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TOWARD DEVELOPING PEPTIDOMIMETICS: SUCCESSFUL REPLACEMENT OF BACKBONE AMIDE BONDS IN TETRAPEPTIDE-BASED CCK-A RECEPTOR AGONISTS

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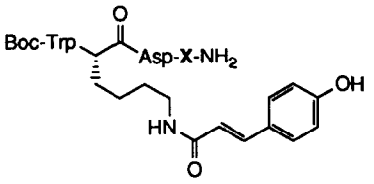
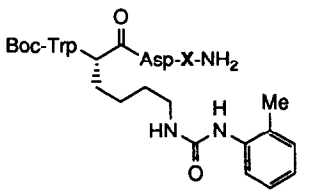
Abstract: The backbone amide bonds in two series of tetrapeptide-based CCK-A receptor agonists were systematically replaced with the methylene amino isostere. Potent and selective pseudopeptides were identified that will facilitate our understanding of how these peptides interact with the CCK receptor and their structural correlations with other CCK ligands. This information will aid in the eventual development of peptidomimetics.

Cholecystokinin (CCK) represents a family of related peptides found in the periphery and the CNS to which a number of biological functions have been attributed.[2] Naturally occurring CCK fragments as well as synthetic peptide[3] and non-peptide ligands[4] can pharmacologically differentiate between the CCK-A receptor, found predominantly in peripheral tissues such as the pancreas, and the CCK-B subtype, prevailing in CNS tissues such as the cortex and possessing a similar ligand binding profile as the peripheral gastrin receptor.[5] Recently, several series of Boc-CCK-4 (Boc-Trp-Met-Asp-Phe-NH_2) derivatives containing a Lys(N^{ε})-amide (1)[6] or -urea (2)[7] side chain in place of Met have been described that are potent and selective CCK-A receptor agonists. When incorporated into Boc-CCK-4, which is 70-fold CCK-B selective, these modified Lys replacements dramatically reversed receptor selectivity of the resulting tetrapeptides to favor the CCK-A subtype by up to 1000-fold (Table 1). Both series of tetrapeptides were full agonists in stimulating amylase release from guinea pig pancreatic acini and were either full or partial agonists in promoting phosphoinositide (PI) hydrolysis; both actions were inhibited by selective CCK-A antagonists. These tetrapeptides represent a significant structural departure from the hepta- and octapeptides bearing an acidic moiety such as a sulfated tyrosine that are the only other class of compounds known to be potent CCK-A receptor agonists.



(1) **a**: X = Phe
b: X = (NMe)Phe
(Hyc = 4-hydroxycinnamoyl)



(2) **a**: X = Phe
b: X = (NMe)Phe
(Tac = 2-tolylaminocarbonyl)

Table 1: Binding and Functional Data of Reference Compounds

Peptide	IC_{50} (nM) cortex	IC_{50} (nM) pancreas	% Max. PI
CCK-8	1.6 ± 0.22 (21)	0.28 ± 0.033 (19)	100
Boc-CCK-4	25 ± 4.5 (6)	1800 ± 630 (5)	100
1a	730 ± 140 (4)	16 ± 3.3 (6)	76 (2)
1b	710 ± 38 (5)	4.2 ± 1.3 (7)	76 (2)
2a	1400 ± 490 (3)	3.8 ± 0.49 (3)	100 (3)
2b	4500 ± 770 (4)	3.7 ± 0.85 (8)	100 (3)

