>Wang-PS</td>
<td>Mmt</td>
<td>C10-23</td>
<td>VII, VIII</td>
<td>XII</td>
</tr>
<tr>
<td>Ir</td>
<td>Wang-PS</td>
<td>Mmt</td>
<td>C10-23</td>
<td>VIII</td>
<td>XV</td>
</tr>
<tr>
<td>Is</td>
<td>Wang-PS</td>
<td>S\text{Bu}, Mmt</td>
<td>C10-23, C17-33</td>
<td>IX, VIII</td>
<td>XV, XV</td>
</tr>
<tr>
<td>It</td>
<td>Wang-PS</td>
<td>S\text{Bu}</td>
<td>C17-33</td>
<td>IX</td>
<td>XVI</td>
</tr>
<tr>
<td>Iu</td>
<td>Wang-PS</td>
<td>S\text{Bu}</td>
<td>C17-33</td>
<td>X</td>
<td>XVII</td>
</tr>
<tr>
<td>Iv</td>
<td>Wang-PS</td>
<td>S\text{Bu}, Mmt</td>
<td>C17-33, C10-23</td>
<td>X, XI</td>
<td>XVIII, XVIII</td>
</tr>
<tr>
<td>Iw</td>
<td>Wang-PS</td>
<td>S\text{Bu}, Trt, Acm</td>
<td>C10-23, C17-33, C3-18</td>
<td>X</td>
<td>XVIII, XIX, XX</td>
</tr>
</tbody>
</table>

2.5.1 First orthogonal strategy

In the first orthogonal strategy S\text{Bu} was used as cysteine side chain protection group for the first disulphide. The first disulphide would be oxidised on resin and the final two in solution after cleavage from resin using Trt and Acm for the final two disulphide pairs. The deprotection conditions, resins, resin linkers and positions attempted are shown in Table 2.6, peptide 1f-1l.

In the first attempt, four different resins were used to evaluate their effect on the deprotection (peptide 1f-1l). The polyethylene glycol (PEG) cross-linked resins used in peptide 1g and 1h were expected to improve the deprotection because of better swelling.
abilities [120,121]. However, the best deprotections and crude synthesis yields were observed for the Wang-polystyrene (PS) resin.

Secondly, the SBu deprotection was evaluated on the three cysteine pairs (peptide 1f, 1j, 1k). The C10-23 position was found superior followed by the C17-33 position. In addition, the mercaptoethanol deprotection (I) was found superior to the DTT deprotection (II). The deprotected cysteines in peptide 1j and 1k were air oxidised on resin using TEA in NMP (XII).

Since not all SBu was deprotected in peptide 1j another PEG cross-linked resin was investigated (peptide 1l), the volume of deprotection mixture was increased and the solvent changed to DCM (III). Various C-terminal deletion peptides were observed using this resin, but the deprotection and oxidation was improved. The synthesis was repeated with triple-coupling of the first residue followed by capping, which reduced the formation of deletion peptides. Surprisingly, the peptide was not deprotected in the repeated synthesis but a +18 Da MW was observed. This was expected to be ring opening of the N-terminal pyroglutamic acid residue induced by DIPEA, since it was only observed when DIPEA was present during the deprotection. The deprotection was ultimately achieved by multiple repeated deprotections with large excess of DTT (IV).

2.5.2 Second orthogonal strategy

Because of the problems with reproducibility of the SBu deprotection Mmt protection was pursued instead (peptide 1m-1r). An initial attempt to deprotect Mmt with HFIP in peptide 1m was unsuccessful. To investigate the deprotection a small amount of resin was reacted with a small amount of 3-maleimidopropionic acid in 10:1 NMP:DIPEA. No alkylation was observed, which most likely was due to no Mmt deprotection.

In a new synthesis, the resin was replaced by a PEG cross-linked resin with a Wang linker, which removed the C-terminal deletions (peptide 1n). Mmt was deprotected with 2% TFA (VI). The colour became dark orange during the deprotections. Between each set of four deprotections the resin was washed with DCM-NMP-NMP-DCM to quench the TFA and remove the colour to better be able to evaluate the deprotection
based on the intensity of the colour. In the oxidation two disulphides were formed, most likely because the multiple TFA deprotections also deprotected the Trt protection groups. In addition, less peptide was observed which could be due to the peptide being cleaved from the resin during the deprotections.

A new synthesis was made (peptide 1o) and the HFIP deprotection was pursued once more followed by oxidation to ensure that the lack of maleimide alkylation observed in peptide 1m in fact was due to no deprotection by HFIP. Since no oxidation was observed, it was confirmed that the HFIP deprotection (V) was unsuccessful. A deprotection with 1% TFA and fewer repetitions was made (VII) and the peptide was oxidised. Using this method, half of the peptide was deprotected and oxidised, while the other half was not deprotected. Consequently, the deprotection and oxidation was repeated, which produced the fully mono-oxidised peptide. The mono-oxidised peptide was cleaved and purified. Because of many by-products only low amounts of pure peptide was isolated. Based on to the resin loading 1% peptide was isolated. The Trt-protected cysteines were deprotected during peptide cleavage, while the Acm-protected cysteines were unaffected. The Trt-deprotected cysteines were oxidised with 4-PDS (XIII) and the peptide was purified. The yield of the second oxidation and purification was 7%. The final oxidation of the Acm-protected cysteines was done using iodine (XIV). However, only low amounts of fully oxidised peptide were observed and it was not possible to isolate the desired product.

The synthesis was repeated using the same resin (peptide 1p) and a Wang-PS resin (peptide 1q). In the latter synthesis, fewer by-products were observed, but no oxidation was observed, since Mmt was not deprotected when using the same conditions as previously (VII). Consequently, the deprotection was repeated without intermediate NMP washes (VIII) and the peptides were oxidised (XII). The peptides were cleaved and purified but no pure fractions were observed.

As an attempt to reduce the by-products and improve the oxidation, a new peptide (1r) was synthesised and deprotected using the same conditions as peptide 1q. The peptide was oxidised with 10 eq. 4-PDS on resin (XV), but two disulphides were formed.
This was suggested to be due to Trt being affected either by the TFA deprotection or by the 4-PDS oxidation.

### 2.5.3 Third orthogonal strategy

Since the conclusion from the experiments described above was that the Mmt and Trt protection groups could not successfully be used orthogonal to each other using the attempted deprotection conditions, a new strategy was proposed. In this strategy, S/Bu was reintroduced as first set of protection groups, followed by Mmt and finally Acm. This choice of protection groups would make three on-resin disulphide formations possible (peptide 1s-1v).

To overcome the previously observed reproducibility issues with mercaptoethanol deprotection of S/Bu a new bottle of mercaptoethanol was purchased. In addition, microwave heating was attempted to facilitate the deprotection (IX). Peptide 1s was successfully S/Bu deprotected using these conditions and oxidised using 10 eq. 4-PDS on resin (XV). Mmt was deprotected using 1% TFA without NMP washes and oxidised on resin (IV). Instead of formation of the second disulphide a product with an extra mass of 220 Da was observed, which indicated that both cysteines had reacted with 4-PDS and thereby disulphide formation was blocked.

It was proposed that the Cys17-33 disulphide was difficult to form on resin because of the close proximity of Cys33 with the resin. To overcome this, the Cys17-33 disulphide was formed first in peptide 1t using only 1 eq. of 4-PDS (XVI). This time the microwave heated deprotection did not fully deprotect S/Bu and consequently the deprotection was repeated twice. This lack of deprotection was probably due to the change in position of the protection groups.

The oxidation produced two disulphides, which was initially considered to be due to the many deprotections at elevated temperature affecting the Mmt protection groups. The synthesis was repeated (peptide 1u), the peptide was deprotected at room temperature (X) and oxidised using 0.5 eq. 4-PDS (XVII). The peptide was not fully S/Bu deprotected and once more two disulphides were formed. Therefore, it was considered that
the formation of two disulphides occurs in solution following test cleavage because of both S\textsubscript{Bu} deprotected cysteines being activated by 4-PDS. To investigate this, the disulphides were reduced with 1 eq. DTT in 10 NMP overnight which reduced the disulphides but did not affect the remaining S\textsubscript{Bu} protection groups. A small amount of resin was reacted with a small amount of iodoacetamide in 100:1 NMP:DIPEA for 10 min at 38°C and subsequently the peptide was test cleaved. In contrast to the previously performed maleimide reaction, alkylated cysteines were observed in this reaction. Since only two alkylated cysteines were observed it was concluded that the Mmt groups were unaffected and that the formation of the two disulphides must occur during the test cleavage when Mmt is deprotected. This also explained the formation of two disulphides in peptide 1\textsuperscript{r}, which were not because of Trt being deprotected as initially thought.

A new synthesis was made (peptide 1\textsuperscript{v}) and S\textsubscript{Bu} deprotected using mercaptoethanol at room temperature (X). The deprotected cysteines were oxidised using DMSO in NMP (XVIII). Mmt was deprotected using 1% TFA with shorter reaction time to reduce the possible cleavage from the resin (XI). Complete Mmt deprotection was confirmed by reaction with iodoacetamide, but the DMSO oxidation (XVIII) was not able to form the second disulphide. Prolonged DMSO oxidation and oxidation in neat DMSO was pursued, but it did not form the second disulphide. A small amount of bis-disulphide peptide was observed when the peptide was oxidised with less than 0.1 eq. of 4-PDS, but it is possible that the second disulphide is formed in solution following the test cleavage.

2.5.4 Fourth orthogonal strategy

Since it was considered to be too difficult to form the second disulphide on resin, and most likely even more difficult to form the third, the initial strategy was pursued once more after it had been shown to be possible to deprotect S\textsubscript{Bu} and form the first disulphide on resin.

Peptide 1\textsuperscript{w} was S\textsubscript{Bu} deprotected (X) and oxidised on resin (VII). The oxidation was done twice before the peptide was fully oxidised. The peptide was also successfully oxidised using 1 eq. 4-PDS. However, formation of two disulphides was observed in another attempt with 1 eq. 4-PDS. The DMSO oxidised mono-disulphide
peptide was cleaved from the resin and purified in 7% yield. Consequently, the yield was greatly increased compared to the mono-oxidised peptide 1o. Since no problems were observed in the second oxidation of peptide 1o using 10 eq. of 4-PDS (XIII) a test oxidation was made with peptide 1w using the same conditions. The second disulphide was formed already after two hours and consequently the remaining peptide was oxidised using these conditions (XIX). The yield of the second oxidation and purification was 69.2%. The final disulphide was formed by iodine oxidation (XX) and the peptide was purified. The yield of the final oxidation and purification was 11.4% and the overall yield of the orthogonal synthesis was 0.55%. An overview of the orthogonal oxidation is depicted in Figure 2.9 where the observed MW of the intermediary products is shown.

**Figure 2.9.** An overview of the orthogonal oxidation. The m/z peak of the quadruplet charged peptide is shown with the total intensity count from the mass spectrometer below. On top is shown the expected MW and the retention time on the HPLC-MS. The gradient was 5-95% acetonitrile containing 0.1% formic acid against 0.1% formic acid in water over 20 min. From left to right are the reduced peptide with two Acm protection groups and two StBu protection groups, the StBu deprotected peptide, the mono-oxidised peptide, the bis-oxidised peptide and the tris-oxidised peptide (1w). The error between the expected and observed MW in three cases was caused by an inaccuracy in the calibration of the mass spectrometer.
**2.5.5 Orthogonal synthesis of a non-native gurmarin disulphide isomer**

Since the thermolysin cleavage of the redox buffer oxidised gurmarin (peptide 1e) showed two possible disulphide connectivities, the possible non-native Cys3-17, Cys10–23, Cys18–33 gurmarin disulphide isomer (9) was synthesised using the orthogonal oxidation strategy. In general, the yields of the synthesis steps were comparable and the overall yield was 0.34%. However, an inseparable +109 Da by-product from the iodine oxidation was present in the final isolated gurmarin isomer.

**2.5.6 UPLC co-injection of gurmarin and gurmarin isomer**

To investigate whether the redox buffer oxidised gurmarin (peptide 1e) was identical with the orthogonally oxidised gurmarin (peptide 1w) or the gurmarin isomer 9, peptide 1e was co-injected on UPLC with the two orthogonally oxidised peptides (see Figure 2.10). One peak was observed when peptide 1e and 1w were co-injected, while two peaks were observed when peptide 1e and 9 were co-injected. This confirms the native disulphide connectivity of peptide 1e. The broader peak of the gurmarin isomer 9 was caused by the by-product described above.

![Figure 2.10](image-url) **Figure 2.10.** Co-injection of peptide 1e and 1w (top) and peptide 1e and the gurmarin disulphide isomer 9 (below). The gradient is 5-95% of acetonitrile containing 0.05% TFA against 0.05% TFA in water over 16 min. The retention times are indicated next to the peaks.
A paper describing the work with the random oxidation of gurmarin, thermolysin cleavage, orthogonal oxidation of gurmarin and the gurmarin isomer along with the confirmation by co-injection on UPLC was accepted for publication (see Appendix D).

2.6 Discussion

In this chapter, different synthesis strategies of gurmarin and work on structure confirmation were described.

In the first strategy pursued, gurmarin was oxidised using a partial orthogonal strategy. The first disulphide was formed selectively using Trt protection of the cysteine pair followed by air oxidation and the final two disulphides were oxidised concurrently by iodine oxidation of Acm protected cysteines. The three possible combinations of this strategy were pursued, but in all cases non-native gurmarin disulphide isomers were obtained as confirmed by thermolysin cleavage. In the combination where the Cys3-18 was formed selectively (peptide 1d), the other native disulphides; Cys10-23 and Cys17-33 were also observed. Consequently, a small amount of native gurmarin was synthesised with this strategy. It was not possible to isolate the native gurmarin from the synthesis though, and it appeared that the iodine oxidation of Acm-protected cysteines formed disulphides between cysteines that were in close proximity in the linear sequence of gurmarin. A similar observation has previously been observed in a random 4-PDS oxidation of an ICK peptide [58]. Therefore, the selective formation of the first disulphide did not induce the formation of native disulphides in the final oxidation.

In the second oxidation strategy, gurmarin was oxidised randomly using a redox buffer containing GSH and cystamine. In an optimised buffer, gurmarin (peptide 1e) was synthesised in an overall yield of 14.1%. The yield of the oxidation and purification was 57.1%. In two previously reported random oxidations of gurmarin the yields were 0.7% and 2.4% [9,41]. Consequently, the yield was significantly increased compared to these results. The major improvement compared to these results was the yield of the reduced gurmarin. This could partly be because of the reduction of aspartimide formation.
by addition of HOBT in the piperidine used for Fmoc-deprotection which was not mentioned in the two cases. It could also be caused by generally improved synthesis and purification yields. The oxidation buffer used in this strategy also appeared to be superior to the ones published, since both yield and progress of the oxidation was improved in this work.

The six cysteines in gurmarin can be connected in 15 possible disulphide combinations. To investigate the disulphide connectivity of peptide 1e, a partial cysteine reduction and cyanylation assay [63] was made. Unfortunately, it was difficult to separate the cyanylated peptides and the concentrations and amounts obtained were very low. This complicated the MS analysis and made the assay very laborious. Two native disulphides were found using this method, but one non-native disulphide was found as well making these results inconclusive. It is possible that lyophilisation of the peptide fractions could have improved the MS analysis. However, it would also have made the assay more laborious and would not affect the low purity and the amount of peptide in the fractions. Another possibility would have been to do the other assay described in chapter 1.2.2 [64] in which aminoethylation of reduced cysteines elegantly introduces trypsin cleavage sites in the peptide. The aminoethylation is preceded by a similar partial reduction and alkylation as done in this work though. Therefore, it is likely that similar complications with separation of the reduced and alkylated peptides would be observed in this assay as well.

