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Positional Scanning for Peptide Secondary Structure by Systematic Solid-Phase Synthesis of Amino Lactam Peptides

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Abstract: Incorporation of amino lactams into biologically active peptides has been commonly used to restrict conformational mobility, enhance selectivity, and increase potency. A solid-phase method using a Fmoc-protection strategy has been developed for the systematic synthesis of peptides containing configurationally defined α- and β-amino γ-lactams. *N*-Alkylation of *N*-silyl peptides with five- and six-member cyclic sulfamidates **9** and **8** minimized bis-alkylation and provided *N*-alkyl peptides, which underwent lactam annulation under microwave heating. Employing this solid-phase protocol on the growth hormone secretagogue GHRP-6, as well as on the allosteric modulator of the IL-1 receptor 101.10, has furnished 16 lactam derivatives and validated the effectiveness of this approach on peptides bearing aliphatic, aromatic, branched, charged, and heteroatomic side chains. The binding affinity IC_{50} values of the GHRP-6 lactam analogues on both the GHS-R1a and CD36 receptors are reported as well as inhibition of thymocyte proliferation measurements for the 101.10 lactam analogues. In these cases, lactam analogues were prepared exhibiting similar or improved properties compared with the parent peptide. Considering the potential for amino lactams to induce peptide turn conformations, the effective method described herein for their supported construction on growing peptides, and for the systematical amino lactam scan of peptides, has proven useful for the rapid identification of the secondary structure necessary for peptide biological activity.

1. Introduction

The secondary structure of proteins and peptides is an essential determinant for their bioactivity. Information concerning the biologically active conformation of a peptide is imperative for understanding function and may be gleaned by employing organic surrogates to mimic different secondary structures. Peptide mimics containing nonpeptidic structural elements have been pursued to imitate the conformation and functional groups of the native peptide.[1−9] Technologies allowing the convenient and rapid introduction of such mimics into peptides constitute powerful tools for studying structure−activity relationships (SARs) and designing biologically active compounds in the drug discovery process.

Methods for systematically scanning peptides for SAR studies have included alanine, enantiomeric amino acid, β-amino acid, proline, macrocyclic lactam, and *N*-alkyl amino acid scanning for identifying the importance of side chain and backbone composition, configuration, conformation, and hydrogen-bonding interactions.[10,13−15] In addition to providing SAR information, such modifications have added benefits

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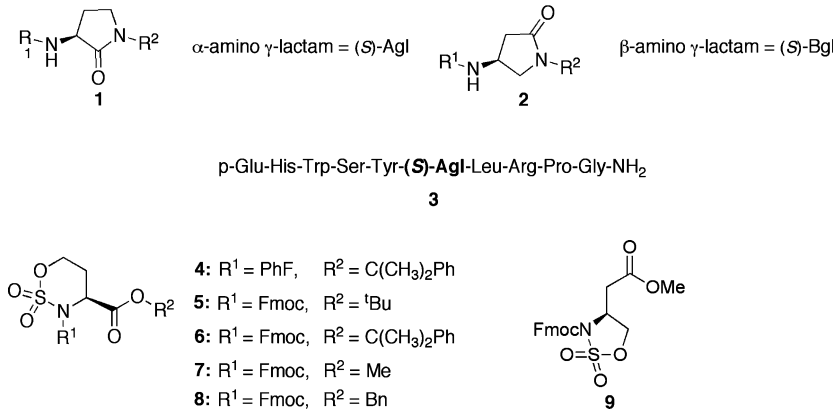

Figure 1. Three letter codes for amino lactam residues, LH−RH analogue containing a Freidinger−Veber lactam **3**, 2,2-dioxo-1,2,3-oxathiazinane esters **4**−**8**, and ((4*S*)-methyl-2-[3-(9-fluorenylmethoxycarbonyl)-2,2-dioxo-1,2,3-oxathiazolidin-4-yl]acetate **9**.

such as enhanced resistance to enzymatic degradation and improved duration of action.[11,12] In our laboratory, the systematic substitution of an α-amino acid by aza-amino acid residues (in which the α-carbon is replaced by nitrogen) within a peptide has proven effective for identifying the importance of turn structures for biological activity,[16] as demonstrated by revealing the importance of a type II′ β-turn centered at Gly[33]-Pro[34] for activity of a calcitonin gene-related peptide 1 receptor antagonist.[17] New solid-phase approaches for introducing conformational constraint into a peptide structure are still needed to expand the power of these methods for systematically studying the relevance of side-chain functionality and backbone conformation for activity.

α-Amino-γ-lactam (Agl, **1**, Figure 1) has been utilized to constrain the backbone conformation of linear peptides to give β-turn mimics.[18−20] The first introduction of a so-called "Freidinger−Veber lactam" into a luteinizing hormone−releasing hormone (LH−RH) resulted in analogue **3** (Figure 1) with 8.9 times greater potency than the parent peptide in an *in vitro* pituitary cell culture assay.[18] Such lactam-containing peptides have since been used as chemotherapeutic and anticancer agents as well as modulators to study the renin-angiotensin-aldosterone (RAAS) system and serine protease inhibition.[21]

In comparison to its α-amino counterpart, β-amino-γ-lactam (Bgl, **2**, Figure 1) has been less frequently used to study peptide structures. The introduction of Bgl into peptides was first reported during studies of the insulin potentiating and hypoglycemic activities of the fragment [6−13] of human growth hormone (hGH) in which replacement of Asp[11] by (*S*)-Bgl conserved biological activity and extended the duration of action.[22−25] In addition, Bgl peptide analogues have exhibited CCK-A receptor agonism with high affinity and selectivity,[26] as well as enhancement of the binding of [^{3}H]*N*-propylnorapomorphine to dopamine receptors.[27]

Systematic scans of a peptide with Agl and Bgl residues may provide information concerning the structural requirements for biological activity by placing a constraint about the ψ-dihedral angle. In this way, lactam scanning complements proline scanning in which the φ-dihedral angle is locked by a structural constraint.[28] Proline restricts the φ-torsion angle to values of $-65 \pm 15°$, yet adds conformational liberty about its N-terminal amide. In Agl peptides, the γ-lactam forces the C-terminal amide into the trans-orientation and restricts the ψ-torsion angle to the range $-125 \pm 10°$,[29] favoring a type II′ β-turn geometry. Conformational analysis of Bgl peptides by NOESY-NMR

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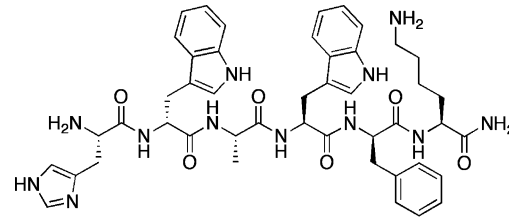
studies in DMSO and molecular dynamics indicated that it may stabilize a type II′ β-turn-like conformation.[25,30,31]

Several synthetic strategies for the synthesis of Agl peptide mimics have been reported.[21,32−38] Most are not suited for scanning peptides for biological activity because they require the synthesis of a dipeptide lactam in solution prior to incorporation into the peptide.[39,40] To date, dipeptides bearing terminal five- and six-membered lactams have only been synthesized on solid phase either without stereocontrol by alkylation of Shiff base protected imino esters with α,ω-dihaloalkanes[40] or by the microwave-assisted *N*-alkylation of resin-bound amino esters with fluorenylmethyl γ-iodo-*N*-(Boc)amino butyrate and intramolecular lactam formation, albeit in moderate yield.[41] Recently, the seven-member lactam Aia (4-amino-1,2,4,5-tetrahydro-indolo[2,3-*c*]-azepin-3-one) was introduced into biologically active peptides containing Trp residues by a solid-phase strategy featuring reductive amination using Fmoc-2′-formyl-tryptophan followed by lactam formation.[42] Although several solution-phase methods have been described,[22–24,43] to the best of our knowledge, no solid-supported methodology has been reported for Bgl peptide synthesis.

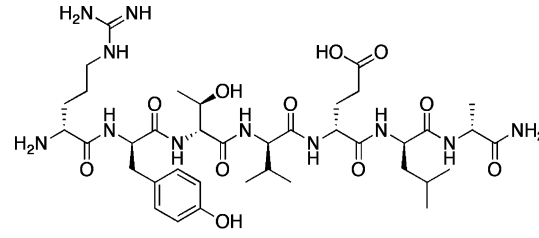
To enhance the application of amino lactams in peptide science and medicinal chemistry, we present a general Fmoc strategy for the solid-phase synthesis of peptides bearing Agl and Bgl residues featuring the regiospecific ring opening of enantiomerically pure five- and six-member cyclic sulfamidates **9** and **8** (Figure 1), followed by microwave-assisted lactam formation. This method has been used to systematically amino-lactam scan the growth hormone secretagogue GHRP-6 (HwAW-fK, Figure 2) and the IL-1 receptor I allosteric modulator 101.10 (rytvela, Figure 3).