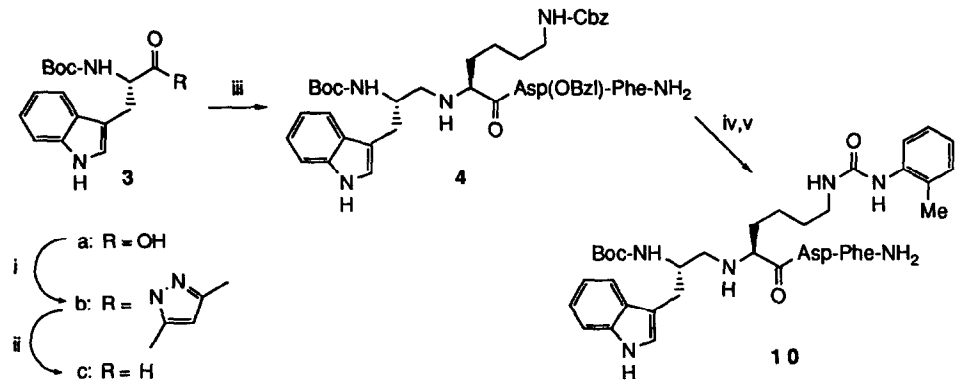
We have been studying these novel tetrapeptides in an attempt to understand their structural relationships with the longer hepta- and octapeptides as well as with the various classes of non-peptide ligands. This understanding would benefit our long-term goal of developing peptidomimetics of these tetrapeptides to improve their physiochemical properties for development purposes. Elucidating the role(s) of the backbone amide bonds would represent a significant step toward realizing these goals. An amide bond can adopt many possible roles,[8] serving simply as a spacer to maintain a correct distance between adjacent side-chain residues or as a rigid chassis to correctly orient side-chain residues in space that enable their critical interactions with the receptor. In addition, they may effect more subtle roles such as participation in intramolecular hydrogen bonding that contributes to a bioactive conformation or in intermolecular hydrogen bonds to the receptor itself. We systematically replaced the amide bonds in both the amide- and urea-bearing tetrapeptides with the methylene amino isostere [$\psi(CH_2NH)$], an amide bond surrogate that maintains the appropriate distance between adjacent side-chain residues as the parent bond but does not preserve the amide's rigid framework nor its hydrogen bonding characteristics, and evaluated the resulting pseudopeptides for CCK activity.

The pseudopeptides were synthesized by modifying a procedure previously described[9] and exemplified in the preparation of **10** (Scheme 1). Peptide couplings were conducted on Boc-protected amino acids and fragments using EDCI (1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride) or isobutylchloroformate and N-methylmorpholine. Boc-deprotection was conducted with HCl (anhydrous) in HOAc. The pyrazolide **3b**, prepared from the Boc-amino acid **3a** via condensation with 3,5-dimethylpyrazole in the presence of EDCI, was reduced with LAH at -78° C to form the amino aldehyde **3c**. Reductive amination of **3c** with the free amino peptide fragment was conducted with $NaCNBH_3$ in AcCN and HOAc to yield pseudopeptide **4**. Removal of Lys(N^{ε}-Cbz) and Asp(OBzl) protecting groups under standard hydrogenolytic conditions and subsequent reaction of the Lys ε-amino with tolylisocyanate in the presence of N-methylmorpholine yielded the final pseudopeptide **10**. All final compounds were fully characterized by their spectral data and satisfactory elemental analysis.

The compounds were evaluated for their ability to displace ^{125}I-Bolton-Hunter-CCK-8 from guinea pig pancreatic (CCK-A) and cortical (CCK-B) tissues by a previously described procedure[10] and is expressed as an

IC_{50}, determined as the concentration of peptide that inhibited 50% of the specific binding of the labeled CCK-8. The protocol for assessing the ability of the compounds to stimulate PI hydrolysis in guinea pig pancreas has been described[10] and is expressed as the % response of the peptide relative to the maximal response elicited by CCK-8. The number of determinations is indicated in parentheses. Each determination was conducted in duplicate; means ± SE are indicated for those compounds with three or more determinations.

Scheme 1:



Reagents: (i) EDCI, 3,5-dimethylpyrazole, CH_2Cl_2; (ii) LAH, THF, -78°C; (iii) Lys(N^{ε}-Cbz)-Asp(OBzl)-Phe-NH_2[7], $NaCNBH_3$, AcCN, HOAc; (iv) H_2, Pd-C, MeOH; (v) tolylisocyanate, N-methylmorpholine, DMF.