Consequently, a different attempt was made to investigate the connectivity of the disulphides. A thermolysin cleavage of peptide 1e produced only two disulphide-containing fragments; one fragment with Cys10-23 (8) and one fragment with either the native Cys3-18, Cys17-33 connectivity (6) or the non-native Cys3-17, Cys18-33 connectivity (7). To investigate which of the fragments 6 or 7 that was obtained in the thermolysin cleavage of peptide 1e, the two fragments were synthesised. It was not possible to distinguish between these two fragments though and as a result thereof the thermolysin cleavage was not able to confirm the disulphide connectivity of peptide 1e. Nonetheless, it was possible to rule out 13 of the 15 possible disulphide combinations. The pH in the thermolysin cleavage was 6.5 and disulphide scrambling was therefore a possibility. However, if scrambling had occurred, it would be anticipated that multiple disulphide-
containing fragments had been observed. Since this was not the case, the occurrence of
disulphide scrambling in the thermolysin cleavage was ruled out.

A third synthesis strategy was attempted. This was a fully orthogonal
oxidation of gurmarin, which ensured that the native disulphide connectivity would be
obtained. A number of resins, protection groups, positions and order of protection groups,
deprotection methods and oxidation methods were pursued. Initially, StBu was deprotected
using mercaptoethanol and air oxidised on resin but the deprotection was not reproducible.
Subsequently, Mmt was used for protection of the first cysteine pair but it appeared that
the deprotection of Mmt also deprotected Trt, since a 4-PDS oxidation of the Mmt
deprotected peptide produced two disulphides. Later it was shown that on-resin oxidation
using 4-PDS in many cases formed two disulphides because both reduced cysteines were
activated by 4-PDS. After test cleavage of the peptide, the activated cysteines reacted with
cysteines that were Trt-deprotected during the TFA treatment used in the test cleavage.
This phenomenon was confirmed using iodoacetamide alkylation, but at the time of the
Mmt deprotections only unsuccessful cysteine alkylation with 3-maleimidopropionic acid
had been made.

Mercaptoethanol deprotection of StBu was reintroduced with a changed
batch, but there were still some problems with reproducibility of both deprotection and
oxidation. In some cases it was possible to oxidise on-resin using 4-PDS and in some cases
both cysteines reacted with 4-PDS even though the same conditions were used. It was
speculated that it could be due to use of different resin batches and possibly inaccurancy in
the determination of the loading of the resin. This was not tested due to lack of time
though. Using StBu, Mmt and Acm protection of the cysteines three on-resin disulphide
formations were attempted, but it was not possible to form the second disulphide using
DMSO or 4-PDS for oxidation. Finally, the orthogonal oxidation of gurmarin succeeded
with peptide 1w using StBu protection to form the first disulphide on resin, followed by
stepwise oxidation in solution of Trt and Acm protected cysteines. The overall yield of the
synthesis was 0.55% and consequently much lower that the random oxidation of peptide
1e. But whereas the random oxidation has 15 possible disulphide combinations, the
orthogonal oxidation strategy produces only the wanted disulphide combination. In
Discussion

another published orthogonal synthesis of an ICK peptide, the overall yield was not provided [58]. In general, the yields of the single steps appeared comparable though, but the synthesis route in this work has the advantage of on-resin disulphide formation and oxidation directly in fractions from preparative HPLC purification. This reduces the handling steps and makes this synthesis route less laborious. It could be possible to use Mmt instead of S/Bu and still form the first disulphide on resin using DMSO. Since the formation of two disulphides when Mmt protection was used in the first step was caused by 4-PDS and not because Trt protection groups were affected by the Mmt-deprotection the DMSO oxidation would most likely be similar in the two cases. This would have further reduced the laboriousness of the orthogonal oxidation of gurmarin.

The orthogonal synthesis route was used to synthesise the non-native gurmarin disulphide isomer (9) which the thermolysin cleavage indicated as a possible product of the random oxidation of peptide 1e. A co-injection on UPLC of peptide 9 and peptide 1e showed a separation of the two peptides, while only one peak was observed when peptide 1e and peptide 1w were co-injected. Previously, partial orthogonal synthesis strategies have been used to confirm disulphide connectivity of cystine knotted peptides [59,60]. However, this result is unique since a fully orthogonal synthetic approach is used to confirm the disulphide connectivity of a randomly redox buffer oxidised ICK peptide.

In the orthogonal synthesis of gurmarin another strategy could have been pursued. In a previously published report [57], a cystine knotted peptide was oxidised orthogonally using a one-pot strategy where the first cysteine pair was Trt-protected. This cysteine pair was deprotected concurrently with cleavage from the resin and oxidised in solution. The final two disulphide pairs are tert-butyl (tBu) protected and 4-methylbenzyl (4-MBzl) protected. These two groups are both deprotected and oxidised using 4% DMSO in TFA, tBu selectively first and 4-MBzl subsequently by heating the solution to 60°C. However, it has been reported that low recovery of Trp-residues are observed after this treatment [122] and since two Trp-residues are present in gurmarin this oxidation method are not likely to be applicable to the synthesis of gurmarin.

Other cysteine protection groups, deprotection conditions and oxidation conditions were considered for the orthogonal strategy but not used due to lack of time or
due to initial results indicating difficulties. Cysteines protected by phenylacetamidomethyl can be oxidised using similar conditions as for oxidation of Acm-protected cysteines and in addition they are also oxidised by the enzyme penicillin aminohydrolase [123]. TCEP was also tried for deprotection of S/Bu, but no deprotection was observed. Instead of using iodine for oxidation of Acm-protected cysteines thallium(III)trifluoroacetate could have been used [124]. The iodine oxidation appeared to produce side products that were not separable as was the case in the orthogonal oxidation of the gurmarin isomer (9). The thallium(III)trifluoroacetate oxidation has also been reported to modify unprotected Trp-residues [124] though, and consequently side products would also be expected for this reaction.

The three on-resin disulphide formation strategy did not succeed since it was not possible to form the second disulphide with DMSO or 4-PDS oxidation. However, only two different positions of the S/Bu and Mmt protection groups were tried. It is possible that by positioning one pair at the Cys3-18 residues the second on-resin oxidation could succeed. The possibility of forming the third disulphide on resin was not investigated. It is possible though, that the formation of the first two disulphides would position the final two cysteines in closer proximity, thereby inducing the formation of the last disulphide. However, it is also possible that protection groups of the other residues in the peptide makes the on-resin folding of the peptide impossible.

2.6.1 Outlook

The development of the stable redox oxidation strategy which provides high yields of gurmarin with the native ICK disulphide connectivity has the potential to be used both for the synthesis of grafted analogues of gurmarin, but also for other ICK peptides. In addition, the orthogonal synthesis strategy can be used for the synthesis of gurmarin, gurmarin analogues, other ICK peptides or gurmarin disulphide isomers. When combined with thermolysin cleavage, the orthogonal synthesis strategy can also be used to confirm disulphide connectivity of randomly oxidised ICK peptides.
2.7 EXPERIMENTAL PROCEDURES

2.7.1 Solid Phase Peptide Synthesis

Peptides were synthesised in 0.1 mmol scale on an Applied Biosystems (Carlsbad, CA) ABI 433A Peptide Synthesizer using standard protocols; piperidine:NMP 20:80 was used for Fmoc deprotection and 4 eq. of amino acid:2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU):HOBr, 8 eq. DIPEA in dimethylformamide (DMF):NMP 2:2.65 for coupling or a CEM Liberty Automated Microwave Peptide Synthesizer (Matthews, NC) using standard protocols; 0.05 M HOBr in 5:95 piperidine:NMP for Fmoc deprotection and 6 eq. of amino acid:Oxyma Pure:1,3-disopropylcarbodiimide (DIC) in NMP for coupling. Standard couplings were 5 min at 75°C. Gln was coupled twice while Cys and His residues were coupled twice at 50°C. Capping was done using acetic anhydride in NMP. Unless otherwise stated, the peptides were synthesised on a preloaded Gly-wang-PS LL resin. Gly-HMPB-Chemmatrix and Gly-Wang-Chemmatrix were purchased at PCAS BioMatrix (St-Jean-sur-Richelieu, Canada). Gly-Wang-TentaGel was purchased at Iris Biotech GmbH (Marktredwitz, Germany). Gly-Wang-PS LL resin, Gly-NovaSynTGT, Gly-2-CITrt-PS and Fmoc-amino acids were purchased from Novabiochem (Darmstadt, Germany) and side chain protection groups used were 7Bu (Asp, Glu, Tyr), Trt (Asn, Gln, His) and Boc (Lys, Trp). Cysteines were protected by Acm, Trt, S7Bu or Mmt. Boc-pyroglutamic acid was purchased from SigmaAldrich (St. Louis, MO).

2.7.2 Peptide cleavage and deprotection of cysteine protection groups

Peptides were cleaved from the resin using 20 ml 92:4:2:2 TFA:ethanedithiol:TIPS:water for 3 hours. Cleaved peptides were precipitated using 200 ml of diethyl ether and filtered. Trt protected cysteines were deprotected concurrently with peptide cleavage from the resin. S7Bu and Mmt protection groups were deprotected as outlined in Table 2.4.
2.7.3 Oxidation of cysteine residues

In the first oxidation strategy and the gurmarin fragment synthesis, Trt-deprotected cysteines were oxidised in solution by air oxidation in 0.1 M Tris-HCl pH 7.8, 2 vol% DMSO. Acm-protected cysteines were oxidised using 20 eq. of iodine in 1:4 water:methanol. The iodine oxidations were stopped by quenching the iodine with ascorbic acid. In both oxidations the peptide concentration was 0.02 mM and the peptide was oxidised for 48 hours. In the fragmentsynthesises the iodine oxidation was quenched after 1 hour.

In the second oxidation strategy, gurmarin was oxidised in solution using a redox buffer. In the first attempt, reduced gurmarin was oxidised for 48 hours in 0.01 mM concentration using 0.1 M Tris-HCl (pH 7.8), 1 mM EDTA, 2 mM GSH, 2 mM cystamine, 35 vol% DMSO, 6 vol% 2-dodecyloxyethanol. Secondly, a buffer optimisation was made using the conditions outlined in Table 2.3. Finally, gurmarin was oxidised for 48 hours in 0.02 mM concentrations using 0.1 M Tris-HCl (pH 7.8), 1 mM GSH, 1 mM cystamine.

In the orthogonal oxidation strategy, deprotected cysteines were oxidised either on resin or in solution as outlined in Table 2.5. Peptide concentrations are provided for the oxidations in solution.

2.7.4 Enzymatic cleavage

Peptide 1b was cleaved with chymotrypsin (SigmaAldrich) in 0.1 M Tris-HCl, 10 mM CaCl$_2$ (pH 7.8) 24 hours at room temperature. The peptide:enzyme ratio was 5:1 w/w, 0.1 mg peptide/ml. Peptides 1b-d were cleaved with thermolysin (SigmaAldrich) in 0.2 M ammonium acetate, 10 mM CaCl$_2$ (pH 6.5) 24 hours at 50°C. The peptide:enzyme ratio was 1:1 w/w, 0.1 mg peptide/ml. Peptide 1c and the gurmarin fragments (10-13) were cleaved with thermolysin using similar conditions, but the cleavage was quenched after 4 hours by adding 10 µl TFA to 200 µl of the cleavage solution.
2.7.5 Partial reduction and cyanylation assay

In the partial reduction and cyanylation assay peptide 1e was incubated with 10 or 30 eq. of TCEP (SigmaAldrich) for 15, 30, 60, 90 or 120 min. Subsequently, the reduced cysteines were cyanylated using 40 eq. of CDAP (SigmaAldrich) over the amount of TCEP for 30 to 180 min. Both reactions were made in 0.1 M sodium citrate buffer (pH 3) containing 6 M guanidinium chloride. The optimal conditions were found to be 30 eq. of TCEP for 30 min followed by 40 eq. (over TCEP) of CDAP for 30 min. The peptides were separated using 20-30% acetonitrile against 0.1% TFA in water over 30 min on a Waters Alliance analytical HPLC system using a Symmetry300, C18, 5 µm, 3.9 x 150 mm column (Waters Corporation, Milford, MA). Fractions were collected manually and analysed by MS. Fractions containing mono-disulphide fragments were cleaved by adding 40% methylamine in water containing 3 M guanidinium chloride. The peptides were cleaved for 60 min and analysed by MS.

2.7.6 Preparative HPLC purification

Peptides were purified on a Waters Delta Prep 4000 using a Waters XBridge Prep C18 OBD column, 5 µm, 30x150 mm. Mobile phases were 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). Peptides 1b-d were purified using 15-35% B over 40 min, 20 ml/min. Peptide 1e was purified using 22-32% B over 40 min, 20 ml/min. Peptide 1w was purified using 23-33% B and a ODDMS 120A, 5 µm, YMC 4x125 mm column (FeF Chemicals, Denmark), 5 ml/min.

2.7.7 Analytical equipment

The peptides were analysed using Waters Acquity UPLC systems. A sulphate buffered system using a BEH Shield RP18 1.7 µm, 2.1 x 150 mm column and a gradient of 20% A in 1 min followed by 20-60% A over 8 min (A: 70% acetonitrile, 30% water. B: 0.2M Na₂SO₄, 0.04M H₃PO₄, 10% acetonitrile in water, pH 3.5), flow: 0.35 ml/min was used for peptides 1b-d and for the aspartimide separation. A TFA system using a BEH130, 1.7 µm,
Synthesis of gurmarin

2.1 x 150 mm column and a gradient of 5-95% of 0.05% TFA in acetonitrile against 0.05% TFA in water over 16 min, flow: 0.4 ml/min was used for peptide 1e, 1w and the gurmarin isomer 9. For the evaluation of the random oxidation a BEH C18, 1.7 μm, 2.1 x 50 mm column and a gradient of 5% of 0.05% TFA in acetonitrile in 0.5 min followed by 5-60% of 0.05% TFA in acetonitrile against 0.05% TFA in water for 3.5 min was used. The MW of the peptides was determined using an Agilent 6230 TOF LC/MS (Agilent Technologies, Santa Clara, CA). Peptide concentration was determined by Chemiluminescent Nitrogen Detection using an Antek 8060 CLND HPLC detector (PAC, Houston, TX) [125]. The analytical data of the peptides are summarised in Table 2.7.

Table 2.7. Analytical data of the peptides. The numbers in brackets refers to the major product obtained using the synthesis procedure, as confirmed by enzymatic cleavage.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>UPLC system</th>
<th>Retention time</th>
<th>Purity</th>
<th>Expected MW&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Observed MW&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b (2)</td>
<td>Sulphate</td>
<td>5.4 min</td>
<td>99%</td>
<td>1052.5</td>
<td>1052.5</td>
</tr>
<tr>
<td>1c (3)</td>
<td>Sulphate</td>
<td>5.3 min</td>
<td>87%</td>
<td>1052.5</td>
<td>1052.5</td>
</tr>
<tr>
<td>1d (4)</td>
<td>Sulphate</td>
<td>5.7 min</td>
<td>100%</td>
<td>1052.5</td>
<td>1052.5</td>
</tr>
<tr>
<td>1e</td>
<td>TFA</td>
<td>5.8 min</td>
<td>97%</td>
<td>1052.5</td>
<td>1052.5</td>
</tr>
<tr>
<td>1w</td>
<td>TFA</td>
<td>5.7 min</td>
<td>77%</td>
<td>1052.5</td>
<td>1052.5</td>
</tr>
<tr>
<td>9</td>
<td>TFA</td>
<td>5.3 min</td>
<td>84%</td>
<td>1052.5</td>
<td>1052.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> the MW are the monoisotopic mass of the quadruple charged mass to charge spectrum.
3. **MELANOCORTIN ANALOGUES OF GURMARIN**

3.1 **INTRODUCTION**

In this chapter, the synthesis of melanocortin analogues of gurmarin will be described. The proposal of synthesising melanocortin analogues of gurmarin is based on the structural similarity between gurmarin and AgRP(87-120; C105A) as depicted in Figure 1.5. Since it has been shown that the melanocortin receptor antagonist AgRP(87-120; C105A) can be turned into an agonist by inserting the His-D-Phe-Arg-Trp pharmacophore instead of Arg-Phe-Phe in the active loop of AgRP(87-120; C105A) [96], a similar substitution was pursued in gurmarin in hope of obtaining MCR agonists (Figure 1.6).