Growth hormone-releasing peptides (GHRPs) are small synthetic peptides, which stimulate growth-hormone (GH) release by a pathway mediated by the G-protein coupled receptor GHS-R1a (the ghrelin receptor).[44] Within this class of peptides, GHRP-6 causes the release of GH from somatotrophs in a dose-dependent manner in several species, including humans,[45] and has been considered as a lead for developing treatments of GH



His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2

Figure 2. Structure and sequence of GHRP-6.



D-Arg-D-Tyr-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2

Figure 3. Structure and sequence of 101.10.

secretory deficiency and of impaired GH secretion related conditions such as cachexia[46] and aging.[47,48] In addition to their endocrine effect, GHRPs have exhibited cardioprotective effects in ischemic conditions,[49,50] which are suggested to be mediated by binding to a multi-ligand scavenger receptor CD36. Expressed in cardiomyocytes and microvascular endothelial cells, CD36 plays a key role in the development of atherosclerosis by binding to oxidized low density lipoproteins (oxLDL). The domains for oxLDL and GHRP binding overlap.[51,52] CD36 also features a distinct CLESH (CD36 LIMP-II Emp sequence homology) binding site for thrombospondin, which is a target for the inhibition of angiogenesis.[52] Upon their modulatory effect on the conformation of the scavenger receptor, GHRPs appear to display a large pharmacologic profile in the regulation of the development of atherosclerosis as well as that of angiogenesis,[53] suggesting their therapeutic potential in angiogenesis-related diseases such as age-related macular degeneration and diabetic retinopathy. The bioactive conformation of GHRP-6 has been suggested to adopt a turn motif based on molecular modeling studies.[54] A lactam scan of GHRP-6 using both Agl and Bgl was thus performed to study the importance of turn

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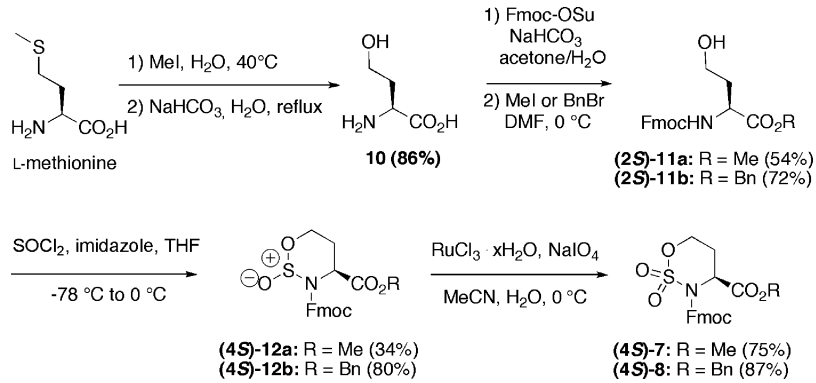
Scheme 1. Synthesis of (4*S*)-Methyl- and (4*S*)-Benzyl-2,2-dioxo-3-*N*-Fmoc-1,2,3-oxathiazinane-4-carboxylates **7** and **8**



geometry for biological activity and to validate the utility of the lactam scanning protocol in the presence of aliphatic (Ala), aromatic (D-Phe, Trp, and His), and polar (Lys) amino acid residues.

The D-heptapeptide 101.10 functions as an allosteric negative modulator of the activity generated from the formation of the interleukin IL-1 pro-inflammatory cytokine/IL-1 receptor I (IL-1RI) complex.[55] Exhibiting activity as a noncompetitive, potent, and selective extracellular IL-1R receptor antagonist, 101.10 blocked IL-1-induced human thymocyte proliferation *in vitro* and inhibited to variable extents different effects induced by IL-1 *in vivo* in established models of inflammation.[56] Seeking analogues with greater potency and understanding of the conformation by which 101.10 exhibits biological activity, a lactam scan was performed in which the robustness of our methodology was further tested in the presence of sterically hindered β-branched residues [Val and Thr(*O*-^{t}Bu)].

2. Results and Discussion

Five-member cyclic sulfamidates (2,2-dioxo-1,2,3-oxathiazolidines) and six-member cyclic sulfamidates (2,2-dioxo-1,2,3-oxathiazinanes) have emerged as effective amino alcohol-derived electrophiles for the synthesis of amino acid and heterocyclic products, in part, because the sulfamidate simultaneously activates the hydroxyl group and protects the amine moiety.[57,58] In particular, six-member cyclic sulfamidates, such as (4*S*)-cumyl-2,2-dioxo-3-*N*-PhF-1,2,3-oxathiazinane-4-carboxylate (**4**, PhF = 9-(9-phenylfluorenyl), Figure 1), have served in the solution-phase synthesis of lactam bridged dipeptides,[37,59] providing access to a greater variety of C-terminal amino acid residues; however, application of the products in peptide synthesis necessitated switching amine protection. (4*S*)-*tert*-Butyl-2,2-dioxo-3-*N*-Fmoc-1,2,3-oxathiazinane-4-carboxylate (**5**) was later employed in the solution-phase synthesis of Fmoc-Agl-Trp, which was later introduced by solid-phase chemistry, as a replacement of the Ala-Trp dipeptide unit, into the growth hormone secretagogue peptide GHRP-6 (HwAWfK, Figure 2).[59,60] With construction of the Agl moiety onto a resin-bound peptide as the next goal, (4*S*)-cumyl- and (4*S*)-methyl-2,2-dioxo-3-*N*-Fmoc-1,2,3-oxathiazinane-4-carboxylates (**6** and **7**, Figure 1) were respectively employed in the solid-phase syntheses of analogues of enkephalin[60] and GNRH.[61]

In these earlier studies, the ester type was found to influence significantly the reactivity of the 2,2-dioxo-3-*N*-Fmoc-1,2,3-oxathiazinane-4-carboxylate in both the peptide alkylation and lactam formation steps. A detailed study of the ester protective group was performed, and a practical solid-phase protocol for making Agl containing peptides using benzyl ester **8** has now been developed and employed in lactam scanning of GHRP-6 as well as the peptide allosteric modulator of the IL-1 receptor 101.10. Moreover, by employing the five-membered cyclic sulfamidate **9** ((4*S*)-methyl-2-[3-(9-fluorenylmethoxycarbonyl)-2,2-dioxo-1,2,3-oxathiazolidin-4-yl]acetate) in a similar protocol, Bgl has also been effectively introduced into peptides on solid phase.

2.1. Synthesis of Sulfamidate Reagents 7, 8, and 9. (4*S*)-Methyl- and (4*S*)-benzyl-2,2-dioxo-3-*N*-Fmoc-1,2,3-oxathiazinane-4-carboxylates **7** and **8** were selected as the key building blocks for the on-resin Agl synthesis and were obtained in solution from L-methionine as a chiral educt (Scheme 1). L-Homoserine **10** was prepared from L-methionine by *S*-alkylation followed by pH controlled intramolecular displacement to form homoserine lactone and subsequent hydrolytic ring opening.[62] *N*-Fmoc-homoserine methyl and benzyl esters **11a** and **11b** were prepared from L-homoserine **10** by acylation with *N*-(9-fluorenyl-methoxycarbonyloxy) succinimide (Fmoc-OSu) in a 1:1 acetone/water mixture containing sodium bicarbonate. After freeze-drying and without further purification, carboxylate *O*-alkylation of the anhydrous *N*-Fmoc-homoserine sodium salt with the appropriate alkyl halide (methyl iodide or benzyl bromide) was achieved in DMF. Purification by flash column