The random redox buffer oxidation was used for the synthesis of these analogues, since the random oxidation in chapter 2 was shown to produce native gurmarin in high yield and purity. Therefore, it was anticipated that the disulphide knot of gurmarin would provide sufficient structural stability to retain the disulphide connectivity despite the amino acid substitutions. In chapter 1 it was described how several cases has shown that native ICK structures are obtained using random oxidation despite engineering of the peptides [31,33-36]. The binding of the analogues to the MC1R, MC3R, MC4R and MC5R were characterised by competition binding studies with radiolabeled NDP-α-MSH. The peptides were not evaluated for their binding to the MC2R, since ACTH is the only endogenous agonist able to bind to this receptor in contrast to the other MCRs [73]. An experimental section is found at the end of this chapter.

3.2 **DESIGN AND SYNTHESIS OF MELANOCORTIN ANALOGUES OF GURMARIN**

Six melanocortin analogues of gurmarin were designed and synthesised. The sequences of the analogues are listed in Table 3.1. The analogues 14 and 15 have the His-Phe-Arg-Trp pharmacophore (L-Phe and D-Phe respectively) inserted in position 27-30 in gurmarin. This position corresponds to the position of the melanocortin binding residues, Arg-Phe-Phe(-Asn) in AgRP(87-120; C105A) based on the structural alignment in Figure 1.6. Analogues
The analogues were synthesised similarly to peptide 1e as described in chapter 2.3 and oxidised using the optimised redox buffer containing 1 mM GSH and 1 mM cystamine to facilitate the folding of the peptides. The oxidation of the analogues is illustrated in Figure 3.1. Samples taken directly after addition of the redox buffer were shown by MS to be fully oxidised immediately. Multiple disulphide connectivities that are separable on UPLC were...
Figure 3.1. Oxidation of the melanocortin analogues 14-19. From left to right are the chromatograms of the reduced peptide, the peptide directly after addition of redox buffer and the peptide after 48 hours of oxidation. Retention times are indicated next to the major peaks. The gradient is 20% A in 1 min followed by 20-60% A against B over 8 min (A: 70% acetonitrile, 30% water. B: 0.2M Na₂SO₄, 0.04M H₃PO₄, 10% acetonitrile in water, pH 3.5).
present as illustrated in Figure 3.1. The retention times of the oxidised peptides are decreased compared to the reduced peptides as it was observed in the random oxidation of gurmarin (Figure 2.4).

### 3.2.1 Design and synthesis of tetra-disulphide analogues of gurmarin

Based on the structural alignment of gurmarin and AgRP(87-120; C105A) (Figure 1.5) it was attempted to design and synthesise gurmarin analogues containing a fourth disulphide surrounding the loop in which the melanocortin pharmacophore was grafted. The sequences of the synthesised analogues are shown in Table 3.2. The disulphide connectivity of the analogues are supposed to be Cys(1-4), Cys(2-5), Cys(3-8) and Cys(6-7) as in AgRP(87-120; C105A) with the cysteines numbered from the position from the N-terminal end. Consequently, the first three disulphides form the ICK, while the fourth disulphide is surrounding the grafting loop.

**Table 3.2.** Sequence alignment of gurmarin, AgRP(87-120; C105A) and the synthesised tetra-disulphide analogues 20 and 21. Amino acids not native to gurmarin are highlighted in bold. D-Phe is indicated by a lower case f. The disulphides in 20 and 21 are indicated with lines. The numbering on top refers to AgRP(87-120; C105A), while the numbering in the bottom refers to gurmarin and the analogues. The yields of reduced and oxidised peptide are shown. The yield of the oxidation is shown in brackets.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Structure</th>
<th>Yields (%)</th>
<th>Reduced</th>
<th>Oxidised</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgRP(87-120;C105A)</td>
<td>CVR LHESC LGQQV PCCDP AATCY CRFFN AFCYC R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gurmarin</td>
<td>*EQCVK KDELCP MYLYL DCCEP LECKR VNWWD HKCIG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>*EQCVK KDELCP MYLYL DCCEP LECYC VNWWD HKCYC IG</td>
<td>13.4</td>
<td>8.5 (63.5)</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>*EQCVK KDELCP MYLYL DCCEP LECYC HFRWN AFCYC IG</td>
<td>12.9</td>
<td>0.78 (6.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 6 11 16 21 26 31 36</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In analogue 20 two Cys-Tyr-Cys fragments present in AgRP(87-120; C105A) were inserted in gurmarin and the C-terminal Ile-Gly residues were preserved thereby
expanding the peptide with two residues. This analogue was designed to investigate the possibility of synthesising tetra-disulphide analogues of gurmarin. In analogue 21, the two Cys-Tyr-Cys fragments were inserted similarly to 20. In addition, the residues from the binding loop of the engineered agonist analogue of AgRP(87-120; C105A) [96] were inserted in hope of obtaining similar binding to the MCRs. Consequently, analogue 21 is a chimeric peptide containing parts from gurmarin, AgRP(87-120; C105A) and NDP-α-MSH.

The two tetra-disulphide analogues were synthesised and oxidised similarly to the other melanocortin analogues. In contrast to the oxidation of gurmarin and the first gurmarin analogues, the UPLC evaluation of the tetra-disulphide oxidation was more complicated. Because of the removal of Lys 24 and 25 in gurmarin the pI of the tetra-disulphide analogues was lower than gurmarin and the other analogues. The lower pI was considered to cause the peptides to precipitate when the oxidation was quenched with TFA in the samples before evaluation by UPLC. Unfortunately, this was not discovered until after the oxidation and consequently the progress of the oxidation was not monitored for the tetra-disulphide analogues. Nonetheless, as can be seen in Figure 3.2 one peak was observed on UPLC after purification of oxidised 20 and 21. This indicates that only one product is formed in the oxidation of the tetra-disulphide gurmarin analogues like in the previous random oxidations.

The synthesis of 20 was also attempted with Acm-protection of the cysteines forming the extra disulphide. In this synthesis route, it was intended to form the ICK in the redox buffer oxidation and subsequently form the fourth disulphide using iodine oxidation of the Acm-protected cysteines. It was not possible to isolate the random oxidised Acm-protected peptide though, and since only one product was formed in the random oxidation of all four disulphides, this alternative synthesis route was dropped.
Melanocortin analogues of gurmarin

Figure 3.2. UPLC chromatograms after purification of reduced (top) and oxidised (below) 20 (left) and 21 (right). Retention times are indicated next to the peaks. The gradient is 5-95% of 0.05% TFA in acetonitrile against 0.05% TFA in water over 16 min.

3.2.2 Thermolysin cleavage of tetra-disulphide analogue

To investigate the disulphide connectivity of the tetra-disulphide analogues a thermolysin digestion of 20 was made. Besides being native to AgRP the two inserted Cys-Tyr-Cys fragments provided extra thermolysin cleavage sites. Therefore, a fragment containing the extra inserted disulphide would be produced after thermolysin cleavage. The cleavage conditions were similar to those described in chapter 2. However, the cleavage was continued for 48 hours before the fragments were observed. The fragments obtained in the thermolysin cleavage are depicted in Figure 3.3.

The thermolysin cleavage indicated two possible disulphide combinations similarly to what was observed in the thermolysin cleavage of randomly oxidised gurmarin (1e). It was shown in chapter 2 that 1e in fact had the native disulphide connectivity. Due to that finding and based on the design of the peptide from the structural alignment, it was anticipated that the disulphide connectivity of 20 is the expected one as illustrated in Figure 3.3 and not the other possible connectivity. However, no final evidence was found of the disulphide connectivity of 20.
Figure 3.3. HPLC-MS analysis of the thermolysin cleavage of 20. The chromatogram and the mass spectra of the highlighted peaks are shown on top. In the table below the expected fragments from the cleavage are listed. The bis-disulphide fragment (23) could also have the non-native Cys3-17, Cys18-35 disulphide connectivity. The asterisk indicates an N-terminal pyroglutamic acid.
### Table 3.3. Binding affinity to the MCRs indicated by average pK<sub>i</sub> ± S.E.M. and number of assays performed in brackets. Transformed K<sub>i</sub> values are indicated in brackets below each pK<sub>i</sub> value.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MC1R pK&lt;sub&gt;i&lt;/sub&gt; (n) (K&lt;sub&gt;i&lt;/sub&gt;/[nM])</th>
<th>MC3R pK&lt;sub&gt;i&lt;/sub&gt; (n) (K&lt;sub&gt;i&lt;/sub&gt;/[nM])</th>
<th>MC4R pK&lt;sub&gt;i&lt;/sub&gt; (n) (K&lt;sub&gt;i&lt;/sub&gt;/[nM])</th>
<th>MC5R pK&lt;sub&gt;i&lt;/sub&gt; (n) (K&lt;sub&gt;i&lt;/sub&gt;/[nM])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gurmarin (1e)</td>
<td>n.a. (4)</td>
<td>n.a. (4)</td>
<td>5.38 ± 0.15 (3)</td>
<td>n.a. (4)</td>
</tr>
<tr>
<td>14</td>
<td>5.05 ± 0.12 (3)</td>
<td>n.a. (3)</td>
<td>6.14 ± 0.01 (2)</td>
<td>n.a. (3)</td>
</tr>
<tr>
<td></td>
<td>(8,800)</td>
<td></td>
<td>(720)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>5.66 ± 0.11 (2)</td>
<td>n.a. (2)</td>
<td>5.47 ± 0.12 (2)</td>
<td>n.a. (2)</td>
</tr>
<tr>
<td></td>
<td>(2,200)</td>
<td></td>
<td>(3,400)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>n.a. (3)</td>
<td>n.a. (3)</td>
<td>5.54 ± 0.18 (3)</td>
<td>n.a. (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2,900)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>6.48 ± 0.02 (3)</td>
<td>5.41 ± 0.07 (3)</td>
<td>6.06 ± 0.26 (3)</td>
<td>5.20 ± 0.05 (3)</td>
</tr>
<tr>
<td></td>
<td>(330)</td>
<td>(3900)</td>
<td>(880)</td>
<td>(6,300)</td>
</tr>
<tr>
<td>18</td>
<td>n.a. (3)</td>
<td>n.a. (3)</td>
<td>5.34 ± 0.01 (2)</td>
<td>n.a. (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(4,600)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>n.a. (3)</td>
<td>n.a. (3)</td>
<td>5.72 ± 0.19 (4)</td>
<td>n.a. (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1,900)</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>-</td>
<td>-</td>
<td>6.30 ± 0.21 (4)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(500)</td>
<td></td>
</tr>
<tr>
<td>α-MSH</td>
<td>9.16 ± 0.05 (3)</td>
<td>7.51 ± 0.04 (4)</td>
<td>7.41 ± 0.26 (4)</td>
<td>6.75 ± 0.16 (3)</td>
</tr>
<tr>
<td></td>
<td>(0.69)</td>
<td>(31)</td>
<td>(39)</td>
<td>(180)</td>
</tr>
</tbody>
</table>

n.a. no activity; indicates that the binding affinity was above 10,000 nM. A dash indicates that no experiments were performed.

### 3.3 Melanocortin receptor binding assays

The six melanocortin analogues of gurmarin (14-19) along with the tetra-disulphide melanocortin analogue of gurmarin (21), gurmarin (1e) and α-MSH as a reference were characterised for their ability to bind to the MC1R, MC3R, MC4R and MC5R. A summary
of the binding data is presented in Table 3.3. Gurmarin was not able to bind to the MC1R, MC3R and MC5R but it bound with low affinity to the MC4R. All synthesised analogues were able to bind to the MC4R. The most potent was the tetra-disulphide analogue (21) that had a binding affinity of 500 nM. This is around ten-fold lower affinity than the endogenous agonist α-MSH, which has low nM binding affinity for the four MCRs evaluated. Since 21 was synthesised at a late stage in the project, its binding to the other MCRs was not determined. Analogue 14 was the second most potent analogue in the binding assays with a binding affinity of 720 nM. It was more than ten-fold selective for the MC4R over the MC1R and did not bind to the MC3R and MC5R. Analogue 17 was the only analogue that bound to all the MCRs and also the only that bound to the MC3R and MC5R.

Figure 3.4. An average of normalised binding curves from the most potent analogues on the MCRs. NDP-α-MSH is included as reference (black line). Gurmarin (1e) (red line), 14 (blue line), 15 (purple line), 17 (green line), 19 (orange line).
In Figure 3.4 is shown a summary of the curves from the binding assays of the most potent peptides on each receptor.

3.4 Discussion

In this chapter, the design, synthesis and functional characterisation of melanocortin analogues of gurmarin were described.

The analogues were designed based on structural alignment of AgRP(87-120; C105A) and gurmarin, and oxidised using the random redox buffer conditions optimised for the synthesis of gurmarin as described in chapter 2. The disulphide connectivity was not determined for the analogues, but in all analogue oxidations one peak was observed after 48 hours of oxidation (Figure 3.1). This indicates the formation of the thermodynamically most favourable conformation, which in the case of gurmarin was shown in chapter 2 to have the native ICK disulphide connectivity. Similarly to the oxidation of gurmarin, all peptide is oxidised immediately after addition of the redox buffer. The UPLC analysis of the oxidation indicates that it is possible to separate the multiple differently disulphide connected species. Because of only one peak being observed and because of the separability of the differently disulphide connected peptides, it was anticipated that the ICK was produced in the oxidation of the melanocortin analogues.

Two tetra-disulphide analogues of gurmarin were designed and synthesised by insertion of two Cys-Tyr-Cys sequences from AgRP(87-120; C105A) in gurmarin based on the structural alignment of the two peptides. In the first analogue 20, only these two sequences were inserted, while the entire active loop from an engineered agonist analogue of AgRP(87-120; C105A) [96] was inserted into the second analogue 21. In both peptides, one peak was observed on UPLC after preparative HPLC indicating the formation of one major product as it was the case with the first synthesised melanocortin analogues. To investigate the disulphide connectivity of 20 the peptide was cleaved with thermolysin similarly to the cleavage of random oxidised gurmarin (1e) described in chapter 2. The cleavage had to be continued for 48 hours before fragments of 20 were observed, which indicates that the tetra-disulphide analogues have higher proteolytic stability than gurmarin.
All disulphide containing fragments observed was expected to be found based on the disulphide connectivity in the design of the peptide. This indicated that the expected tetra-disulphide peptide in fact was formed in the redox buffer oxidation. Nonetheless, it is possible that the bis-disulphide fragment (23) observed could have the non-native Cys3-17, Cys18-35 disulphide connectivity. A similar fragment was observed in the thermolysin cleavage of redox buffer oxidised gurmarin (1e) as shown in chapter 2. Since it was proven that the native disulphide connectivity was produced in the gurmarin oxidation, it was assumed that this was also the case for 20, despite no complete evidence was established.

The yields of the melanocortin analogues of gurmarin were lower than in the synthesis of gurmarin. This is expected to be caused by the synthesis of the reduced gurmarin being optimised more compared to the synthesis of the analogues. In addition, the amino acid substitutions made in the analogues could affect the yield of the oxidation negatively. In general, the yields of the analogues were comparable. In two cases, 15 and 20 the oxidation yields were very high. In contrast, the oxidation yield of 21 was very low. No explanation was found to this observation, since everything was done similarly and nothing unusual was observed during the oxidation or purification. Only, the complications when evaluating the oxidation of the tetra-disulphide analogues by UPLC were observed. However, since one of these oxidations gave a high yield, while the other gave a low yield, this is not likely to have caused the observed difference in the oxidation.