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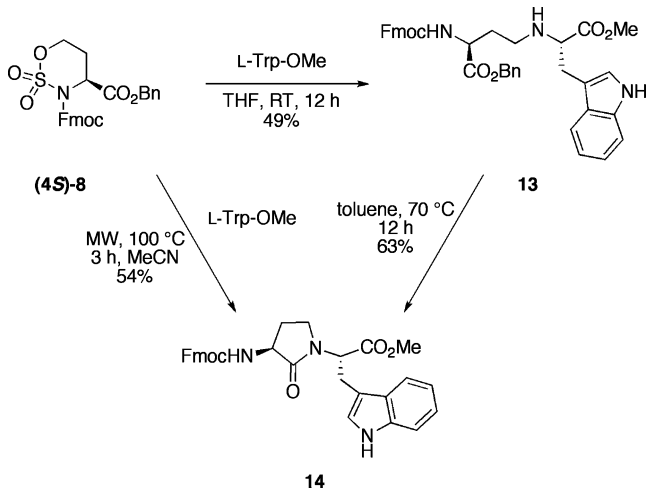
Scheme 2. Solution Phase Synthesis of Lactam Dipeptide **14**



chromatography[63] gave, respectively, methyl and benzyl *N*-(Fmoc)homoserine esters **11a** and **11b** in 54% and 72% yield over two steps. Methyl and benzyl *N*-(Fmoc)amino esters **11a** and **11b** were susceptible to lactonization and were purified immediately by chromatography and stored at −4 °C. Homoserinate **11a** needed to be used quickly before decomposition occurred; benzyl ester **11b** was relatively stable and could be stored as a solid for a longer time. Cyclic sulfamidites (2-oxo-1,2,3-oxathiazinanes) **12a** and **12b** were produced as a 2:1 and 4:1 mixtures of diastereomers, respectively, by treating *N*-Fmoc-homoserine esters **11a** and **11b** with a dilute premixed solution of thionyl chloride and imidazole in THF to minimize formation of sulfite dimer.[64] Subsequent oxidation to 2,2-dioxo-1,2,3-oxathiazinanes **7** and **8** was performed using catalytic ruthenium(III) trichloride hydrate (2 mol %) and sodium metaperiodate.[65] On a larger scale (>1 g), successful ruthenium catalyzed oxidation required higher catalyst loading (10 mol %) and efficient stirring of the biphasic medium in a Morton flask.[66] (4*R*)-Benzyl-2,2-dioxo-3-*N*-Fmoc-1,2,3-oxathiazinane-4-carboxylate [(*R*)-**11b**] was prepared from D-Met as described for the (4*S*)-enantiomer.

A study by ^{1}H NMR spectroscopy of the relative stability of cyclic sulfamidates **7** and **8** revealed that both compounds were stable as solutions in $CDCl_3$ for 4 weeks. However, when stored neat at 4 °C methyl ester **7** decomposed totally within 8 days as observed by TLC (50% EtOAc/Hexanes). Crystalline benzyl ester **8** was stable at 4 °C for 3 weeks before decomposition began to be observed by TLC (complete decomposition required longer than 2 months). Although preliminary studies demonstrated methyl ester **7** to be suitable for solid-phase lactam synthesis,[61] the limited stability of **7** relative to its benzyl ester counterpart **8** made the latter the reagent of choice for use in further studies.

The Agl-bridged dipeptide **14** was prepared in solution to examine the reactivity of sulfamidate **8** (Scheme 2). Nucleophilic addition of tryptophan methyl ester to cyclic sulfamidate **8** at room temperature in THF gave *N*-(aminopropyl)-amino ester **13** in 49% yield, after chromatography. Formation of **14** required 3 days in THF and acetonitrile, but only 12 h in the less polar solvent toluene at 70 °C. Lactam **14** was made next in one pot by microwave heating of Trp-OMe and **8** in acetonitrile for 3 h at 100 °C in 54% yield after chromatography.

For the synthesis of Bgl peptides, five-member cyclic sulfamidate **9** was synthesized from L-aspartic acid **15** as a chiral educt (Scheme 3). β-Methyl *N*-(Fmoc)aspartate (**16**)[67,68] was converted to alcohol **17** in 75% yield by activation as a mixed anhydride, using *iso*-butylchloroformate and *N*-methylmorpholine (NMM) and *in situ* reduction with sodium borohydride in THF/MeOH at low temperature (from −78 to −20 °C) to prevent lactone and diol formation.[69] 2,2-Dioxo-1,2,3-oxathiazolidine (**9**) was prepared from **17** in a two-step process as described for the preparation of its six-member counterpart above, without purification of the sulfamidite intermediate. Cyclic sulfamidate **9** was purified by flash chromatography and proved to be unstable if kept as an oil, degrading within a few days at 4 °C. Crystallization from diethyl ether after chromatography afforded **9** in 76% yield as white crystals, which were stable for several months at 4 °C. The reaction of **9** and Trp-OMe under microwave heating in acetonitrile at 100 °C for 3 h gave Fmoc-Bgl-Trp-OMe (**18**) in 78% yield.

2.2. Incorporation of Lactams into Peptides Using Solid-Phase Fmoc Chemistry. With five- and six-member cyclic sulfamidates **9** and **8** in hand, and their effectiveness in solution-phase chemistry demonstrated, the solid-phase synthesis of Agl and Bgl peptides was pursued on Rink amide resin. *N*-Alkylation was initially tested on Lys(ϵ-Boc) bound to Rink resin **19** using sulfamidate **8** (2 equiv) in THF for 12 h at room temperature; however, low amounts of the desired product were observed by LC−MS due to bis-alkylation of the amine. Several strategies to overcome this bis-alkylation issue were examined without success, including varying reaction temperature, sulfamidate stoichiometry, and rate of addition, as well as adding acid to protonate the secondary amine.

o-Nitrobenzenesulfonamide was then used as a temporary protecting group.[70−72] Resin-bound Lys(ϵ-Boc) **19** was reacted with *o*-nitrobenzenesulfonyl chloride (*o*-NBS-Cl) and 2,4,6-collidine in DCM to give *o*-nitrobenzenesulfonamide **20** (Scheme 4). Attempts to *N*-alkylate **20** using sulfamidate **8** (5 equiv) in THF for 24 h gave incomplete reactions. On the other hand, Mitsunobu reaction of nitrophenylsulfonamide **20** with Fmoc-Hse-OBn **11b** using triphenyl phosphine and diisopropyl azodicarboxylate (DIAD) in THF proceeded quantitatively in 24 h as determined by LC−MS analysis. Removal of the *o*-nitrobenzenesulfonyl group from *N*-alkyl sulfonamide **21** using different thiols and conditions failed; however, this is likely due to the use of the Rink amide linker, which was previously

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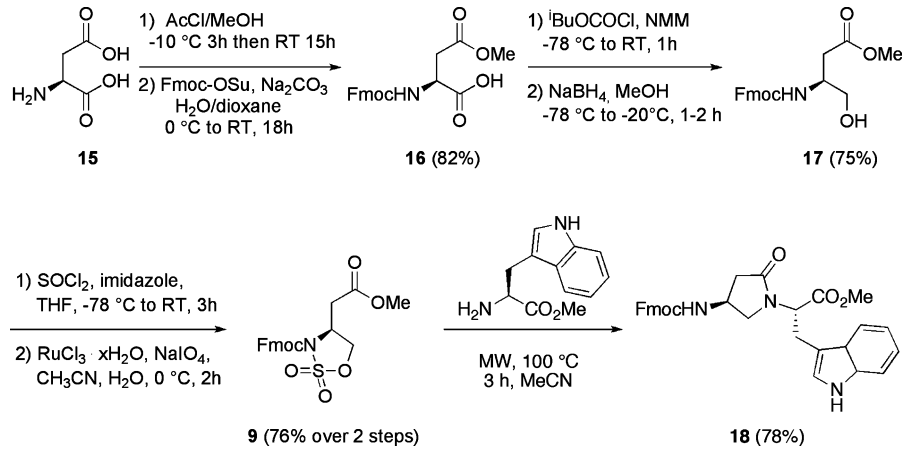
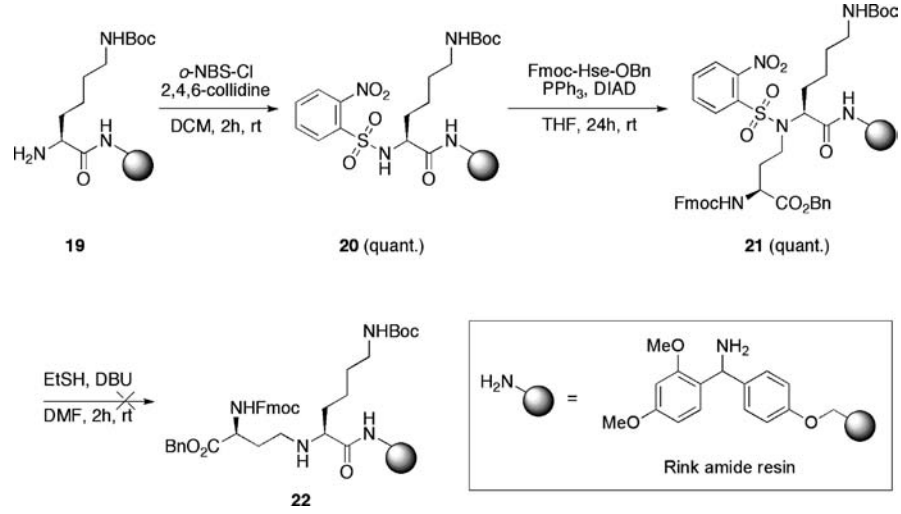
Scheme 3. Synthesis and Employment of Cyclic Sulfamidate **9** To Prepare Lactam Dipeptide **18**



Scheme 4. Temporary Amine *o*-NBS Protection Strategy



suggested to prevent *o*-NBS removal during the synthesis of diketopiperazines.[73]

Alkylation of *N*-trimethylsilyl lithium amides with alkylhalides and epoxides has been used to selectively prepare secondary amines after solvolysis of the aminosilane.[74] Peptide alkylation was thus pursued by silylation with *N*,*O*-bis(trimethylsilyl)acetamide (BSA), reaction with sulfamidates **8** and **9**, and silyl group removal by washing the resin with methanol. Resin-bound Lys(ϵ-Boc) **19** was thus treated with BSA and shaken for 12 h (which caused an observable color change of the resin from yellow to lighter yellow), filtered under argon, and treated with a solution of the appropriate sulfamidate (5 equiv) in dry THF. After 24 h, **9** and **8** gave, respectively, monoalkylated products in 64% and 97% conversion, with <2% bis-alkylated product, as assessed by LC−MS analysis of material cleaved from resin samples (Scheme 5).

Lactam formation was next achieved by heating secondary amine resins **22a** and **22b** at 100 °C in DMF over 3 days. This time was reduced to 3 h in the case of Bgl and 75 min in the case of Agl by microwave heating at 100 °C in DMF, conditions which gave cleaner products and >95% conversion in both cases (Scheme 5).

At the lactam forming step, the resulting resins **24a** and **24b** exhibited positive Kaiser tests,[75] indicating the presence of primary amines, which were considered to be due to loss of the Boc group from the Lys residue by thermolysis (Fmoc-Bgl/Agl-Lys-NH-Rink) as well as deprotected and unreacted starting peptide (Lys-NH-Rink). This was verified by treating an aliquot of resin **22b** with Fmoc-OSu in the presence of DIEA in DCM. After TFA cleavage, analysis by LC−MS indicated Fmoc-Bgl-Lys(Fmoc)-NH_2 as the major compound (60%) along with Fmoc-Lys(Fmoc)-NH_2 (30%). A capping step was therefore introduced after the lactam formation by treating the resin with di-*tert*-butyl dicarbonate (Boc_2O) and DIEA in DMF for 1.5 h, after which time resins **25a** and **25b** gave negative Kaiser tests.

2.3. Enantiomeric Purity Investigations. RP-HPLC analysis of crude Agl peptides **34**−**38** and **44**−**49** (*vide infra*) showed small quantities (<10%) of an isobaric species. In the synthesis of the model Agl tripeptides L-Ala-(*S*)-Agl-L-Ala-NH_2 and D-Ala-(*S*)-Agl-L-Ala-NH_2 (see Supporting Information), no epimerization was detected during the alkylation and lactam formation steps; however, chiral supercritical fluid chromatography (chiral SFC, see Supporting Information for conditions) analyses demonstrated that the cyclic sulfamidate **8** had an enantiomeric ratio of 9:1, explaining the level of epimers observed in crude Agl peptides. The synthesis of homoserine

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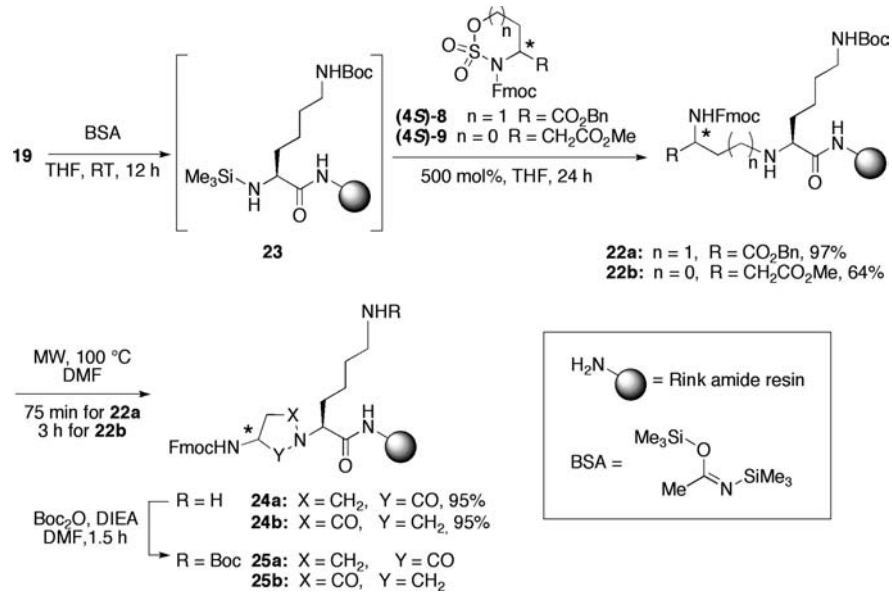
Scheme 5. Solid-Phase Synthesis of Lactam Bridged Dipeptide **25**



from methionine, which has previously been shown to cause racemization,[76] accounts for the preparation of **8** in 80% ee. Enantiomerically pure sulfamidate **8** was also synthesized from commercially available homoserine (99% ee, see Supporting Information); however, using sulfamidate **8** prepared from methionine was not considered as a drawback, as epimeric impurities were easily separated by preparative RP-HPLC from the desired Agl peptides. Only one isomer of crude Bgl peptides **39**−**43** (*vide infra*) could be detected by RP-HPLC under various conditions. The purity of sulfamidate **9** prepared from L- and D-aspartate was investigated by chiral SFC analysis, which showed sulfamidates (4*S*)- and (4*R*)-**9** to have, respectively, 95.8% and 97.9% enantiomeric excess.

2.4. Lactam Scan of GHRP-6. Using sulfamidates **8** and **9** in a general protocol for solid-phase lactam-bridged peptide synthesis, 10 analogues of GHRP-6 were synthesized (Scheme 6). Standard conditions for solid-phase peptide synthesis with Fmoc protection were first employed to synthesize the peptide chain **27** on Rink amide resin (see Supporting Information for protocols).[77] At the appropriate residue for lactam formation, the N-terminal Fmoc group was removed and the resin was dried *in vacuo*. Using the optimized conditions described earlier, the N-terminal amine was silylated, alkylated with the respective sulfamidate (**8** or **9**), and converted to the lactam by microwave irradiation. After Fmoc removal, the remaining amino acid residues were coupled to the amino lactam residue **30** and **31** using standard coupling conditions. Reaction progress was monitored by LC−MS analyses of material cleaved from a small resin sample (3−5 mg). After solid-phase synthesis, the terminal Fmoc group of lactam peptide **32** was removed, and the peptide was cleaved in a mixture of TFA/TES/H_2O (95:2.5:2.5, v/v/v). The crude lactam peptide **33** was precipitated in cold diethyl ether, analyzed by analytical RP-HPLC for crude purity, and purified by preparative RP-HPLC to give the desired peptides **34**−**43** as white foams (Table 1).

2.5. Lactam Scan of 101.10. Lactam peptides were made in higher yield and purity using Rink amide MBHA resin in the synthesis of 101.10 analogues, instead of Rink amide resin (Table 2). The solid-phase protocol was performed using (*R*)-cyclic sulfamidate (4*R*)-**8** as described above for the preparation of GHRP-6 lactam analogues. The desired lactam peptides **44**−**49** were recovered in 36−81% crude purity as assessed by analytical RP-HPLC (Table 2). Exploring alternative microwave conditions, we found that improved conversion to lactam was achieved using 1% acetic acid in DMSO. Nevertheless, microwave-assisted intramolecular acylation required longer reaction times (>4 h) in the synthesis of analogues **45** and **46**, presumably due to the difficulties of cyclization on sterically bulky β-alkyl branched amino acids. For example, formation of Agl on the D-Thr(*O*-*t*Bu) residue required 10 h of microwave heating of resin bound amine **28** at 110 °C in 1% acetic acid in DMSO and gave at best 50% conversion. Longer reaction times affected significantly the purity of the lactam peptides. The linear secondary amine could be isolated after the sequence of reactions by RP-HPLC and was subjected to lactam formation conditions in solution to provide additional lactam peptide. The main impurity observed by analytical RP-HPLC of the crude material after cleavage from resin was typically the deletion sequence from incomplete sulfamidate alkylation.