The analogues 14-19, 21 and gurmarin (1e) were characterised for their binding to the MCRs. Gurmarin were able to bind with low affinity to the MC4R. This is surprising, since gurmarin does not contain the melanocortin pharmacophore. All melanocortin analogues of gurmarin were also able to bind to the MC4R, however no peptide had had similar binding affinity as the endogenous agonist α-MSH. The tetra-disulphide analogue 21 had the highest affinity with a $K_i$ of 500 nM, but was not evaluated at the other MCRs. Since the entire binding loop was replaced with the corresponding loop from the engineered AgRP agonist that had low nM activity on the MC4R [96], a higher binding affinity was expected for 21. It is possible though, that 21 would be more potent for activation of the MC4R. Only EC$_{50}$ values describing activation of the MCRs were determined for the reported engineered AgRP agonist, while only $K_i$ values describing
Melanocortin analogues of gurmarin

binding to the MCRs were determined for the analogues synthesised in this work. Analogue 14 had the second highest affinity for the MC4R, a $K_i$ of 720 nM and was more than ten-fold selective for the MC4R over the MC1R. The binding to the MC3R and MC5R could not be determined since they were higher than 10,000 nM. Analogue 17 was the only analogue that was able to bind to all MCRs. It bound with around three-fold higher affinity to the MC1R than the MC4R and around ten- and twenty-fold higher affinity to the MC1R compared to the MC3R and MC5R respectively. Analogue 15, one of the D-Phe analogues like 17 bound with slightly higher affinity to the MC1R than the MC4R. Finally, analogues 16, 18 and 19 only bound to the MC4R and 18 was the only analogue that did not show improved binding to the MC4R compared to gurmarin.

The binding experiments of the melanocortin analogues confirmed the hypothesis that it was possible to synthesise analogues of gurmarin which bound to the MCRs. The difference observed in binding affinities of these highly similar analogues are affected by four factors; the position of the pharmacophore in the loop, the size of the loop, the residues adjacent to the pharmacophore and whether the phenylalanine residue in the pharmacophore is in D or L conformation.

In the least potent analogues 18 and 19, the pharmacophore was shifted one position towards the C-terminal. Consequently, this seems to be a less favourable position in the loop, but it could also be due to different residues being adjacent to the pharmacophore. In 18 and 19 Asn and Lys are adjacent, in 14 and 15 Val and His are adjacent, in 16 and 17 Val and Asp are adjacent and in 21 Cys and Asn are adjacent. As shown in Figure 1.4b, the pharmacophore of the three MSH peptides are surrounded by either Glu or Gly on the N-terminal and Gly or Asp on the C-terminal. A comparison with the natural melanocortin agonists thereby indicates that the positively charged Lys residue on the C-terminal side of the pharmacophore in 18 and 19 could affect the binding to the MCRs negatively. A Lys residue is also present on the C-terminal side of the subnanomolar agonist MT-II [92] though. However, in MT-II the positive charge in the side chain of the Lys residue is removed by an amide bond formation with an Asp side chain on the N-terminal side of the pharmacophore. On the other hand 17, the only analogue with activity on all MCRs, has an Asp residue on the C-terminal side of the pharmacophore like γ-MSH.
It is possible that this improves the binding compared to the other analogues, but it is not known to what extent. In another report, several analogues were made with both positively charged, negatively charged, hydrophobic and short side chains on the N-terminal side of the pharmacophore [126]. These changes only affected the binding to the MC5R, which indicates that the residue on the N-terminal side of the pharmacophore only has a minor effect on MCR binding.

It has previously been shown that loop sizes of cyclised melanocortin analogues can affect MCR selectivity. In one example, a peptide was reversed from an agonist to an antagonist by increasing the ring one carbon atom [127]. In another example, various modifications of ring sizes affected MCR affinity and selectivity [126]. This means that the increased loop in 16 and 17 might have a significant effect on both binding and selectivity.

Finally, the higher binding affinity for the MC1R for the D-Phe analogues 15 and 17 compared with their respective L-Phe analogues 14 and 16 indicates that the D-Phe substitution has highest effect on the MC1R. On the MC4R, the d-Phe analogues 17 and 19 has higher binding affinity than their respective L-Phe analogues 16 and 18 while the opposite is the case for 14 and 15 where the L-Phe analogue 14 has the highest affinity. In previously reported studies it appears the D-Phe substitutions affect the binding to the MC1R to a lower extent than the other MCRs in contrast to what was observed in this work [128-130]. However, in most studies found on the development of melanocortin analogues only D-Phe analogues were evaluated. This means that it is hard to make a general conclusion on how the D-Phe substitution affects MCR selectivity. In addition, this novel incorporation of the melanocortin pharmacophore in an ICK framework could very well behave differently compared to usual short linear or side chain cyclised melanocortin analogues.

3.4.1 Outlook

The work described in this chapter demonstrated the possibility of turning unrelated peptides into melanocortin receptor analogues. Since only binding assays on the MCRs
were performed and no functional assays, it is not known whether the synthesised peptides are agonists, partial agonists or antagonists. It would have been interesting to elucidate this, but the time and resources did not allow for this. In addition, further investigation of which position in the loop that favours binding to the MCRs most could have been made. For example by synthesising analogues with the pharmacophore at various positions, but surrounded by identical residues. This could be two Gly residues since these are native to the MSH peptides and their lack of side chains would rule out the possible effect of side chain interactions from the residues adjacent to the pharmacophore. In general, additional engineering of the structures could have produced even more potent and selective compounds.

Interestingly, it was possible to design and synthesise tetra-disulphide analogues of gurmarin based on structural alignment with AgRP(87-120; C105A), which appeared to have the expected disulphide connectivity. These analogues could provide the basis for a novel approach in the design of melanocortin analogues for obesity treatment. Other engineering work on ICK peptides and cyclotides has indicated the possibility for oral bioavailability [29,35,131]. Additional exploration of this opportunity for the peptides synthesised in this work should be investigated before anything can be concluded. Nonetheless, oral treatment of obesity is a highly valuable concept with the emerging worldwide prevalence of obesity [132] and the current deficiency in treatment options [133].

3.5 EXPERIMENTAL PROCEDURES

3.5.1 Synthesis of melanocortin analogues of gurmarin

The peptides were synthesised on a CEM Liberty as described for the synthesis of gurmarin in chapter 2.7 using similar side chain protection groups. Arginine was protected with 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf). All cysteines were Trt protected, except for Cys25 and Cys33 which were Acm protected only in the alternative synthesis attempt of 20. The peptides were oxidised using the redox buffer described in chapter 2.7. The peptide peptides were purified using the preparative HPLC system.
described in chapter 2.7. 14 was purified using 10-30% B, 15 using 8-28% B, 16-19 using 20-30% B, 20 using 27-37% B and 21 using 23-43% B. The analytical data of the melanocortin analogues are summarised in Table 3.4. The analytical equipment used were the same as described in chapter 2.7.

Table 3.4. Analytical data of the melanocortin analogues of gurmarin.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>UPLC system</th>
<th>Retention time</th>
<th>Purity</th>
<th>Expected MWa</th>
<th>Observed MWa</th>
</tr>
</thead>
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<tr>
<td>14</td>
<td>TFA</td>
<td>4.7 min</td>
<td>81%</td>
<td>1058.7</td>
<td>1058.8</td>
</tr>
<tr>
<td>15</td>
<td>TFA</td>
<td>4.7 min</td>
<td>89%</td>
<td>1058.7</td>
<td>1058.8</td>
</tr>
<tr>
<td>16</td>
<td>TFA</td>
<td>5.1 min</td>
<td>83%</td>
<td>1087.5</td>
<td>1087.5</td>
</tr>
<tr>
<td>17</td>
<td>TFA</td>
<td>5.0 min</td>
<td>91%</td>
<td>1087.5</td>
<td>1087.5</td>
</tr>
<tr>
<td>18</td>
<td>TFA</td>
<td>4.9 min</td>
<td>83%</td>
<td>1053.0</td>
<td>1053.0</td>
</tr>
<tr>
<td>19</td>
<td>TFA</td>
<td>4.7 min</td>
<td>83%</td>
<td>1053.0</td>
<td>1053.0</td>
</tr>
<tr>
<td>20</td>
<td>TFA</td>
<td>5.8 min</td>
<td>93%</td>
<td>1121.0</td>
<td>1121.0</td>
</tr>
<tr>
<td>21</td>
<td>TFA</td>
<td>6.3 min</td>
<td>99%</td>
<td>1119.2</td>
<td>1119.2</td>
</tr>
</tbody>
</table>

a the MW are the monoisotopic mass of the quadruple charged mass to charge spectrum.

3.5.2 Enzymatic cleavage

Peptide 20 was cleaved with thermolysin using the same conditions as described in chapter 2.7. The cleavage was continued for 48 hours.

3.5.3 Melanocortin receptor assays

The binding assays for the MCRs were performed by Novo Nordisk laboratory technicians as described previously [134] and each binding assay was performed in duplicate. Baby Hamster Kidney cells stably expressing the different MCRs were generated by transfection with an expression vector. The synthesised peptides were evaluated by incubation with homogenised membranes expressing the MCRs and a radiolabeled $^{125}$I-NDP-$\alpha$-MSH (Perkin-Elmer, Waltham, MA) using a filtration system to separate bound radioligand from unbound. The peptides were initially dissolved in DMSO to a concentration of 4 mM and the peptides were evaluated in a range from 10 pM to 10 µM.
MC1R assay: The assay was performed in a total volume of 250 µL with 100 pM $^{125}$I-NDP-α-MSH, test peptide and 200 µL cell membrane (35 µg/mL) added. The compounds were diluted in 25 mM HEPES pH 7.4, 0.1 mM CaCl$_2$, 1 mM MgSO$_4$, 1 mM EDTA, 0.1% ovalbumin, 0.005% Tween-20, 5% hydroxypropyl-β-cyclodextrin buffer. The samples were incubated in a microtiter plate for 90 min at 30°C. The incubation was stopped by filtration of the plate through a polyethyleneimine treated filter plate using a Packard Filtermate harvester (Packard Instrument Company, Meriden, CT). After filtration, the filters were washed 10 times with ice cold 0.9% NaCl and dried at 50°C for 30 min. Finally, 30 µL of Microscint 0 (Packard Instrument Company, Meriden, CT) were added to each well and the plate was counted (1 min/well) in a Topcounter (Packard Instrument Company, Meriden, CT).

MC3R and MC5R assays: The assays were performed in a total volume of 100 µL with 250 pM $^{125}$I-NDP-α-MSH, test peptide and 30 µL cell membrane (30 µg/mL) added. The compounds were diluted in 25 mM HEPES pH 7.4, 1 mM CaCl$_2$, 5 mM MgSO$_4$, 0.1% ovalbumin, 0.005% Tween-20, 5% hydroxypropyl-β-cyclodextrin buffer. The samples were incubated in a microtiter plate for 60 min at room temperature. The remaining procedure was performed similarly to the MC1R assay.

MC4R assay: The assay was performed in a total volume of 200 µL with 79 pM $^{125}$I-NDP-α-MSH, test peptide and 50 µL cell membrane (150 µg/mL) added. The compounds were diluted in 25 mM HEPES pH 7.0, 1 mM CaCl$_2$, 1 mM MgSO$_4$, 1 mM ethylene glycol tetraacetic acid, 0.02% bacitracin, 0.1% ovalbumin, 0.005% Tween-20, 5% hydroxypropyl-β-cyclodextrin buffer. The samples were incubated in a filter plate for 120 min at room temperature. The incubation was stopped by filtration of the plate using a Millipore vacuum manifold (Merck Millipore, Billerica, MA). After filtration, the filter was washed with 100 µL ice cold 0.9% NaCl and air dried. Radioactivity retained on the filter was counted using a Cobra II auto γ-counter (Packard Instrument Company, Meriden, CT).

The data analyses from the receptor assays were performed using the program GraphPad Prism, GraphPad Software, USA. pIC$_{50}$ values were calculated using a nonlinear regression model and pK$_i$ values were calculated from the pIC$_{50}$ values using the Cheng–Prusoff equation $K_i = IC_{50}/[1 - L/K_d]$, where L is the concentration of the
Experimental procedures

radioligand, K_d is the dissociation constant of NDP-α-MSH calculated from saturation analysis and IC_{50} values are the concentration of test peptide when 50% of the radioligand is displaced.

To determine K_d of the radioligand for the MCRs, a saturation analysis was performed. For the MC1R, 10 concentrations of ^{125}I-NDP-α-MSH in the range of 0.1 pM to 100 pM were used, for the MC3R and the MC5R, 12 concentrations of ^{125}I-NDP-α-MSH in the range of 12.5 pM to 5 nM were used, and for the MC4R 11 concentrations of ^{125}I-NDP-α-MSH in the range of 2.6 pM to 800 pM were used. Nonspecific binding was determined in the presence of 10 μM ^{125}I-NDP-α-MSH. K_d: MC1R 45 ± 12 pM (n = 3), MC3R 1.46 ± 0.34 nM (n = 3), MC4R 0.34 ± 0.13 nM (n = 5), and MC5R 0.96 ± 0.23 nM (n = 4). K_d values were calculated using the one site binding model (hyperbola) in GraphPad Prism.
4. MELANOCORTIN ANALOGUES OF KALATA B1

4.1 INTRODUCTION

This chapter will briefly introduce and discuss work that was conducted during a four month stay at Prof. David Craik’s lab at the Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia during this Ph.D. project.

The project proposal was to design and synthesise melanocortin analogues of the cyclotide kalata B1 by grafting the melanocortin pharmacophore into one of the inter-cysteine loops. The analogues were characterised for their binding to the MC1R, MC3R, MC4R and MC5R and the analogue that bound with highest affinity to the MC4R was also evaluated for its ability to activate the MC4R. In addition, the ability to obtain the native kalata B1 cyclotide fold were investigated using NMR spectroscopy and a NMR derived structure of the most potent analogues were calculated.

A paper describing the work and results from the stay was submitted and can be found in Appendix E. Since all work and results are presented in the appendix, only the most important findings are repeated in this chapter. These will be discussed compared with the results obtained for the melanocortin analogues of gurmarin.

4.2 DISCUSSION

In contrast to the most MC4R potent melanocortin analogue of gurmarin (21), the most potent melanocortin analogue of kalata B1, kB1[GHFRWG;23-28] (25) bound with higher affinity to the MC4R than α-MSH. As the name of the peptide indicates, the melanocortin pharmacophore is surrounded by two Gly residues as discussed in chapter 3. A Gly residue is found adjacent to the N-terminal of the pharmacophore in γ-MSH, while Gly residues are found adjacent to the C-terminal of the pharmacophore in α-MSH and β-MSH. This could be one explanation to the improved binding observed in kB1[GHWRWG;23-28] compared to the gurmarin analogues. Similarly to the observation of the gurmarin analogues, a D-Phe substitution in the pharmacophore of kB1[GHFRWG;23-28] had most
effect on the MC1R, and decreased the binding around ten-fold at the MC4R. In addition, this analogue was the only analogue that bound to all four MCRs evaluated similarly to 17.

The most potent analogue in binding to the MC4R, kB1[GHFRWG;23-28] (25) was evaluated for its ability to activate the MC4R. Despite being more potent than α-MSH in binding to the MC4R, it was more than ten-fold less potent in activating the MC4R. The D-Phe analogue of kB1[GHFRWG;23-28] had similar binding affinity to the MC4R as the tetra-disulphide gurmarin analogue 21. As discussed in chapter 3, the binding affinity of 21 to the MC4R was lower than expected compared to the engineered AgRP agonist [96]. It was suggested though, that the D-Phe substitution could make analogue 21 more potent at activating the MC4R as observed previously in the discovery of NDP-α-MSH [90]. Similarly, it is possible that the D-Phe analogue of kB1[GHWRWG;23-28] is more potent at activating the MC4R compared to kB1[GHFRWG;23-28] despite its lower binding affinity for the MC4R.

4.2.1 Outlook

The melanocortin grafting work in kalata B1 confirms the potential of using cyclotides as molecular scaffolds in which pharmaceutically interesting sequences can be grafted. The analogues were confirmed to be highly resistant to chymotrypsin digestion, highlighting the potential of engineered cyclotides as orally available drugs as was recently demonstrated [35]. Similar to the gurmarin analogues, further structure-activity work is necessary to improve the functionality of these analogues and especially the D-Phe/L-Phe question needs to be elucidated in more detail. Nonetheless, it has been demonstrated that it is possible to design and synthesise active melanocortin agonist analogues based on these highly stable disulphide frameworks. These analogues provide a novel approach for the development of peptide drugs targeting obesity treatment.
5. **CONCLUSIONS AND FUTURE PERSPECTIVES**

In this PhD thesis the design, synthesis and functional characterisation of gurmarin and gurmarin analogues has been described. It was showed to be possible to synthesise grafted melanocortin analogues of gurmarin that were able to bind to the MCRs. The highest binding affinities were found at the MC4R which is responsible for regulation of appetite and energy expenditure. Consequently, these gurmarin analogues introduce a novel group of compounds with the possibility for treatment of obesity. Due to the stability of ICK peptides like gurmarin, they have been shown to have possibility for oral delivery. The analogues synthesised in this work need further optimisation, but the possibility for an oral treatment are a very promising feature of these peptides.