2.6. Biological Examinations. 2.6.1. Binding Affinity IC_{50} Values of GHRP-6 Lactam Analogues 34−43 on GHS-R1a and CD36 Receptors. The affinity of Agl- and Bgl-containing GHRP-6 analogues **34**−**43** for the ghrelin receptor was assessed in a competition binding study using radiolabeled ghrelin$^{1-28}$ as a radioligand and membranes from GHS-R1a transfected cells as a binding partner.[78] The affinity of amino lactam derivatives for the scavenger receptor CD36 has been assessed by a covalent competition binding study using a radiolabeled photoactivatable hexarelin derivative as a radioligand and rat cardiac membranes as a source of CD36 receptor as previously reported.[79] GHRP-6 lactam analogues were used as competitive ligands in the concentration range from 10^{-12} to 10^{-6} M, and IC_{50} values of competition binding

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Scheme 6. General Protocol for Solid-Phase Synthesis of Freidinger−Veber Lactam-Bridged Peptides

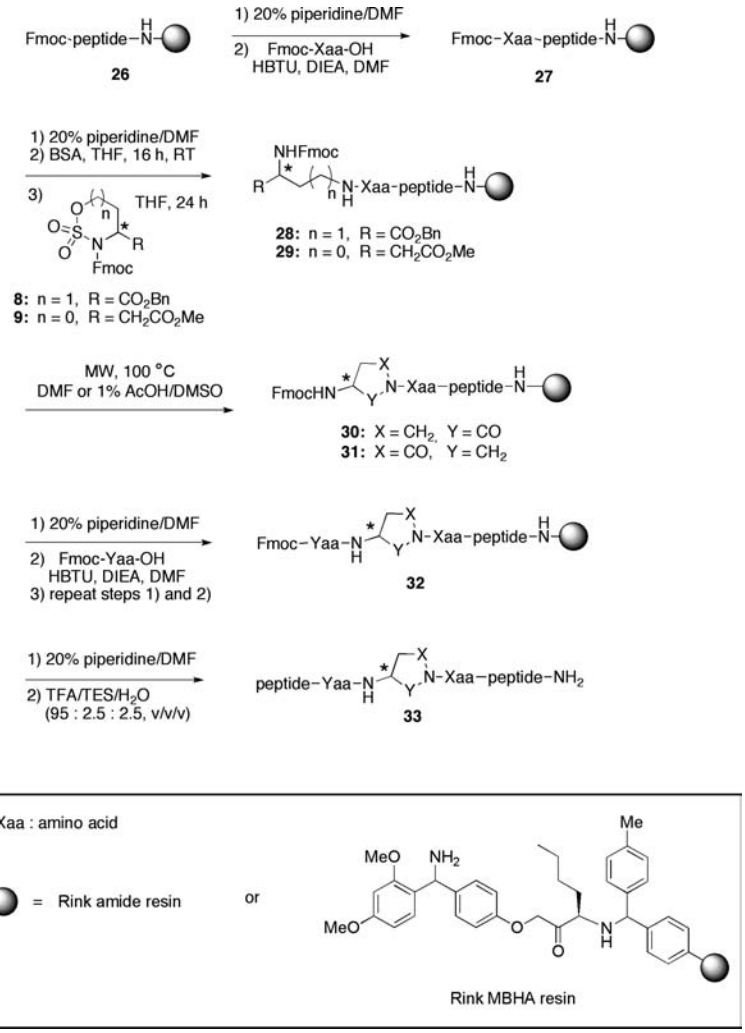

Table 1. Yields and Purities of Lactam-Bridged GHRP-6 Peptides **34**−**43**

peptide[a]	crude purity, %[b]	purity, %[c]	yield, %[d]	HRMS *m/z* (calcd)	HRMS *m/z* (obsd)
(*S*)-Agl-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 **34**	30	>99	8	819.4300	819.4291
His-**(*S*)-Agl-**Ala-Trp-D-Phe-Lys-NH_2 **35**	36	>99	22	385.7085	385.7094
His-D-Trp-**(*S*)-Agl-**Trp-D-Phe-Lys-NH_2 **36**	22	>99	11	443.2304	443.2296
His-D-Trp-Ala-**(*S*)-Agl-**D-Phe-Lys-NH_2 **37**	66	>99	11	770.4096	770.4090
His-D-Trp-Ala-Trp-**(*S*)-Agl-**Lys-NH_2 **38**	44	>99	13	405.2139	405.2149
(*S*)-Bgl-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 **39**	39	92	22	819.4300	819.4298
His-**(*S*)-Bgl-**Ala-Trp-D-Phe-Lys-NH_2 **40**	26	91	12	770.4096	770.4097
His-D-Trp-**(*S*)-Bgl-**Trp-D-Phe-Lys-NH_2 **41**	46	>99	25	885.4518	885.4511
His-D-Trp-Ala-**(*S*)-Bgl-**D-Phe-Lys-NH_2 **42**	52	>99	6	770.4097	770.4089
His-D-Trp-Ala-Trp-**(*S*)-Bgl-**Lys-NH_2 **43**	92	>99	5	809.4205	809.4198

[a] Bold lettering indicates lactam residues. [b] RP-HPLC purity at 214 nm of the crude peptide. [c] RP-HPLC purity at 214 nm of the purified peptide. [d] Yields after purification by RP-HPLC are based on Fmoc loading test for Rink resin.

curves were calculated by an iterative nonlinear curve-fitting program (Table 3, Prism 3 Graph Pad software).

Most lactam analogues lost binding affinity for both the GHS-R1a and CD36 receptors typically with the substitution of Trp residues by Agl or Bgl. However, lactam **36**, in which Ala^3 was replaced by (*S*)-Agl, retained a similar binding affinity for the CD36 receptor, with a loss of binding ability to the GHS-R1a receptor by a factor of 10^3 relative to GHRP-6. In the cases of **38** and **43**, in which D-Phe^5 was, respectively, replaced by (*S*)-Agl and (*S*)-Bgl, and **35** where D-Trp^2 was substituted by (*S*)-Agl, the influence of the lactam moiety was similarly more significant on the GHS-R1a receptor, for which the binding affinity decreased by a factor of 10^3-10^4, compared to that on the CD36 receptor with a reduction of binding affinity by a factor of 15. Considering that both Agl and Bgl may induce turn conformations, the affinity of lactam analogues of GHRP-6 supports the hypothesis for a β-turn conformation about residues 2−4, which may be responsible for binding and differentiating the CD36 receptor from the GHS-1Ra receptor. Finally, replacement of the His^1 residue with (*S*)-Agl and (*S*)-Bgl

Table 2. Yields and Purities of Lactam Containing Analogues of 101.10 **44**−**49**

peptide[a]	crude purity, %[b]	purity, %[c]	yield, %[d]	HRMS *m/z* (calcd)	HRMS *m/z* (obsd)
(***R***)-**Agl**-D-Tyr-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2 **44**	55	>99	19	777.4141	777.4138
D-Arg-(***R***)-**Agl**-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2 **45**	41	>99	15	770.4519	770.4514
D-Arg-D-Tyr-(***R***)-**Agl**-D-Val-D-Glu-D-Leu-D-Ala-NH_2 **46**	58	>99	7	832.4675	832.4676
D-Arg-D-Tyr-D-Thr-(***R***)-**Agl**-D-Glu-D-Leu-D-Ala-NH_2 **47**	50	>99	11	833.4468	833.4458
D-Agl-D-Tyr-D-Thr-D-Val-(***R***)-**Agl**-D-Leu-D-Ala-NH_2 **48**	36	>99	21	803.4726	803.4724
D-Arg-D-Tyr-D-Thr-D-Val-D-Glu-(***R***)-**Agl**-D-Ala-NH_2 **49**	81	>99	7	820.4311	820.4306

[a] Bold lettering indicates lactam residue. [b] RP-HPLC purity at 214 nm of the crude peptide. [c] RP-HPLC purity at 214 nm of the purified peptide. [d] Yields after purification by RP-HPLC are based on Fmoc loading test for Rink resin.

Table 3. IC_{50} Binding Values for GHS-R1a and CD36 Receptors for Lactams **34**−**43**

entry	compound	peptide	IC_{50} binding GHS-R1a	IC_{50} binding CD36
1	GHRP-6	His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2	6.08×10^{-9} M	
2	hexarelin	His-D-2-Me-Trp-Ala-Trp-D-Phe-Lys-NH_2		3.33×10^{-6} M
3	**34**	(***S***)-**Agl**-D-Trp-Ala-Trp-D-Phe-Lys-NH_2	4.09×10^{-8} M	1.45×10^{-5} M
4	**35**	His-(***S***)-**Agl**-Ala-Trp-D-Phe-Lys-NH_2	2.86×10^{-5} M	1.34×10^{-5} M
5	**36**	His-D-Trp-(***S***)-**Agl**-Trp-D-Phe-Lys-NH_2	2.39×10^{-6} M	7.45×10^{-6} M
6	**37**	His-D-Trp-Ala-(***S***)-**Agl**-D-Phe-Lys-NH_2	$\gg 10^{-5}$ M	$\gg 10^{-5}$ M
7	**38**	His-D-Trp-Ala-Trp-(***S***)-**Agl**-Lys-NH_2	3.10×10^{-6} M	2.14×10^{-5} M
8	**39**	(***S***)-**Bgl**-D-Trp-Ala-Trp-D-Phe-Lys-NH_2	3.71×10^{-7} M	$\gg 10^{-5}$ M
9	**40**	His-(***S***)-**Bgl**-Ala-Trp-D-Phe-Lys-NH_2	$\gg 10^{-5}$ M	$\gg 10^{-5}$ M
10	**41**	His-D-Trp-(***S***)-**Bgl**-Trp-D-Phe-Lys-NH_2	6.54×10^{-7} M	$\gg 10^{-5}$ M
11	**42**	His-D-Trp-Ala-(***S***)-**Bgl**-D-Phe-Lys-NH_2	$\gg 10^{-5}$ M	$\gg 10^{-5}$ M
12	**43**	His-D-Trp-Ala-Trp-(***S***)-**Bgl**-Lys-NH_2	2.45×10^{-5} M	2.65×10^{-5} M

(compounds **34** and **39**) appears to affect relatively less the binding affinity toward the ghrelin receptor than it does with CD36. This flexibility observed with modifications of the His^1 for binding on the GHS-1Ra receptor is consistent with previous analogues featuring substitutions at this position.[80]

2.6.2. Inhibition of Thermocyte TF-1 Proliferation by 101.10 Lactam Analogues 44−49. The efficacy of Agl peptides **44**−**49** was ascertained by measuring their influence on IL-1 induced human thymocyte TF-1 proliferation as assessed by incorporation of [^{3}H]thymidine as previously described.[55a] Among the analogues tested, five maintained some inhibitory effect on TF-1 proliferation (Figure 4). Peptide **46** failed to block proliferation of TF-1 cells treated with IL-1. Relative to 101.10, lactams **45**, **47**, **48**, and **49** all exhibited similar efficacy, suggesting that the balance between side chain removal and conformational constraint at positions 2 and 4−6 does not perturb activity. Replacement of the N-terminal D-Arg residue by (*R*)-Agl in compound **44** led to a 2.2-fold increase in efficacy compared to 101.10, suggesting that the Arg^1 side chain may not be necessary for activity.

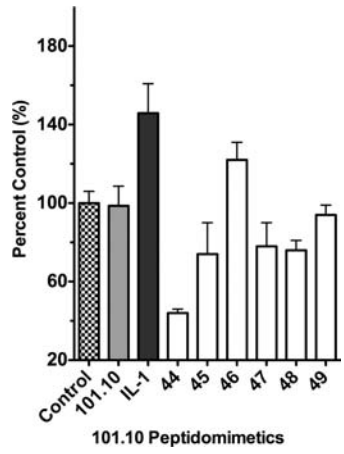

Figure 4. Inhibition of IL-1 induced TF-1 cell proliferation by compounds **44**−**49**.

3. Conclusion

An efficient solid-phase methodology was developed for the synthesis of α- and β-amino γ-lactam peptides by regioselective ring opening of six- and five-member cyclic sulfamidates, followed by microwave irradiation to effect lactam formation. A proof-of-concept was demonstrated by the synthesis of amino lactam tryptophan methyl ester dipeptides **14** and **18** in solution. In the alkylation step on solid phase, peptide amine bis-alkylation was overcome by using a temporary silyl amine protection strategy. Lactam scanning was performed on both GHRP-6 and the heptapeptide 101.10 giving the peptide lactams in good overall purified yields and high crude purity. Evaluation of the lactam analogues of GHRP-6 showed that peptides bearing Agl and Bgl at position 3 maintained an affinity for the CD36 receptor and lost affinity toward the GHS-R1a receptor, in support of a β-turn conformation for achieving affinity and selectivity of GHRP-6 analogues. The synthesis of six Agl-containing 101.10 analogues and the assessment of their efficacy in inhibiting IL-1 induced TF-1 cell proliferation led to the discovery of a more efficient compound than 101.10 itself. Lactam scanning has thus proven useful for rapidly identifying peptide secondary structures required for affinity and biological activity.

4. Experimental Section

See Supporting Information for general chemistry methods.

4.1. (*S*)-Benzyl-4-[(*S*)-3-(1*H*-indol-3-yl)-1-methoxy-1-oxopropan-2-ylamino]-2-[*N*-(9-fluorenylmethoxycarbonyl)amino]-butanoate (13). Tryptophan methyl ester hydrochloride (70 mg, 0.27 mmol) was dissolved in water (5 mL), saturated with potassium

carbonate, and extracted with chloroform (5 × 10 mL). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to give the free amino ester, which was subsequently dissolved in THF (1 mL) and added to a solution of (4*S*)-benzyl-2,2-dioxo-3-(9-fluorenylmethoxycarbonyl)-1,2,3-oxathiazinane-4-carboxylate [(4*S*)-**8**, 45 mg, 0.09 mmol] in THF (1 mL). The reaction mixture was stirred at room temperature for 12 h and treated with 1 M monopotassium phosphate (4 mL). The layers were separated. The aqueous layer was extracted with EtOAc (4 × 10 mL). The combined organic layers were dried ($MgSO_4$) and concentrated to a solid that was purified by flash column chromatography (30% EtOAc in hexanes). Evaporation of the collected fractions gave (*S*)-benzyl 4-[(*S*)-3-(1*H*-indol-3-yl)-1-methoxy-1-oxopropan-2-ylamino]-2-[*N*-(9-fluorenylmethoxycarbonyl)-amino]butanoate (**13**, 28 mg, 49%) as a white foam: R_f 0.29 (50% EtOAc/Hexanes): $[\alpha]_D^{20}$ −7.3 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.80−1.97 (m, 2H, 2′-H_2), 2.51 (m, 1H, 1′-*H*H), 2.72 (m, 1H, 1′-H*H*), 3.06 (dd, 1H, *J* = 14.4, 5.5 Hz, 3-*H*H), 3.15 (dd, 1H, *J* = 14.4, 7.1 Hz, 3-H*H*), 3.56 (t, 1H, *J* = 6.1 Hz, 2-H), 3.63 (s, 3H, OMe), 4.23 (t, 1H, *J* = 6.5 Hz, Fmoc-CH), 4.34−4.49 (m, 3H, 2′-H and Fmoc-CH_2), 5.11 (d, 1H, *J* = 12.2 Hz, OC*H*HPh), 5.17 (d, 1H, *J* = 12.2 Hz, OCH*H*Ph), 6.55 (br d, 1H, *J* = 7.4 Hz, 3′-NH), 6.96−7.76 (m, 19H, ar-H) and 7.94 (s, 1H, Trp-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.9 (CH_2), 30.7 (CH_2), 44.1 (CH_2), 47.1 (CH), 51.8 (CH), 53.1 (CH), 61.3 (CH_3), 66.6 (CH_2), 66.9 (CH_2), 111.1 (ar-CH), 118.4 (ar-CH), 119.3 (ar-CH), 119.8 (ar-CH), 121.9 (ar-CH), 123.0 (ar-CH), 124.9 (ar-CH), 125.0 (ar-CH), 126.9 (ar-CH), 127.1 (ar-C), 127.6 (ar-CH), 128.1 (ar-CH), 128.4 (ar-CH), 135.3 (ar-C),136.0 (ar-C), 141.1 (ar-C), 143.7 (ar-C), 143.9 (ar-C), 156.1 (ar-C), 172.0 (C═O) and 174.3 (C═O); ν_{max}/cm^{-1} (NaCl): 3338 (NH), 2952 (CH), 1723 (CO), 1451 (C═C), 1070, 959 and 741; HRMS Calcd *m*/*z* for $C_{38}H_{38}O_6N_3$ $[M + H]^+$ 632.2755, found 632.2755.