In addition, melanocortin analogues of the cyclotide kalata B1 were synthesised. Similarly to the gurmarin analogues, they were able to bind to the MCRs and one analogue bound with higher affinity to the mC4R than the endogenous agonist α-MSH. In addition, this analogue was shown to activate the MC4R, although it was not as potent as α-MSH. The kalata B1 analogue was shown to be almost completely stable towards chymotrypsin cleavage highlighting the potential for oral bioavailability for the ICK peptides and cyclotides. Similarly to the melanocortin analogues of gurmarin, the kalata B1 analogues need further optimisation in order to achieve more MC4R potent peptides.

Besides the synthesised analogues in this work an improved redox buffer oxidation strategy of gurmarin was developed. This strategy produced gurmarin in high yields and will most likely be applicable to other ICK peptides as well. In addition, an orthogonal oxidation strategy was established to control the disulphide connectivity of gurmarin. This procedure was used to synthesise both gurmarin and a gurmarin disulphide isomer, and in combination with thermolysin cleavage of redox buffer oxidised gurmarin it was used to confirm the disulphide connectivity of this randomly oxidised gurmarin. This principle could also be used for the synthesis of other ICK peptides or disulphide isomers.
6. **REFERENCE LIST**


Reference list


**APPENDIX A: TABLE OF COMPOUNDS**

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<thead>
<tr>
<th>Peptide</th>
<th>Structure</th>
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<tr>
<td>1</td>
<td>*EQCVK KDEL C IPYYL DCCEP LECKK VNW WD HKCIG</td>
</tr>
<tr>
<td>2</td>
<td>*EQCVK KDEL C IPYYL DCCEP LECKK VNW WD HKCIG</td>
</tr>
<tr>
<td>3</td>
<td>*EQCVK KDEL C IPYYL DCCEP LECKK VNW WD HKCIG</td>
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<td>8</td>
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<td>CGETC VGGT C TNPCC TCSWF VCQGF RWGV</td>
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*E* is pyroglutamic acid. Disulphides are indicated with lines. In compound 25, the cyclic backbone is indicated with lines below the amino acid sequence.
APPENDIX B: CONFERENCE POSTER

Poster presented at the 31st European Peptide Symposium, Copenhagen, 5-9 September 2010.
Background

- Gurmarin is a 35-residue peptide from the plant Gymnema sylvestre. It functions as a sweet taste inhibitor in rodents (Ninomiya et al., Am J Physiol-Reg I, 1995; 268(4):R1019). The structure of Gurmarin (Figure 1) is defined by three disulfides in a common fold known as a knotted cysteine knot.

Aims

- To do three syntheses of Gurmarin by partial orthogonal protection group routes differing in the position of protection groups, and confirm the disulfide connections of the synthesized peptides by enzymatic cleavage (Figure 2).

Methods

- The peptides were synthesized by standard Fmoc chemistry on a CEM Liberty peptide synthesizer. HOBt was added to the piperidine deprotection to minimize aspartimide formation.

  - The first disulfide was oxidized in 0.1M Tris-HCl buffer (pH 7.8, 0.02mM peptide), 2 vol% DMSO with air bubbled through until complete oxidation was confirmed by UPLC.

  - The final two disulfides were oxidized with 20 eq. iodine in 1:4 water:methanol (0.02 mM peptide) until complete oxidation was confirmed by MS.

  - The peptides were cleaved with 1 mass eq. chymotrypsin in 0.1M TrisHCl (pH 7.8), 10 mM CaCl2 for 24h at RT or with 1 mass eq. thermolysin in 0.2M ammonium acetate (pH 6.5), 10 mM CaCl2 for 24h at 50°C.

Results

- The synthesis of the peptide with trityl protected cysteines 3 and 18 produced a mixture of two products. One of these was identified as the native peptide by the fragment in Figure 3 and another fragment containing two native disulfides (second fragment, Table 1). In this fragment one disulfide was oxidized selectively in the air oxidation of the trityl protected cysteines and consequently all three disulfides in this peptide are native.

- In all three peptides non-native disulfides were produced in the iodine oxidation as the main product. Disulfide formation between cysteines that are close in the primary sequence are not favoured.

Conclusions

- The position of the protection groups are critical for the outcome of the disulfide oxidation.
- The synthesis of native Gurmarin is possible with this synthesis route.
APPENDIX C: PROCEEDINGS PAPER

Proceedings paper from the 31st European Peptide Symposium, Copenhagen, 5-9 September 2010.

Synthesis and Structure Confirmation of the Cysteine Knotted Peptide Gurmarin by Selective Disulphide Formation

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Introduction
Gurmarin is a 35-residue peptide from the plant Gymnema sylvestre [1]. The peptide has been shown to function as a sweet taste inhibitor in mice [2]. The structure of Gurmarin (Figure 2) is defined by three disulphides in a common fold known as an inhibitor cysteine knot. Folding of such peptides is often achieved by equilibrium driven folding in a redox buffer [2]. In this study, the synthesis of Gurmarin was carried out in three different ways, where one of the three cysteine-pairs was trityl-protected, while the other two pairs were acetyldimethyl-protected. This allowed for selective formation of the first disulphide by air oxidation, followed by iodine oxidation of the last two disulphides. The structures of the peptides were confirmed by cleavage with thermolysin. In this way it was possible to determine the disulphide connection.

Results and Discussion
The peptides were synthesized by standard Fmoc chemistry on a CEM Liberty peptide synthesizer. HOBr was added to the piperidine deprotection to minimize aspartimide formation. The first disulphide was oxidized in 0.1 M Tris-HCl buffer (pH 7.8, 0.02mM peptide), 2 vol% DMSO with air bubbled through until complete oxidation was confirmed by UPLC. The final two disulphides were oxidized using 20 eq. iodine in 1:4 water:methanol (0.02 mM peptide) until complete oxidation was confirmed by MS. The peptides were cleaved with 1 mass eq. thermolysin in 0.2 M ammonium acetate (pH 6.5), 10 mM CaCl₂ 24h at 50°C.

The synthesis of the peptide with trityl protected Cys-3 and Cys-18 produced a mixture of two products. One of these was identified as the native peptide by the fragment in Figure 1 and another fragment containing two native disulphides (second fragment, Table 1). In the latter fragment the disulphide between Cys-3 and Cys-18 was oxidized selectively first in the air oxidation of the trityl protected cysteines and consequently all three disulphides in this peptide are native.

The cleavage of the peptide synthesized with trityl protected Cys-10 and Cys-23 produced one fragment with the native disulphide between Cys-10 and Cys-23, as expected. However no other fragments containing native disulphides were found from this cleavage.

Fig. 1. Sequence and mass spectrum of a native disulphide fragment obtained by cleavage with thermolysin.

Fig. 2. The structure (PDB: 1C4E) of Gurmarin (left) and the primary sequence (right).
Table 1. Fragments obtained from cleavage with thermolysin of the three differently synthesized peptides. The three peptides are identified by the trityl protected cysteines in the first column. <E is pyroglutamate. Disulphides are indicated with lines.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Fragment</th>
<th>Theoretical mass</th>
<th>Observed mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3-C18</td>
<td>LCIP LECKK</td>
<td>531.78 (M+2H⁺)</td>
<td>531.79</td>
</tr>
<tr>
<td>Trityl</td>
<td>&lt;EQC LDCEP</td>
<td>861.80 (M+2H⁺)</td>
<td>861.91</td>
</tr>
<tr>
<td>protected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C10-C23</td>
<td>LCIP LECKK</td>
<td>531.78 (M+2H⁺)</td>
<td>531.79</td>
</tr>
<tr>
<td>Trityl</td>
<td>LDCEP</td>
<td>677.22 (M+1H⁺)</td>
<td>677.22</td>
</tr>
<tr>
<td>protected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C17-C33</td>
<td>&lt;EQC LCIP</td>
<td>803.34 (M+1H⁺)</td>
<td>803.35</td>
</tr>
<tr>
<td>Trityl</td>
<td>LDCEP LECKK</td>
<td>982.40 (M+2H⁺)</td>
<td>982.42</td>
</tr>
<tr>
<td>protected</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The peptide fragment contains only native disulphides*

The third peptide was synthesized with trityl protected Cys-17 and Cys-33. The only native disulphide found in this cleavage was the selectively formed disulphide between Cys-17 and Cys-33. Only two other non-native disulphides were found.

In all three peptides non-natural disulphides were produced as the main product, when using the iodine oxidation. Disulphide formation between cysteines that are close in the primary sequence seems to be favoured. Despite the prevalence of non-native disulphide formation, it is possible to synthesize native Garrmin with this synthesis route. The position of the protection groups are critical for the outcome of the disulphide oxidation, since only the synthesis route with trityl protected Cys-3 and Cys-18 produced native Garrmin.

References
Appendix D: Accepted manuscript

APPENDIX D: ACCEPTED MANUSCRIPT

Manuscript accepted for publication.

Handling a tricycle: orthogonal versus random oxidation of the tricyclic inhibitor cystine knotted peptide gurmarin

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Abstract

Gurmarin is a 35 amino acid peptide with three disulfide bridges in an inhibitor cystine knot. It is found in the plant Gymnema sylvestre, and has been identified as a sweet taste inhibitor in rodents. In this article we provide an efficient route for the synthesis of gurmarin by a controlled random oxidation strategy. We compared two oxidation procedures to form the three disulfide bridges. In the first, based on random oxidation, reduced gurmarin was synthesized using trityl for cysteine protection, and oxidized for 48 hours in a Tris-HCl buffer containing cystamine and reduced glutathione to facilitate disulfide scrambling. The second was based on step-wise deprotection followed by oxidation in which the cysteine pairs are orthogonally protected with tert-Butylthio, trityl and acetamidomethyl. To verify that the native gurmarin oxidation product was obtained, thermolysin cleavage was used. Cleavage of random oxidized gurmarin showed two possible disulfide combinations; the native and a non-native gurmarin disulfide isomer. The non-native isomer was therefore synthesized using the orthogonal deprotection-oxidation strategy and the native and the non-native gurmarin isomers were analyzed using UPLC. It was found that the random oxidation procedure leads to native gurmarin in high yield. Thus, the synthetic route was simple and significantly more efficient than previously
reported syntheses of gurmarin and other cysteine rich peptides. Importantly, native gurmarin was obtained by random oxidation, which was confirmed by a synthetic approach for the first time.

**Highlights**
The inhibitor cystine knotted peptide gurmarin is synthesized using a random oxidation strategy and an orthogonal oxidation strategy.
A thermolysin cleavage of random oxidized gurmarin shows two possible disulfide combinations.
The non-native gurmarin disulfide isomer is synthesized using the orthogonal strategy and compared with random oxidized gurmarin using UPLC.
A synthetic approach is used for the first time to confirm the structure of a random oxidized inhibitor cystine knotted peptide.

**Keywords**
Gurmarin
Peptide synthesis
Inhibitor cystine knot
Disulfide formation
Orthogonal oxidation

**Abbreviations**
4-PDS, 4,4-dithiodipyridine; Acm, acetamidomethyl; DIPEA, diisopropylethylamine; DMSO, dimethylsulfoxide; EDT, ethanedithiol; ICK, inhibitor cystine knot; NMP, N-methylpyrrolidone; Pyr, pyroglutamic acid; S\text{Bu}, \textit{tert}-Butylthio; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; Trt, trityl; UPLC, Ultra Performance Liquid Chromatography.
1 Introduction

Gurmarin is a 35 amino acid peptide isolated from leaves of the plant Gymnema sylvestre [10]. The tertiary structure of gurmarin is determined by three disulfides in an inhibitor cystine knot (ICK) (figure 1) [7,8]. Gurmarin was identified as a sweet taste suppressor in rodents [10,16,17] and in Ayurvedic system of medicine the leaves and root of Gymnema sylvestre has been used for treating diabetes and obesity [14]. This effect is believed to be caused by gymnemic acids, a mixture of at least 17 saponins, but gurmarin is also expected to be part of the effect [10,14]. Cystine knotted peptides have been suggested as scaffolds for drug development, due to their increased stability induced by the disulfide knot and their potential oral bioavailability [6,23].

The folding of cystine knotted peptides can essentially be done in two different ways. Using a redox buffer in which the disulfides scramble until they reach the thermodynamically most favorable conformation [4,17] or by using orthogonal cysteine side chain protection groups during solid phase peptide synthesis. The latter allow for selective formation of one disulfide at a time [3,12,19]. When using a redox buffer to oxidize a cystine knotted peptide, the peptide is dissolved in a slightly basic buffer containing a mixture of low molecular weight disulfides and sulfides, which facilitate scrambling of non-native disulfides combinations into the thermodynamically most favorable conformation [1,2,18]. The selective disulfide formation requires three pairs of orthogonal side chain protection groups. Cysteine protection groups can be labile to acid, base, metal ions or reducing agents [2,11]. The most commonly used cysteine protection group in Fmoc-SPPS is trityl [11], which can be removed with 95% TFA in the presence of scavengers [24]. Another common protection group is acetamidomethyl (Acm) [11], which is stable towards TFA. When treated with iodine, the Acm-protected cysteine is activated and forms a disulfide concurrently [20]. The two mentioned protection groups are stable towards reducing agents, and consequently tert-Butylthio (S/Bu) can be used in combination with these, since it can be removed with thiols or phosphines [11,22]. The free thiols can be oxidized using a variety of methods, both in solution and on-resin. It is important to maintain acidic pH during the oxidations to minimize disulfide scrambling. DMSO or 4,4-dithiodipyridine (4-PDS) can be used to oxidize thiols at acidic pH via activated disulfide
intermediates [5,21]. By using 4-PDS it is possible to oxidize cysteines directly in the acidic acetonitrile/water solvent typically used in preparative HPLC purification.

The selective disulfide formation method ensures that the right disulfide combination is obtained. However, the procedure is often laborious and side reactions can occur during the continuous deprotections and oxidations [15]. The random oxidation method has the disadvantage that multiple disulfide combinations can be obtained. Nonetheless, it is expected that the native product will be formed in a random redox buffer oxidation, since the native product most likely has the highest thermodynamic stability [15]. However, this is not always the case [12]. Consequently, structure confirmation is essential when random oxidation is used. Because of the neighboring cysteines in ICK peptides, the structure confirmation cannot be done by an enzymatic fragmentation alone, since no cysteine specific proteases exist.

**Figure 1.** The structures of and numbering of gurmarin, iso-gurmarin (1) and peptide fragments obtained in a thermolysin cleavage of random oxidized gurmarin.