4.2. (*S*)-Methyl-2-{(*S*)-3-[*N*-(9-fluorenylmethoxycarbonyl)amino]-2-oxopyrrolidin-1-yl}-3-(1*H*-indol-3-yl)propanoate (14). (*S*)-Benzyl 4-[(*S*)-3-(1*H*-indol-3-yl)-1-methoxy-1-oxopropan-2-ylamino]-2-[*N*-(9-fluorenyl-methoxycarbonyl)amino]butanoate (**13**, 9 mg, 0.02 mmol) was dissolved in toluene (3 mL) and stirred at 70 °C for 12 h, when LC−MS analysis indicated complete consumption of starting material: analytical RP-HPLC using 20−80% MeCN (0.1% FA) in water (0.1% FA) over a 4 min gradient (Gemini 5u C18 110A 50 mm × 4.6 mm, 5 μm column from Phenomenex Inc., Torrance, California); amine **13**, t_R 5.35; lactam **14**, t_R 7.69. The reaction mixture was concentrated on a rotary evaporator and purified by column chromatography (30% EtOAc/hexanes) to give (*S*)-methyl 2-{(*S*)-3-[*N*-(9-fluorenylmethoxycarbonyl)amino]-2-oxopyrrolidin-1-yl}-3-(1*H*-indol-3-yl)propanoate **14** as a white foam (5 mg, 63%): R_f 0.29 (50% EtOAc/Hexanes); $[\alpha]_D^{20}$ −1.3 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.75−1.95 (m, 1H, 4-*H*H), 2.51 (m, 1H, 4-H*H*), 3.25 (dd, 2H, *J* = 15.6, 11.2 Hz, 5-H_2), 3.45 (m, 2H, 3′-H_2), 3.78 (s, 3H, OMe), 4.05−4.21 (m, 2H, Fmoc-H and 3-H), 4.35 (d, 2H, *J* = 6.4 Hz, Fmoc-H_2), 5.20 (dd, 1H, *J* = 11.2, 4.2 Hz, 2′H), 5.40 (d, 1H, *J* = 4.0 Hz, 3-NH), 7.00 (br s, 1H, 2-NH), 7.11−7.42 (m, 12H, ar-H) and 8.12 (br s, 1H, NH-Trp); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.2 (CH_2), 27.5 (CH_2), 41.4 (CH_2), 47.3 (CH_3), 52.8 (CH), 54.6 (CH), 67.3 (CH_2), 110.6 (ar-C), 111.5 (ar-CH), 116.8 (CH), 118.4 (ar-CH), 119.9 (ar-CH), 120.1 (ar-CH), 122.0 (ar-CH), 122.6 (ar-CH), 125.3 (ar-CH), 127.3 (ar-CH), 127.9 (ar-CH), 136.3 (ar-C), 141.5 (ar-C), 144.0 (ar-C), 144.1 (ar-C) and 171.1 (C═O); ν_{max}/cm^{-1} (NaCl): 3335 (NH), 2952 (CH), 1692 (CO), 1524, 1450, 1248, 1053 and 741; HRMS Calcd *m*/*z* for $C_{31}H_{30}O_5N_3$ $[M + H]^+$ 524.2180, found 524.2191.

4.3. (*S*)-Methyl-2-{(*S*)-3-[*N*-(9-fluorenylmethoxycarbonyl)amino]-2-oxopyrrolidin-1-yl}-3-(1*H*-indol-3-yl)propanoate (14). Tryptophan methyl ester hydrochloride (133 mg, 0.61 mmol) was converted to its free amino ester as described above, dissolved in acetonitrile (2 mL), and the solution was added to (4*S*)-benzyl-2,2-dioxo-3-(9-fluorenylmethoxycarbonyl)-1,2,3-oxathiazinane-4-carboxylate [(4*S*)-**8**, 100 mg, 0.2 mmol] in a 2 mL microwave vessel. The reaction mixture was heated to 100 °C under microwave irradiation for 3 h and treated with 1 M monopotassium phosphate (4 mL). The two layers were separated, and the aqueous layer was extracted with EtOAc (4 × 10 mL). The combined organic layers were dried ($MgSO_4$) and concentrated to a solid that was purified by flash column chromatography (30% EtOAc/hexanes) to give (*S*)-methyl 2-{(*S*)-3-[*N*-(9-fluorenylmethoxycarbonyl)amino]-2-oxopyrrolidin-1-yl}-3-(1*H*-indol-3-yl)propanoate (**14**, 56 mg, 54%) as a white foam.

4.4. (*S*)-Methyl-2-{(*S*)-4-[*N*-(9-fluorenylmethoxycarbonyl)amino]-2-oxopyrrolidin-1-yl}-3-(1*H*-indol-3-yl)propanoate (18). Tryptophan methyl ester hydrochloride (157 mg, 0.72 mmol) was converted to its free amino ester as described above and dissolved in acetonitrile (2 mL), and the solution was added to (4*S*)-methyl-2-[3-(9-fluorenylmethoxycarbonyl)-2,2-dioxo-1,2,3-oxathiazolidin-4-yl]acetate (**9**, 100 mg, 0.24 mmol) in a 2 mL microwave vessel. The reaction mixture was heated to 100 °C under microwave irradiation for 3 h and treated with 1 M monopotassium phosphate (4 mL). The layers were separated. The aqueous layer was extracted with EtOAc (4 × 10 mL). The combined organic layers were dried ($MgSO_4$) and concentrated using a rotary evaporator to give a yellow oil that was purified by flash column chromatography (60% EtOAc/hexanes) to yield (*S*)-methyl 2-{(*S*)-4-[*N*-(9-fluorenylmethoxycarbonyl)amino]-2-oxopyrrolidin-1-yl}-3-(1*H*-indol-3-yl)propanoate (**18**, 98 mg, 78%) as a white foam: R_f 0.26 (80% EtOAc/Hexanes); $[\alpha]^{23}_D$ 20.5 (*c* = 1.95 in $CHCl_3$); ^{1}H NMR (300 MHz, acetone-d_6) δ 2.28 (dd, 1H, C*H*H−C═O lactam, *J* = 5.6, 16.7 Hz), 2.62 (dd, 1H, CH*H*−C═O lactam, *J* = 8.1, 16.7 Hz), 3.22 (dd, 1H, C*H*H Trp, *J* = 10.1, 15.0 Hz), 3.37 (m, 1H, C*H*H−N lactam), 3.42 (dd, 1H, CH*H* Trp, *J* = 5.4, 15.0 Hz), 3.69 (s, 3H, CH_3), 3.74 (dd, 1H, CH*H*−N lactam, *J* = 9.2, 16.8 Hz), 4.19 (t, 1H, Fmoc−CH, *J* = 6.8 Hz), 4.28 (m, 1H, *CH*−N), 4.33 (d, 2H, Fmoc−CH_2, *J* = 6.8 Hz), 5.13 (dd, 1H, α-H Trp, *J* = 5.7, 9.6 Hz), 6.68 (d, 1H, NH, *J* = 6.1 Hz), 7.02 (td, 1H, H_5 indole, *J* = 1.2, 6.9 Hz), 7.11 (td, 1H, H_6 indole, *J* = 1.2, 6.9 Hz), 7.18 (s, 1H, H_2 indole), 7.30 (t, 2H, 2 × ar-H Fmoc, *J* = 7.1 Hz), 7.40 (t, 2H, 2 × ar-H Fmoc, *J* = 7.1 Hz), 7.37 (d, 1H, H_7 indole, *J* = 7.8 Hz), 7.61 (d, 1H, H_4 indole, *J* = 7.5 Hz), 7.64 (d, 2H, 2 × ar-H Fmoc, *J* = 7.4 Hz), 7.84 (d, 2H, 2 × ar-H Fmoc, *J* = 7.5 Hz), 10.01 (s, 1H, NH indole);^{13}C NMR (75 MHz, acetone-d_6) δ 25.6 (CH_2), 37.7 (CH_2), 45.9 (CH), 48.0 (CH), 51.1 (CH_2), 52.4 (CH_3), 54.7 (CH), 66.8 (CH_2), 109.9 (ar-CH), 111.5 (ar-CH), 118.3 (ar-CH), 119.7 (ar-CH), 120.1 (ar-CH), 122.2 (ar-CH), 122.4 (ar-CH), 124.9 (ar-CH), 126.7 (ar-CH), 127.3 (ar-C), 128.0 (ar-C), 136.0 (ar-C), 141.4 (ar-C), 143.8 (ar-C), 155.6 (C═O), 171.1 (C═O), 173.2 (C═O); HRMS calcd for $C_{31}H_{30}N_3O_5$ $[M + H]^+$: 524.2180, found: 524.2180; ν_{max}/cm^{-1} (NaCl): 3432 (N−H), 3019 (C−H arom.), 1738, 1714, 1693 (C═O), 1514, 1479, 1450 (C═C arom.), 1435 (C−H alkyl), 1213 (C−O ester), and 753.

4.5. General Experimental Procedures for Lactam Synthesis on Resin. 4.5.1. N-Terminal Amine Silylation and Alkylation with Sulfamidates. After the Fmoc protecting group was removed using a 20% piperidine solution, the resin was dried *in vacuo* for at least 3 h. The anhydrous resin, in a plastic filtration tube with a polyethylene frit, was then flushed with argon, swollen in dry THF under argon, and treated with BSA (5 equiv., to reach a concentration of 0.15 M in dry THF), which caused an observable color change of the resin (from yellow to lighter yellow), and shaken for 16 h in the filtration tube tightly sealed with stoppers and parafilm. The resin was then filtered under argon, treated with a solution of sulfamidate **8** or **9** (5 equiv, 0.15 M in dry THF) under argon, and shaken for 24 h in the filtration tube tightly sealed with stoppers and parafilm. The resin was then filtered and washed under argon with THF (3×), MeOH (3×), and DCM (3×) and dried *in vacuo*.

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4.5.2. Microwave Assisted Annulation. A 2 mL glass V-shaped microwave vial was charged with resin (not more than 100 mg to ensure efficient stirring and homogeneous microwave irradiation), a triangular stirring bar, and either DMF (2 mL) (conditions A) or a freshly prepared 1% acetic acid/DMSO solution (2 mL) (conditions B). The vial was sealed, heated in the microwave at 100 °C (pressure 1 bar) for 75 min−10 h (see Supporting Information for residue-specific time), and then cooled using a jet of air. The resin was then washed from the microwave vessel into a plastic filtration tube with a polyethylene frit and washed by shaking for 1 min with DMF (3×), MeOH (3×), and DCM (3×) and then dried *in vacuo*.

4.6. Membrane Preparation for GHS-R1a Receptor. 4.6.1. Transfection. LLC-PK1 cells were seeded at 1.5×10^6 cells/10 cm Petri dishes and grown for 24 h in DMEM high-glucose (4.5 g/L) with 10% fetal bovine serum supplemented with penicillin (10 000 Units/mL) and streptomycin (10 000 μg/mL) and cultured at 37 °C, with 5% CO_2. The medium was then replaced for another 4−5 h before $Ca_3(PO_4)_2$ calcium phosphate transfection. The DNA solution consists of 40 μg of DNA in a volume of 500 μL in which were added 500 μL of 2 mM Tris-HCl pH 8.0, 0.2 mM EDTA pH 8.0 containing 500 mM $CaCl_2$, to a final volume of 1 mL. Then 1 mL of 50 mM Hepes, 280 mM NaCl, 1.5 mM Na_2HPO_4 pH to 7.1 (HBSS) was added by alterning 1 drop/2 air bubbles. The transfection mix reaction was incubated at RT for 30 min. After the incubation period, 1 mL of the mix was added to each plate and distributed evenly for incubation. The media was replaced with standard DMEM-high glucose media for another 24 h, and cells were collected for membrane preparation.

4.6.2. Membrane Preparation. The experiment was carried out at 4 °C unless specified. Cells were washed 2 times with PBS and with the homogenization buffer (HB) consisting of 50 mM Tris, 5 mM $MgCl_2$, 2.5 mM EDTA, 30 μg/mL bacitracin at pH 7.3 and were scraped in Eppendorf tubes. Cells were lyzed with 2 cycles of freeze/thawing using liquid nitrogen and were then centrifuged at 4 °C for 20 min at 10 000 *g* to collect the membranes. The membranes were resuspended in a small volume of HB and aliquoted for storage at −80 °C.

4.7. GHS-R1a Receptor Binding Assay. The competitive binding assay consists of 200 μL of HB, 100 μL of ^{125}I-Ghrelin (40 000 cpm), 100 μL of competitive ligand (from 10^{-12} to 10^{-5} M), and 100 μL of GHS-R1a transiently transfected in LLC-PK1 cells as source of binding sites (10 μg protein/tube). The nonspecific binding was determined by the excess of competitive ligand at 10^{-5} M. The reaction was carried out at RT for 1 h. After the incubation period, the separation of bound from free fraction was performed by filtration over a GF/C filter presoaked with 0.5% polyethyleneimine. The filters were washed with 4 mL of HB consisting of 50 mM Tris, 10 mM $MgCl_2$, 2.5 mM EDTA and 3 mL of wash buffer consisting of 50 mM Tris-10 mM $MgCl_2$, 20 mM EDTA, 0.015% Triton X100 (pH 7.3) and were then collected for radioactivity counting using a gamma counter (LKB Wallac 1277, Turku Finland).

4.8. Membrane Preparation for CD36. Animal use was in accordance with the Institutional Animal Ethics Committee and the Canadian Council on Animal Care guidelines for the use of experimental animals. Sprague−Dawley (275−350 g) rats were anaesthetized with sodium pentobarbital, and their hearts were promptly removed in ice-cold saline and the cardiac membranes were prepared according to Harigaya and Schwartz.[81]

4.9. Competitive Covalent CD36 Binding Assay Using Photoactivatable [^{125}I]-Tyr-Bpa-Ala-Hexarelin as Radioligand. The radioiodination procedure of the photoactivatable ligand and the receptor binding assays were performed as previously described by Ong et al.[82] Briefly, the rat cardiac membranes (200 μg) as a source of CD36 were incubated in darkness, in 525 μL of 50 mM Tris-HCl pH 7.4 containing 2 mM EGTA (Buffer A) in the presence of a fixed concentration of [^{125}I]-Tyr-Bpa-Ala-Hexarelin (750 000 cpm) in Buffer B (50 mM Tris-HCl pH 7.4 containing 2 mM EGTA and 0.05% Bacitracin) and with increasing concentrations of competitive ligands (ranging from 0.1 to 50 μM). Nonspecific binding was defined as binding not displaced by 50 μM peptide. After an incubation period of 60 min at 22 °C, membranes were submitted to UV irradiation at 365 nm for 15 min at 4 °C. After centrifugation at 12 000 *g* for 15 min, the pellets were resuspended in 100 μL of sample buffer consisting of 62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 15% 2-mercapto-ethanol, and 0.05% bromophenol blue and boiled for 5 min prior to being subjected to electrophoresis on 7.5% SDS-PAGE. The SDS/PAGE gels were fixed, colored in Coomassie Brilliant Blue R-250, dried, exposed to a storage phosphor intensifying screen (Amersham Biosciences), and analyzed by using a Typhoon PhosphorImager (Amersham Biosciences) and ImageQuant 5.0 software to establish competition curves. Protein bands corresponding to the specifically labeled protein of 87 kDa were quantified by densitometry analysis.

4.10. [^{3}H]Thymidine Incorporation for TF-1 Cell Proliferation Measurements. Human TF-1 cells (5×10^4 cells/well) were cultured in complete RPMI medium (GIBCO RPMI Medium 1640, Invitrogen) supplemented with GM-CSF (Granulocyte Macrophage Colony Stimulating Factor, 2 ng/mL; BD Biosource). Cells were deprived of growth factors for 18 h before preincubation with lactam peptide (1 μM) followed by treatment with IL-1β (10 or 25 ng/mL). After 24 h of incubation at 37 °C, [^{3}H]thymidine (1 μCi/mL; Amersham) was added and the cells were incubated for another 24 h. Cells were harvested, washed two times with PBS (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4), and lyzed with a 0.1 N NaOH/ 0.1% Triton X-100 solution. A scintillation cocktail (Fisher Scientific, 8 mL/sample) was added to the lysate, and after 3 h, radioactivity was measured (Beckman Multi-Purpose Scintillation Coulter Counter LS6500). Results were analyzed by one- or two-way ANOVA factoring for concentration or treatments. Postanova comparisons among means were performed using the Tukey−Framer method. Statistical significance was set at $p < 0.05$. Data are presented as mean ± SEM.

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Supporting Information Available: Procedures for the syntheses and characterizations of all substrates and products, synthesis of the model Agl tripeptides L-Ala-(*S*)-Agl-L-Ala and D-Ala-(*S*)-Agl-L-Ala, and complete ref 55a with all authors. This material is available free of charge via the Internet at http://pubs.acs.org.

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