In this work gurmarin was synthesized both by random oxidation and by selective formation of the three disulfides using orthogonal cysteine side chain protection groups. The two different oxidation routes are illustrated in figure 2. To confirm the combination of the disulfides, the random oxidized gurmarin was cleaved with thermolysin to obtain one fragment (4) containing one disulfide from Cys10 to Cys23 and one fragment
(3) containing two disulfides from Cys3 to Cys18 and Cys17 to Cys33. To ensure that fragment 3 in fact has the native conformation and not the non-native Cys3-Cys17 and Cys18-Cys33 conformation (5), a non-native gurmarin disulfide isomer (1) with this disulfide combination was synthesized using the orthogonal oxidation strategy. Finally, the two orthogonal oxidized peptides were compared with the random oxidized gurmarin by co-injection of the peptides on UPLC. Consequently, the disulfide pattern was confirmed by a synthetic approach.

\[
\begin{align*}
&\text{PyrQCVKDELCPYLYLDCPELECKKVNWWDHKCIG} \\
&\text{Acm SBu TrtAc TrtSH Acm SH TrtSH} \\
&\text{90:20 1 NMP-mercaptoethanol, DIPEA} \\
&\text{2.4 x 2.4 h, r.t.} \\
&\text{PyrQCVKDELCPYLYLDCPELECKKVNWWDHKCIG} \\
&\text{Acm SH TrtAc TrtSH} \\
&\text{4:1 NMP-DMSO} \\
&\text{2 x 4 h, r.t.} \\
&\text{PyrQCVKDELCPYLYLDCPELECKKVNWWDHKCIG} \\
&\text{Acm TrtAc} \\
&\text{92:4:2 2 TFA/EDT.TIPS/water} \\
&\text{3 h, r.t.} \\
&\text{PyrQCVKDELCPYLYLDCPELECKKVNWWDHKCIG} \\
&\text{Ac SH} \\
&\text{10 eq. 4-PDS} \\
&\text{2 h, r.t.} \\
&\text{PyrQCVKDELCPYLYLDCPELECKKVNWWDHKCIG} \\
&\text{Ac} \\
&\text{100 eq. i, in 4:1 acetic acid/water} \\
&\text{2 h, r.t.} \\
&\text{PyrQCVKDELCPYLYLDCPELECKKVNWWDHKCIG} \\
&\text{SH SH HS SH SH} \\
&\text{0.1 M Tris-HCl, pH 7.8,} \\
&\text{1 mM glutathione, 1 mM cysteamine} \\
&\text{48 h, r.t.} \\
&\text{PyrQCVKDELCPYLYLDCPELECKKVNWWDHKCIG} \\
&\text{SH SH} \\
&\text{92:4:2 2 TFA/EDT.TIPS/water} \\
&\text{3 h, r.t.} \\
&\text{PyrQCVKDELCPYLYLDCPELECKKVNWWDHKCIG} \\
&\text{SH SH} \\
&\text{92:4:2 2 TFA/EDT.TIPS/water} \\
&\text{3 h, r.t.} \\
&\text{PyrQCVKDELCPYLYLDCPELECKKVNWWDHKCIG} \\
&\text{SH SH} \\
&\text{0.1 M Tris-HCl, pH 7.8,} \\
&\text{1 mM glutathione, 1 mM cysteamine} \\
&\text{48 h, r.t.} \\
&\text{PyrQCVKDELCPYLYLDCPELECKKVNWWDHKCIG} \\
&\text{SH SH} \\
&\text{92:4:2 2 TFA/EDT.TIPS/water} \\
&\text{3 h, r.t.} \\
&\text{PyrQCVKDELCPYLYLDCPELECKKVNWWDHKCIG}
\end{align*}
\]

**Figure 2.** Illustration of the two oxidation strategies. From the top is the orthogonal oxidation and from the bottom is the random oxidation. Gurmarin is highlighted by the dashed box. The numbers to the left are used to refer to synthesis intermediates.
2 Materials and methods

2.1 Materials
Resin and amino acids were purchased at Novabiochem, Germany. Thermolysin, glutathione, cystamine and 4-PDS were purchased at Sigma Aldrich, Germany.

2.2 General Solid Phase Peptide Synthesis
The peptides were synthesized using Fmoc-chemistry using a preloaded Fmoc-Gly-Wang polystyrene LL resin in 0.1 mmol scale on a CEM Liberty microwave peptide synthesizer (CEM Corporation, NC) using standard protocols. Oxyma Pure, diisopropylcarbodiimide and amino acids were used for coupling in 5-10 fold excess over theoretical loading, and 5% piperidine in NMP with 0.05 M HOBt was used for Fmoc-deprotection. HOBt was added to reduce aspartimide formation of Asp16 [13]. Glu2 was coupled twice. Cys-residues and His31 were coupled twice at 50 °C.

2.3 Redox buffer oxidation
The peptide was synthesized as described in section 2.2. Cysteines were protected with trityl. The peptide was cleaved with 92:4:2:2 TFA:ethanedithiol:water:trisopropylsilane, precipitated in diethyl ether (crude purity 54%) and purified on a Waters Delta Prep 4000 using a Waters XBridge Prep C18 OBD column (Waters Corporation, MA), 5 µm, 30x150 mm. Mobile phases were 0.1% TFA in water and 0.1% TFA in acetonitrile. Peptide concentration was determined by Chemiluminescent Nitrogen Detection using an Antek 8060 CLND HPLC detector (PAC, TX) [9]. Reduced gurmarin (10) was dissolved at 0.02 mM in a 0.1 M Tris-HCl (pH 7.8), 1 mM reduced glutathione, 1 mM cystamine redox buffer and stirred for 48 hours. Gurmarin was purified as described above and fractions were analyzed on Waters Acquity UPLC using a BEH, 1.7 µm, 2.1 x 150 mm column and a gradient of 5-95 % of acetonitrile containing 0.05 % TFA against 0.05 % TFA in water over 16 min, flow: 0.4 ml/min. Peptide MW was confirmed using Agilent 6230 TOF LC/MS (Agilent Technologies, CA). Peptide purity: 97.3 %, retention time 5.8 min. Observed MW 1052.5 (M+4H⁺)/4 (expected 1052.5).
2.4 Orthogonal oxidation

The peptide was synthesized as described in section 2.2. Cys3 and Cys18 were Acm-protected, Cys10 and Cys23 were S\textsubscript{Bu}-protected, while Cys17 and Cys33 were trityl-protected. S\textsubscript{Bu} was removed (7) using 2-4 x 24 h 80:20:1 NMP:mercaptoethanol:diisopropylethylamine. The first disulfide was formed using 4:1 NMP:DMSO for 2 x 4 h. Peptide 7 was cleaved from the resin using the same conditions as described in section 2.3, which also removed the trityl-protection groups. Peptide 8 was purified after cleavage from the resin. The second disulfide was formed using 10 eq. of 4-PDS for 2 h and a peptide concentration of 0.05 mM directly in pooled fractions from the preparative HPLC diluted with water. After purification, the final disulfide was formed using 100 eq. of I\textsubscript{2} in 4:1 acetic acid:water for 2 h and a peptide concentration of 0.05 mM (small amounts of acetonitrile and TFA from the preparative HPLC are included in the water part for simplicity. Exact amounts were not determined). The oxidation was stopped by adding ascorbic acid, the reaction mixture was diluted to 20% acetic acid and purified using a ODDMS 120A, 5 μm, YMC 4x125 mm column (FeF Chemicals, Denmark). All reactions were done at room temperature. The peptides were analyzed as described above. Synthesis of gurmarin. Purity: 76.6 %, retention time 5.7 min. Observed MW 1052.5 (M+4H\(^{+}\))/4 (expected 1052.5). Synthesis of iso-gurmarin (1). Purity: 83.7 %, retention time 5.3 min. Observed MW 1052.6 (M+4H\(^{+}\))/4 (expected 1052.5).

2.5 Thermolysin cleavage

A thermolysin cleavage was done with 0.1 mg of random oxidized gurmarin and 0.1 mg thermolysin in 0.6 ml 0.2 M ammonium acetate (pH 6.5), 10 mM calcium chloride at 50 degrees centigrade for 4 h. The fragments obtained from the cleavage were analyzed by LC/MS.

3. Results

3.1 Redox buffer oxidation of gurmarin

Peptide 10 was synthesized using trityl as protection group for all cysteines. Peptide 10 was oxidized for 48 h in a redox buffer until the major peak observed on UPLC did not
increase as depicted in figure 3, which shows the progress of the oxidation. The yields and MW of peptide 10 and gurmarin are summarized in table 1. When peptide 10 was oxidized without prior purification very little oxidized peptide was isolated (data not shown).

![UPLC chromatograms showing the progress of the random oxidation. The gradient is 5-60 % of acetonitrile containing 0.05 % TFA against 0.05 % TFA in water for 3.5 min. A Waters BEH column, 1.7 μm, 2.1 x 50 mm was used.](image)

**Figure 3.** UPLC chromatograms showing the progress of the random oxidation. The gradient is 5-60 % of acetonitrile containing 0.05 % TFA against 0.05 % TFA in water for 3.5 min. A Waters BEH column, 1.7 μm, 2.1 x 50 mm was used.

### 3.2 Orthogonal oxidation of gurmarin

Peptide 6 was synthesized with orthogonal cysteine side chain protection groups as illustrated in figure 2. Cys10 and Cys23 was S/Bu deprotected on resin 2-4 x 24 h until full deprotection was determined by LC/MS in a test cleavage. The amount of time required for the deprotection changed with different batches of the same resin. In some batches it
was possible to deprotect S\textsubscript{Bu} in 5x60 min at 60 °C using a microwave oven. Following the deprotection, the cysteines were oxidized with DMSO, and peptide 8 was cleaved from the resin and purified. When 4-PDS was used instead of DMSO in the first oxidation, it was observed that both cysteines reacted with 4-PDS instead of being oxidized even though sub-stoichiometric amounts of 4-PDS were used. Peptide 9 was formed using 4-PDS in pooled preparative HPLC fractions containing peptide 8. The Acm-protected cysteines were oxidized with iodine and finally gurmarin was purified. The yield and MW of the intermediates and gurmarin are summarized in table 1 and depicted in figure 4.

<table>
<thead>
<tr>
<th>Oxidation</th>
<th>Product</th>
<th>Expected MW\textsubscript{a} (M+4H\textsuperscript{+})/4</th>
<th>Observed MW\textsubscript{a} (M+4H\textsuperscript{+})/4</th>
<th>Yield (%)</th>
</tr>
</thead>
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<td>Reduced gurmarin</td>
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<td>1054.0</td>
<td>24.7</td>
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<td>Oxidized gurmarin</td>
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<td>1052.5</td>
<td>57.1</td>
</tr>
<tr>
<td>Orthogonal</td>
<td>S\textsubscript{Bu} deprotected\textsuperscript{b}</td>
<td>1089.5</td>
<td>1089.7\textsuperscript{c}</td>
<td>7.0</td>
</tr>
<tr>
<td>Orthogonal</td>
<td>Mono-disulfide</td>
<td>1089.0</td>
<td>1089.2\textsuperscript{c}</td>
<td>69.2</td>
</tr>
<tr>
<td>Orthogonal</td>
<td>Bis-disulfide</td>
<td>1088.5</td>
<td>1088.7\textsuperscript{c}</td>
<td>69.2</td>
</tr>
<tr>
<td>Orthogonal</td>
<td>Tris-disulfide</td>
<td>1052.5</td>
<td>1052.5</td>
<td>11.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} monoisotopic mass. \textsuperscript{b} the mass was found in a TFA test cleavage and consequently only Acm protection groups were not removed \textsuperscript{c} the error in the observed MW was caused by an inaccuracy in the instrument calibration.
Figure 4. LC-MS analysis of the intermediates and final product of the orthogonal synthesis. The intermediates are indicated with numbers, expected MW (M+4H⁺)/4 and observed retention time from the LC-MS. The mass spectrum and chromatogram for each peptide is shown below. The MW observed for 6 and 7 corresponds to the Trt deprotected products from a TFA test cleavage.

Three orthogonal on-resin deprotections and disulfide formations were attempted using monomethoxytrityl as protection group instead of trityl. This protection group was deprotected using 1% TFA for 10 x 2 min. Unfortunately, it was not possible to form the second disulfide on resin using DMSO. When 4-PDS was used, both cysteines reacted with 4-PDS instead of forming the disulfide as observed in the first disulfide formation. Only when using less than 0.1 eq. of 4-PDS, a small amount of the second disulfide was observed.

3.3 Thermolysin cleavage of gurmarin

The random oxidized gurmarin was cleaved with thermolysin, which cleaves the N-terminal peptide bond of hydrophobic amino acids. When the cleaved peptide was evaluated on LC-MS, four major peaks appeared (figure 5). Peak 1 contained fragment 2 (exp. MW 418.20 (M+1H⁺)), peak 2 the bis-disulfide fragment 3 or 5 (exp. MW 574.86 (M+3H⁺)/3), peak 3 the mono-disulfide fragment 4 (exp. MW 531.78 (M+2H⁺)/2), while
peak 4 contained non-cleaved gurmarin (exp. MW 1052.47 \((M+4\text{H}^+)/4\)). All MW are monoisotopic masses.

![Figure 5](image)

**Figure 5.** LC-MS analysis of thermolysin cleaved gurmarin. On top is the chromatogram and below are mass spectra of the major m/z peaks observed in the four highlighted peaks on the chromatogram.

### 3.4 Orthogonal oxidation of non-native iso-gurmarin (1)

The thermolysin cleavage of the random oxidized gurmarin indicated that two disulfide combinations were possible. The bis-disulfide peptide fragment observed in peak 3 in figure 5 can be the native fragment 3 or the non-native fragment 5. To investigate which of the two combinations that were produced in the random oxidation, the orthogonal oxidation strategy was used to synthesize the non-native iso-gurmarin (1). In general, the yields of the synthesis steps were comparable to the synthesis of gurmarin, and the overall yield was 0.34 %. However, an inseparable by-product from the iodine oxidation with an additional MW of 109 Da was present in the finished product, which broadened the peak observed on UPLC.
3.5 Comparison of orthogonal oxidized and random oxidized gurmarin

Random oxidized gurmarin was co-injected with both orthogonal oxidized gurmarin and peptide 1 on UPLC, to investigate whether the products were separable. One sharp peak was observed when gurmarin was co-injected (figure 6a), while two clearly separable peaks appeared when the peptide 1 was co-injected (figure 6b).

![Figure 6. UPLC chromatograms of the two co-injection runs. The gradient is 5-95 % of acetonitrile containing 0.05 % TFA against 0.05 % TFA in water for 16 min. A Waters BEH column, 1.7 μm, 2.1 x 150 mm was used. Retention times are indicated next to the peaks. a) Co-injection of random oxidized gurmarin and orthogonal oxidized gurmarin. b) Co-injection of random oxidized gurmarin and orthogonal oxidized peptide 1.](image)

4 Discussion

In this work, the ICK gurmarin was synthesized using two different oxidation strategies. One in which the peptide was oxidized randomly in a redox buffer and one using three orthogonal cysteine side chain protection groups that allowed the selective formation of one disulfide at a time. The orthogonal oxidation route was also used to synthesize a non-native disulfide isomer 2 that was separable by analytical UPLC.
The yield in the random oxidation strategy was 14.1 %. In previous reported random oxidations of gurmarin the yields were 0.7 % and 2.4 % [8,17]. Thus, the yield in this synthesis is significantly increased. This is mainly due to an increase in the yield of reduced gurmarin (10), which could be a consequence of reduction of aspartimide formation of Asp16 by adding HOBT to the piperidine deprotection. In previously reported orthogonal oxidations of peptides with three disulfides, overall yields were not reported [3,12]. However, compared to these examples the orthogonal oxidation method in this work is less laborious and time consuming. The reason why deficit 4-PDS was able to react with both cysteines in the first disulfide formation is considered to be due to the resin loading being lower than stated on the product.

When three disulfides are formed by six cysteine residues, 15 possible disulfide combinations can be obtained. A thermolysin cleavage of the random oxidized gurmarin showed that only one of two combinations, the native and a non-native, was possible. These two disulfide isomers of gurmarin were synthesized using the orthogonal oxidation route. When orthogonal oxidized gurmarin was co-injected with the redox buffer oxidized gurmarin on analytical UPLC only one peak appeared. This confirms the native disulfide combination of the random oxidized gurmarin. To our knowledge, this is the first fully orthogonal synthetic approach to confirm the structure of an ICK peptide.

A previous attempt to synthesize an ICK peptide by both random and orthogonal oxidation was unsuccessful [12]. In this example the native conformation was only obtained using the orthogonal strategy. In the random oxidation one equivalent of 2-PDS was used, compared to 16.7 equivalents of the glutathione/cystamine redox pair in this work. It is likely that excess of a redox pair is necessary to allow the peptide to scramble until the most thermodynamically favorable conformation is obtained.

In the orthogonal strategy, three on-resin disulfide formations were attempted without success. If the protection groups had been placed at different cysteine pairs, thereby changing the order in which the disulfides were formed, it is possible that three on-resin disulfide formations could have succeeded. However, it is also possible that the peptide is not able to fold into the disulfide knot while attached to the resin due to steric hindrance by the resin polymer and side chain protection groups.
4.1 Conclusion
In the synthesis of gurmarin using the two oxidation strategies described herein, the random oxidation is superior to the orthogonal oxidation regarding yield, time and simplicity. However, it is difficult to determine the disulfide combination when oxidizing ICK peptides randomly in a redox buffer. In this work the combination was determined by a synthetic approach, which proved that the same product was obtained in both strategies. This has been reported not always to be the case when synthesizing ICK peptides. The orthogonal oxidation strategy is more laborious, but it removes the uncertainty regarding the disulfide combination. The on-resin oxidation of the first disulfide reduces the number of purifications, and consequently increases the overall yield. The final two oxidations are simple and could be made directly in diluted HPLC fractions, thereby reducing the overall synthesis time.

Acknowledgements
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References


APPENDIX E: Submitted manuscript

Submitted manuscript.

Design, synthesis, structural and functional characterization of novel melanocortin agonists based on the cyclotide kalata B1

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* Running title: Novel melanocortin agonists based on the kalata B1 scaffold

Keywords: cyclic peptides, cystine knot, obesity, structure

**Capsule**

**Background:** Cyclotides are useful scaffolds to stabilize bioactive peptides.

**Results:** Four melanocortin analogues of kalata B1 were synthesized. One is a selective MC4R agonist.

**Conclusion:** The analogues retain the native kalata B1 scaffold and introduce a designed pharmacological activity, validating cyclotides as protein engineering scaffolds.

**Significance:** A novel type of melanocortin agonist has been developed, with potential as a drug lead for treating obesity.

**Abstract**

Obesity is an increasingly important global health problem that lacks current treatment options. The melanocortin receptor 4 (MC4R) is a target for obesity therapies since its activation triggers appetite suppression and increases energy expenditure. Cyclotides have been suggested as scaffolds for the insertion
and stabilization of pharmaceutically active peptides. In this study we explored the development of appetite reducing peptides by synthesizing MC4R agonists based on the insertion of the His-Phe-Arg-Trp sequence into the cyclotide kalata B1. The ability of the analogs to fold similarly to kalata B1 but display MC4R activity was investigated. Four peptides were synthesized using Boc peptide chemistry with a C-terminal thioester to facilitate backbone cyclization. The structures of the peptides were found to be similar to kalata B1, evaluated by Hα NMR chemical shifts. KB1[GHFRWG;23-28] had a $K_i$ of 29 nM at the MC4R and was 107 or 314 times more selective over this receptor than MC1R or MC5R respectively, and had no detectable binding to MC3R. The peptide had higher affinity for the MC4R than the endogenous agonist, α-melanocyte stimulation hormone (MSH), but was less potent at the MC4R, with an EC$_{50}$ of 580 nM for activation of the MC4R. In conclusion, we synthesized melanocortin analogs of kalata B1 that preserve the structural scaffold and display receptor binding and functional activity. KB1[GHFRWG;23-28] is potent and selective for the MC4R. This compound validates the use of cyclotides as scaffolds, and has the potential to be a new lead for the treatment of obesity.

## Introduction

Obesity is an increasing problem world-wide with 1.5 billion adults estimated to be overweight (BMI above 25 kg/m$^2$) and 500 million obese (BMI above 30 kg/m$^2$) in 2008 [1]. These statistics are alarming given that obesity is associated with a risk of developing a variety of debilitating diseases, including type 2 diabetes, cardiovascular disease and hypertension [2-4]. To prevent or reduce obesity and the risk of related diseases, regular exercise and a healthy diet are very effective [5], but in high risk patients drug treatment is also necessary [6]. Currently only two drugs are approved for the treatment of obesity, i.e., orlistat and phentermine. The FDA withdrew sibutramine in 2010 because of increased risk of cardiovascular events [7]. Although drugs currently on the market for other indications are undergoing clinical trials for their potential to treat obesity, there is still an urgent need for new drugs targeting the world-wide obesity problem.

One approach to the development of drugs for the treatment of obesity is to target the melanocortin system, which comprises a family of five G-protein coupled receptors, the melanocortin receptors (MCR), named MCR1-5 in the order of their discovery [8-12]. The endogenous peptide agonists, adrenocorticotropic hormone (ACTH) and α-, β- and γ- melanocyte stimulating hormone (MSH), all derived from the precursor protein pro-
opiomelanocortin, form part of the system, in addition to the inverse agonists agouti protein and agouti-related protein (AGRP).

The five MCR receptors are distributed throughout the body and are associated with a variety of physiological effects. MC1R is mainly present in melanocytes and melanoma cells [8,9]. MC2R is present in the adrenal cortex, where it regulates steroidogenesis, and is only activated by ACTH, in contrast to the other MCRs [8]. MC3R is mainly expressed in the arcuate nucleus [13], but is also present in the gut [10]. MC4R is found throughout the brain [14] and in the spinal cord and dorsal root ganglia [15]. Stimulation of MC4R reduces food intake and increases the metabolic rate in mice [16], which makes it an interesting target for the treatment of obesity. MC5R is distributed throughout the body with high levels found in skeletal muscle, brain and exocrine glands, and has a variety of functions [12,17,18].

The MSH peptides and ACTH share the tetrapeptide His-Phe-Arg-Trp pharmacophore, which is the shortest peptide active on the MCRs [19]. Various modifications to this tetrapeptide have been made to explore the role of individual amino acids. Substitution of Met⁴ with norleucine (Nle) and modifying the conformation of Phe⁷ to D-Phe in α-MSH (see Table 1), yielded a stable subnanomolar agonist named NDP-α-MSH [20]. Additionally it has been shown that the His-D-Phe-Arg-Trp tetrapeptide alone has nanomolar activity on the MC4R [21]. In another study a truncated analogue of the inverse agonist AGRP was turned into a full agonist by exchanging the binding sequence with His-D-Phe-Arg-Trp [22]. These findings suggest that it might be possible to graft the His-Phe-Arg-Trp sequence and related modified sequences into stable peptide frameworks to develop novel MCR agonists. The motivation for the grafting is to place the bioactive tetrapeptide in a molecular context where it will be less susceptible to proteolysis and will have more favorable biopharmaceutical properties than the isolated peptide sequence.

The concept of developing MC4R agonists for the treatment of obesity has been around for more than a decade, as reviewed by Emmerson et al. [23] and Wikberg and Mutulis [24]. Despite strenuous efforts, no compounds have reached clinical trials for the treatment of obesity. The similarity between the various MCR receptors, which makes it challenging to make selective agonists, and the generally low half-lives of peptides in vivo [23] have probably contributed to this lack of success.

Recent discoveries of naturally occurring stable peptides offer the potential to overcome some of the limitations of peptides as drug leads. In the mid 1990’s several ultra-stable peptides that had both a cyclic backbone and three disulfide bonds forming a cystine knot were discovered [25-28]. The most extensively
studied of these is the 29 residue peptide kalata B1 found in the African plant *Oldenlandia affinis* [26], which originally attracted attention because of its reported uterotonic activity [29]. More recently it has been reported to have antimicrobial and cancer cell cytotoxic activity [30] as well as a range of other activities. Its biological function in the plant is thought to be as a host defense pesticidal agent, since it reduces the growth of the larvae of the crop pest *Helicoverpa punctigera* [31]. Following additional discovery efforts [32-36] since these early studies, the group of cyclic cystine knotted (CCK) peptides now comprises several hundred sequences and been given the name cyclotides [33,37]. They are exceptionally stable towards enzymatic or thermal degradation because of their cyclic backbone and cystine knot [26,28,38]. Due to their stability and large sequence variation in nature, cyclotides have been suggested as promising scaffolds for the grafting of pharmaceutically interesting peptides to stabilize them [33,39]. There are six backbone loops decorating the cystine knot core, and these offer opportunities for conformational control of a variety of peptide epitopes. In addition to being amenable to solid phase peptide synthesis [30,40], cyclotide libraries have recently been expressed in bacterial cells [41] thus providing alternative routes to their manufacture.

There are currently only a few examples demonstrating the grafting of biologically active sequences into cyclotides or related acyclic cystine knot scaffolds. For example, Clark et al. demonstrated that it was possible to substitute selected amino acids in loop 5 of kalata B1 to remove intrinsic hemolytic activity but retain the native fold [42] and later studies on this framework demonstrated efficacy for grafted antiangiogenic sequences [43] and bradykinin antagonists [44]. Thongyoo et al. changed the specificity of the trypsin inhibitor cyclotide, MCoTI-II, towards other proteases with one to three amino acid substitutions [45] and Chan et al. reported a novel angiogenic agent based on this framework [46]. In acyclic cystine knotted peptides, Reiss et al. successfully grafted platelet aggregation inhibiting sequences [47], and as mentioned above, AGRP was turned into a full agonist on the melanocortin receptors [22].

In the current study, kalata B1 was used as a scaffold for the insertion (grafting) of melanocortin receptor activating sequences. The grafted sequences are shown in Figure 1 along with the NMR-derived structure of kalata B1. These analogues have the potential to become leads for a new class of melanocortin receptor agonists for the treatment of obesity. More broadly, they will expand knowledge on the applicability of cyclotides as scaffolds.
Experimental procedures

Peptide synthesis

Peptides were synthesized manually by Boc solid phase peptide synthesis on a Boc-Gly-PAM resin using 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) or 2-(6-chloro-1-H-benzotriazol-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU) as coupling reagents, as described previously [42]. Mercaptopropionic acid was used as a linker to facilitate backbone cyclization by native chemical ligation. Peptides were cleaved from the resin using hydrogen fluoride/cresol/thiocresol (50:4:1 v/v/v), precipitated in diethylether and lyophilized. The crude peptides were purified using HPLC with a gradient of 25-45% solvent B (90% acetonitrile, 0.1% TFA in water) against solvent A (0.1% TFA in water) over 40 minutes. A Phenomenex C18 column was used and absorbance measured at 280 nm. Backbone cyclization and disulfide bonds were formed using 0.1 M ammonium bicarbonate (pH 8.2), isopropanol (50:50, v/v) buffer for 48 hours, at a peptide concentration of 0.2 mg/ml. At 0 h and 24 h, 1 mM reduced glutathione was added. The oxidized peptides were purified on HPLC with 30-50% solvent B over 60 minutes. The peptides were characterized using electrospray ionization mass spectrometry; the purity was determined by RP-UPLC and the peptides were quantified using a chemiluminescent nitrogen detector [48].

NMR

Peptides were dissolved at a concentration of approximately 1 mM in 10% D2O and 90% H2O. Spectra were recorded at 298 K on a Bruker AVANCE 600 MHz NMR spectrometer. Two-dimensional spectra recorded included TOCSY, NOESY and DQF-COSY. The mixing times for the TOCSY and NOESY spectra were 80 ms and 200 ms respectively. The processed spectra were analyzed using the program CcpNMR Analysis, University of Cambridge, UK. Three-dimensional structures were calculated from the recorded two-dimensional spectra using CYANA as described previously [49]. Structures were analyzed using Molprobity and PROMOTIF [50] and displayed using MolMol [51] and PyMol [52]. Structures and chemical shifts have been deposited in the protein data bank (PDB ID 2lur) and Biomagnetic Resonance Bank (BMRB ID 18536) respectively.

Melanocortin assays

Melanocortin receptor binding assays were conducted as previously described [53]. Each assay was performed in duplicate and three assays were done on each receptor. Peptides (3pM to 3µM) were incubated with homogenized membranes from baby hamster kidney cells expressing the various MCRs and
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I-NDP-α-MSH (around 60,000 counts/min). The incubation was stopped after 1 hour by filtration through polyethylenimine treated filter plates. Scintillation liquid was added to the plates and the radioactivity was counted.

The cAMP activity assays were conducted on baby hamster kidney cells expressing the MC4R, using the FlashPlate™ assay as previously described [53]. Cells and peptide (100 fM to 10 µM) were incubated and shaken for 30 min at room temperature. Detection mix was added according to FlashPlate protocol, shaken for 30 min and incubated overnight. The amount of cAMP produced was measured by displacement of radiolabeled cAMP bound to anti-cAMP antibodies in accordance with the FlashPlate protocol.

The data analyses from the receptor assays were performed as described previously [53], using the program GraphPad Prism, GraphPad Software, USA. IC\textsubscript{50} values were calculated using a nonlinear regression model and K\textsubscript{i} values were calculated from the IC\textsubscript{50} values. The potency at the MC4R was determined as EC\textsubscript{50} values, calculated by nonlinear regression of the activity assay data.

Results

Design, synthesis and characterization of the grafted peptides

Four melanocortin analogues of kalata B1 were synthesized using solid phase Boc-chemistry. The sequences of the peptides are shown in Table 1. They are named to reflect the grafted epitopes incorporated into the kalata B1 (kB1) sequence. For example, kB1[HFRW;24-27] indicates replacement of residues 24-27 in loop 6 of kalata B1 (kB1) sequence. In this case the tetrapeptide is flanked by the remaining native amino acids in loop 6, i.e., T at the N-terminal side and PV at the C-terminal side, making the total composition of loop 6 THFRWPV in place of the native TRNGLPV. To account for the possibility that direct replacements might constrain the bioactive tetrapeptide in a non-active conformation other grafts were made where flanking glycine residues were used in place of the native kalata B1 residues.

The peptides were assembled attached to a C-terminal mercaptopropionic acid linker to facilitate subsequent cyclization of the termini. The linear precursor peptides were synthesized

Enzymatic cleavage assays

Peptides were incubated with chymotrypsin (1:5 w/w enzyme/peptide ratio) at 37°C. For α-MSH the ratio was 1:10 w/w enzyme/peptide in order to have close to equal molar ratios for all peptides. Samples were aliquotted out and quenched with TFA before analysis on UPLC. Peak heights were used to calculate the amount of intact peptide.
with V21 as the C-terminus, since this allowed the synthesis of one batch of resin to which was coupled the first 22 identical amino acids. This batch was subsequently split to synthesize the four analogues. Loop 6 was chosen as the site for grafting the melanocortin sequence, since it is the largest of the inter-cysteine loops in kalata B1. In addition, the structure of kalata B1 shown in Figure 1 indicates that the other loops are not in close proximity with loop 6 and therefore are not likely to interfere with the presentation of the grafted melanocortin sequence to the MCRs.

The peptides were oxidized and cyclized in a single step at 0.2 mg/ml concentration in a 0.1 M ammonium bicarbonate (pH 8.2), isopropanol (50:50, v/v) buffer for 48 hours with 1 mM reduced glutathione added at 0 and 24 hours. The chromatograms from the purification of the folded peptides are shown in Figure 2.

The late eluting peaks were purified and analyzed by NMR spectroscopy to confirm that the native fold was present. A comparison of the Hα chemical shifts of the grafted peptides with the native peptide is shown in Figure 4. Only the amino acids that are common with kalata B1 are shown and the comparison indicates that the overall structures of the peptides are similar. The only Hα chemical shift differences larger than 0.2 ppm were found for Val29, which is the C-terminal neighbor to the grafted sequence, Cys1 which is bound to Val29 and Cys15 which forms a disulfide with Cys1. The peptides kBl[HFRW;24-27] and kBl[HfRW;24-27] had additional peaks in the NMR spectra (data not shown) as a result of cis/trans isomerization at the Trp27/Pro28 peptide bond. The major conformation, judging from the intensity of the peaks, was the cis-Pro conformation. The two conformers of kBl[HfRW;24-27] were distinguished by a strong NOE cross-peak between Hα-Hα protons of Trp27 and Pro28 (4.24 ppm; 3.33 ppm) in the major conformation and a weaker NOE cross-peak between Hα-Hδ protons (5.01 ppm; 3.91 ppm) in the minor conformation.

**Melanocortin assays**
The binding affinities of the four synthesized melanocortin analogs of kalata B1 to the melanocortin receptors 1 and 3-5 were determined by competition binding assays using radiolabeled NDP-α-MSH. Kalata B1 and α-MSH were also evaluated as controls. The results are given in Table 2 and results from binding studies of kalata B1, kBl[GHFRWG;23-28] and α-MSH at the MC4R are shown in Figure 3. KB1[GHFRWG;23-28] had a Ki of 29 nM at the MC4R and was 107 or 314 times selective for the MC4R over the MC1R or MC5R respectively. The peptide was not able to bind to the MC3R. The Ki of α-MSH at the MC4R receptor was 39 nM. KB1[GHFRWG;23-28] was also tested in a functional assay to
evaluate the potency at the MC4R. The EC\textsubscript{50} of kB1[GHFRWG;23-28] was determined to be 580 nM, while α-MSH had an EC\textsubscript{50} of 3.7 nM (Table 2).

**Three-dimensional structure of kB1[GHFRWG;23-28]**

Given the potency and selectivity observed for kB1[GHFRWG;23-28], the three-dimensional structure was determined to provide insight into structure activity relationships. Structures were determined using torsion angle dynamics in the program CYANA and the 20 structures with the lowest target function chosen to represent the global fold. Energetic and geometric statistics are given in Table 3. Analysis of the structures with PROMOTIF [50] revealed that the major element of secondary structure is a β-hairpin involving residues 16-18 and 21-23. A third strand comprising residues 3-4 is associated with the hairpin to form a triple-stranded β-sheet as shown in Figure 5. In addition, residues 5-8 form a type I β-turn, and residues 10-12 and 12-14 form inverse γ-turns. Comparison of the structure of kB1[GHFRWG;23-28] with the native kalata B1 framework indicates that the overall fold is maintained. The cyclic cystine knot motif and β-hairpin are the defining structural features of the cyclotides and both are present in kB1[GHFRWG;23-28], despite the sequence changes in loop 6. The introduction of non-native sequences into the CCK scaffold can sometimes lead to disordered loops [43,46]. However, for kB1[GHFRWG;23-28] the grafted loop 6 is as well defined as the other loops, with order parameters >0.93 for the ϕ and ψ angles over the whole molecule.

The grafted sequence has several large hydrophobic residues, including His, Phe and Trp and it was of interest to determine how their presence might influence the nature of the cyclotide surface. Analysis of the surface of kB1[GHFRWG;23-28] shows that a set of surface-exposed hydrophobic residues are punctuated by polar or charged residues. In addition, the arginine in loop 6 is highly solvent exposed. A similar distribution of surface residues is evident in kalata B1, with the exception of loop 6, as illustrated in Figure 6.

**Proteolytic stability**

To investigate the stability of the peptides kalata B1, kB1[GHFRWG;23-28] and α-MSH were incubated with chymotrypsin. As shown in Figure 7, more than 95% of kalata B1 remained intact for five hours. KB1[GHFRWG;23-28] was slightly less stable but around 80% of kB1[HFhRW;24-27] was intact after five hours. After 24 hours, the amounts of intact peptide had not changed further, but this is probably due to auto-cleavage of chymotrypsin by this time. Compared to kalata B1, kB1[GHFRWG;23-28] has two additional potential chymotrypsin cleavage sites in the grafted sequence, so the
observed resistance to proteolysis is a significant finding. Finally, α-MSH completely degraded within ten minutes.

**Discussion**

In this study we used the kalata B1 cyclotide scaffold to design a novel agonist for the MC4R. This analogue (kB1[GHFRWG;23-28]) also showed selectivity over other MCRs tested, highlighting the potential of the CCK scaffold for targeting specific interactions implicated in regulation of appetite and energy homeostasis, and providing a possible lead molecule for the treatment of obesity. Interestingly, kB1[GHFRWG;23-28] showed higher affinity for the MC4R compared to the native agonist α-MSH but was not as potent functionally. It appears likely that kB1[GHFRWG;23-28] has stronger binding to the active site than α-MSH but due to the placement of the pharmacophore within the kalata B1 scaffold and possible steric clashes with the receptor it is not able to induce a similar conformational change in the receptor, which is required for full activation of the cAMP cascade. Nevertheless, significant (submicromolar) activation of the cAMP response was observed. This successful example of grafting a function into a cyclotide adds to a growing body of evidence that the CCK framework can accommodate a diverse range of bioactivities.

Four analogues of kalata B1 were synthesized using Boc chemistry and generally folded well using the oxidation conditions previously established for native kalata B1 (Figure 2). All of the analogues retained the native fold based on analysis with NMR spectroscopy. Even though six out of the seven residues in loop 6 were replaced, the CCK holds the remaining loops in place and allows these changes in loop 6. These results confirm the plasticity of the cyclotide scaffold previously reported [42], and in particular the tolerance of loop 6 to changes. This tolerance is further demonstrated by observations that loop 6 can be truncated and opened to produce acyclic cystine knot derivatives that maintain the same global fold as kalata B1 [54]. In essence, the exposed loops of the CCK framework can be regarded as ‘plug and play’ cassettes that can be substituted to introduce a desired biological activity.

Interestingly, kB1[GHfRWG;23-28], the D-Phe25 analogue of kB1[GHFRWG;23-28] bound with lower affinity to the MCRs than the all-L graft. This was surprising, since D-Phe analogues of short linear peptides are known to have higher affinity for the MCRs compared to L-Phe analogues [20]. The locked conformation of the pharmacophore in loop 6 of kalata B1 could change the presentation to the active site of the receptors and consequently be an explanation for this observation. There is clearly potential for further optimization of the bioactive
epitope and these studies are planned in our laboratory.

The NMR spectra for kB1[HFRW;24-27] and kB1[HfRW;24-27] revealed that two conformations were present. This conformational heterogeneity appears to be related to cis/trans isomerization around Pro28. The major conformation, based on inter-residue cross-peaks was the cis conformation. This was confirmed by a strong Hα-Hα cross-peak between Trp27 and Pro28. The cis-Pro conformation is consistent with what has previously been described for the Trp19–Pro20 bond in kalata B1 [33,55]. The strong Hα-Hα cross peak between Trp19 and Pro20 was observed in all peptides, whereas a Hα-Hδ cross peak was observed for the Thr12-Pro13 bond. The cis-Pro20 bond causes a conceptual twist in the backbone, which defines the Möbius family of cyclotides [33]. The additional twist caused by the cis-Pro28 bond changes the topology of kB1[HFRW;24-27] and kB1[HfRW;24-27]. This could be part of the reason why they did not bind to the MCRs, since a twist in the backbone around the pharmacophore changes the presentation of the side chains at the active site of the receptors. Besides the conformational change, it is also possible that the side chains of Thr23 and Pro28 in kB1[HFRW;24-27] and kB1[HfRW;24-27] affect the binding of the pharmacophore to the active site of the MCRs negatively compared to Gly23 and Gly28 in kB1[GHFRWG;23-28] and 2, which due to their lack of side chain would interfere less with the active site.

The intrinsic resistance of α-MSH to proteolytic degradation is very low but can be somewhat increased by engineering a single disulfide in the sequence [56]. Kalata B1 has been demonstrated to have significantly increased stability towards enzymatic degradation compared to non-cystine knotted analogues, linear cystine knotted peptides and non-cyclic peptides [38]. It was of interest to determine if the target grafting epitope would have enhanced resistance to proteolysis when incorporated into the cyclotide framework. Chymotrypsin cleavage assays for kB1[GHFRWG;23-28], kalata B1 and α-MSH confirmed this expectation. Whereas α-MSH was completely degraded within ten minutes under the conditions studied, kalata B1 was 95% intact after five hours and remained stable even after 24 hours, probably due to auto-cleavage of chymotrypsin. KB1[GHFRWG;23-28] was only slightly less stable than kalata B1, being 80% intact after five hours. This marginal loss of stability is surprising on one hand since the grafted sequence in loop 6 contains two potential chymotrypsin cleavage sites and hence the sequence is intrinsically susceptible to proteolysis. On the other hand, the results provide clear confirmation of the ability of the cyclotide framework to stabilize even a

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'susceptible’ epitope. The observation that kB1[GHFRWG;23-28] was more than 80% intact after extended incubation with chymotrypsin confirms the high stability towards enzymatic degradation of both kalata B1 and grafted analogues thereof.

Cyclotides and other cystine knot microproteins have been suggested to have the potential for oral delivery, partly because of their high stability [57,58]. This potential was recently demonstrated in the development of an analogue of kalata B1 that is orally active in a mouse model of inflammatory pain [44], suggesting that similar exciting results for the oral route of drug delivery might be achieved with other examples of kalata B1 grafting. The analogues of kalata B1 reported here represent a new group of selective melanocortin agonists. Because of their cyclotide framework they are more stable than previously reported linear and mono-cyclic melanocortin analogs, and with further optimization this group of peptides could provide new peptide-based leads with the potential to treat obesity.
References


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Footnotes
The abbreviations used are: AGRP, agouti related protein; ACTH, adrenocorticotropic hormone; CCK, cyclic cystine knot; MCR, melanocortin receptor; MSH, melanocyte stimulating hormone.

Figure 1. a) The primary sequence of kalata B1. The disulfide bonds and circular backbone are indicated with lines, and the inter-cysteine-loops labeled below the sequence. Loop 6, in which the melanocortin sequences are grafted, is highlighted in blue. b) The four sequences grafted into loop 6. The lower case f refers to D-Phe. Non-native kalata B1 residues are highlighted in red. c) The structure of kalata B1 (PDB ID 1nb1), with disulfides indicated and loop 6 highlighted in blue [59].
Figure 2. Chromatograms from the preparative HPLC purifications of the grafted peptides. The peaks corresponding to the correctly folded peptides are marked with an asterisk.

Figure 3. MC4R binding assays for kB1[GHFRWG;23-28], kalata B1 and α-MSH. Each assay was performed in duplicate. Cell membranes expressing the MC4R were incubated with $^{125}$I-NDP-α-MSH and the synthesized peptides. The amount of radioactivity from bound $^{125}$I-NDP-α-MSH was counted and $K_i$ values were calculated. Data points are average values from the assays and SEM values are indicated with error bars.

Figure 4. The Hα chemical shifts of the four synthesized peptides and kalata B1. The numbers corresponding to cysteine residues are highlighted in bold.

Figure 5. a) Overlay of the 20 minimum energy structures of kB1[GHFRWG;23-28]. b) Ribbon model of the NMR derived structure of kB1[GHFRWG;23-28]. PDB ID 2lur. c) Ribbon model of kalata B1. PDB ID 1nb1.

Figure 6. Surface representation of kB1[GHFRWG;23-28] (left) and kalata B1 (right). The only major differences are observed in the grafted loop 6, i.e. residues 23-28. Hydrophobic residues are highlighted in green, positively charged in blue, negatively charged in red, cysteines in yellow and polar in cyan. The top views have similar orientation as the ribbon models in Figure 5. The residues F25, R26 and W27 in kB1[GHFRWG;23-28] are part of the melanocortin pharmacophore grafted into loop 6 of kalata B1.

Figure 7. Enzymatic stability of kalata B1, kB1[GHFRWG;23-28] and α-MSH towards chymotrypsin cleavage. The peptides were incubated with chymotrypsin for five hours at 37°C. The enzyme to peptide ratio was 1:5 w/w (1:10 w/w for α-MSH to have equal molar ratios). The insert shows the degradation of α-MSH the first ten minutes of incubation.
### Table 1. Nomenclature, sequences and disulfide pattern of grafted cyclotides with their corresponding masses. The sequence of α-MSH is shown below.

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<th>Peptide</th>
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<th>Calculated mass (Da)*</th>
<th>Observed mass (Da)*</th>
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<tr>
<td>kB1[HfRW;24-27]</td>
<td>CGETCVGGTCNTPGCTCSWPVCTHfRWVPV</td>
<td>3076.2</td>
<td>3076.1</td>
</tr>
<tr>
<td>α-MSH</td>
<td>Ac-SYSMEHFRWGKPV</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*monoisotopic mass

### Table 2. Binding affinity of grafted cyclotides for melanocortin receptors 1 and 3-5 and functional activity at MCR4 of the most potent binder.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>hMC1R</th>
<th>hMC3R</th>
<th>hMC4R</th>
<th>hMC4R cAMP</th>
<th>hMC5R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pK_i</td>
<td>pK_i</td>
<td>pKi</td>
<td>pEC_{50}</td>
<td>pK_i</td>
</tr>
<tr>
<td></td>
<td>(K_i/[nM])</td>
<td>(K_i/[nM])</td>
<td>(K_i/[nM])</td>
<td>(EC_{50}/[nM])</td>
<td>(K_i/[nM])</td>
</tr>
<tr>
<td>kB1[GHFRWG;23-28]</td>
<td>5.50 ± 0.09</td>
<td>Na.</td>
<td>7.53 ± 0.28</td>
<td>6.24 ± 0.08</td>
<td>5.04 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>(3,100)</td>
<td>(29)</td>
<td>(580)</td>
<td>(9,100)</td>
<td></td>
</tr>
<tr>
<td>kB1[GHHRWG;23-28]</td>
<td>6.37 ± 0.06</td>
<td>5.11 ± 0.03</td>
<td>6.59 ± 0.15</td>
<td>5.30 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(420)</td>
<td>(7,700)</td>
<td>(250)</td>
<td>(5,000)</td>
<td></td>
</tr>
<tr>
<td>α-MSH</td>
<td>9.16 ± 0.05</td>
<td>7.51 ± 0.04</td>
<td>7.41 ± 0.26</td>
<td>8.44 ± 0.26</td>
<td>6.75 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>(0.69)</td>
<td>(31)</td>
<td>(39)</td>
<td>(3.7)</td>
<td>(180)</td>
</tr>
</tbody>
</table>

Na: no activity (i.e. K_i > 10,000 nM). Data represents an average pK_i of three replicates ± SEM. K_i value (in brackets) is calculated from the average pK_i value. Potency at the MC4R was determined only for kB1[GHFRWG;23-28].
Table 3. NMR and refinement statistics for the structure of kB1[GHFRWG;23-28].

<table>
<thead>
<tr>
<th>NMR distance and dihedral constraints</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Distance constraints</strong></td>
<td></td>
</tr>
<tr>
<td>Total NOE</td>
<td>291</td>
</tr>
<tr>
<td>Short-range (</td>
<td>i-j</td>
</tr>
<tr>
<td>Medium-range (1&lt;</td>
<td>i-j</td>
</tr>
<tr>
<td>Long-range (</td>
<td>i-j</td>
</tr>
<tr>
<td><strong>Dihedral angle restraints</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td><strong>Structure statistics</strong></td>
<td></td>
</tr>
<tr>
<td>CYANA target function</td>
<td>1.02±0.07</td>
</tr>
<tr>
<td>Violations (mean and s.d.)</td>
<td></td>
</tr>
<tr>
<td>Distance constraints, RMSD (Å)</td>
<td>0.01±0.002</td>
</tr>
<tr>
<td>Dihedral angle constraints, RMSD (°)</td>
<td>1.45±0.12</td>
</tr>
<tr>
<td>Max. distance constraint violation (Å)</td>
<td>0.26</td>
</tr>
<tr>
<td>Max. dihedral angle violation (°)</td>
<td>1.01</td>
</tr>
<tr>
<td><em><em>Average pairwise r.m.s. deviation</em> (Å)</em>*</td>
<td></td>
</tr>
<tr>
<td>Backbone</td>
<td>0.41±0.11</td>
</tr>
<tr>
<td>Heavy</td>
<td>0.84±0.18</td>
</tr>
<tr>
<td><strong>Ramachandran statistics</strong></td>
<td></td>
</tr>
<tr>
<td>Residues is most favored regions</td>
<td>49.5%</td>
</tr>
<tr>
<td>Residues in additionally allowed regions</td>
<td>50.5%</td>
</tr>
</tbody>
</table>

*Pairwise RMSD was calculated from the 20 structures with the lowest target function.
Figure 1

a

<table>
<thead>
<tr>
<th>CGETCVGGTCNTPGCTCSWPVCTRNLNPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>loop 1</td>
</tr>
</tbody>
</table>

b

Graft 1: GHFRWGV
Graft 2: GHfRWGV
Graft 3: THFRWPV
Graft 4: THfRWPV
Figure 2
Figure 3
Figure 4

[Graph showing chemical shift (ppm) against residue number with various markers indicating different samples or conditions.]

- Kalata B1
- kB1[GHRW;24-27]
- kB1[GHRW;23-28]
- kB1[HFRW;24-27]
- kB1[HfRW;24-27]
Figure 5
Figure 6
Figure 7