Replacement of the Trp-Lys amide bond in peptide **1a** with the methylene amino isostere yielded pseudopeptide **6** that possessed 50-fold weaker binding affinity for the CCK-A (pancreatic) receptor (Table 2). Weaker binding for the cortical receptor also was observed and compound **6** demonstrated significantly reduced efficacy in stimulating PI hydrolysis relative to CCK-8. However, substitution of the Lys-Asp bond with the methylene amino isostere resulted in pseudopeptide **7** that demonstrated only a 2.5-fold decrease in binding affinity over the parent compound **1a** for the pancreatic receptor. In addition, **7** maintained a significant degree of efficacy in promoting PI hydrolysis relative to **1a**. The pseudopeptide **8**, which contains the amide bond surrogate between the Asp-Phe bond, possessed roughly 8-fold weaker affinity for the pancreatic receptor than **1a**. Compound **8** did not elicit any measurable response in the PI hydrolysis assay up to peptide concentrations of 10^{-5} M. Previous studies[6] indicated that substitution with (NMe)Phe could improve pancreatic binding affinity in some instances. Pseudopeptide **1b** possesses nearly 4-fold higher binding affinity for the CCK-A type receptor over the parent compound **1a**; cortical binding affinity and PI efficacy were not affected. Thus, (NMe)Phe was incorporated into pseudopeptide **7**, the most potent compound in this particular amide-substituted series, to

produce **9**. However, this substitution significantly weakened pancreatic binding affinity and reduced efficacy in the PI hydrolysis assay.

Table 2: Binding and Functional Data of Amide-bearing Pseudopeptides of 1a and 1b

No.	Pseudopeptide	IC_{50} (nM) cortex	IC_{50} (nM) pancreas	% Max PI
6	Boc-Trp-ψ(CH_2NH)-Lys(Hyc)-Asp-Phe-NH_2	3600 (2)	930 ± 34 (3)	5.6 ± 1.7 (3)
7	Boc-Trp-Lys(Hyc)-ψ(CH_2NH)-Asp-Phe-NH_2	3800 ± 790 (3)	40 ± 9.8 (5)	44± 7 (3)
8	Boc-Trp-Lys(Hyc)-Asp-ψ(CH_2NH)-Phe-NH_2	7,000 (2)	130 ± 30 (3)	0 (3)
9	Boc-Trp-Lys(Hyc)-ψ(CH_2NH)-Asp-(NMe)Phe-NH_2	5000 ± 210 (3)	250 ± 63 (3)	28 ± 3.5 (3)

An analogous series of pseudopeptides was synthesized that derived from the urea-bearing tetrapeptides **2a** and **2b**. As observed in the previous series, replacement of the amide bond between Trp-Lys resulted in pseudopeptide **10** that possessed significantly weaker affinity for the CCK-A type receptor than **2a** (Table 3). The efficacy of **10** relative to **2a** in eliciting PI hydrolysis also was reduced dramatically. Substitution of the Lys-Asp bond with the isostere yielded pseudopeptide **11**, which maintained a considerable degree of binding affinity and functional efficacy with respect to the parent compound **2a**. Introduction of the methylene amino isostere for the Asp-Phe bond produced compound **12**, which demonstrated reduced pancreatic binding affinity and efficacy. The incorporation of (NMe)Phe in the most potent urea-substituted pseudopeptide **11** was not tolerated, the resulting compound **13** having lost significant binding affinity and now devoid of functional activity in the PI hydrolysis assay. In general, a parallel trend is observed in the biological activities of both the amide and the urea-based pseudopeptides as a function of the site of the amide bond replacement.

Table 3: Binding and Functional Data of Urea-bearing Pseudopeptides of 2a and 2b

No.	Pseudopeptide	IC_{50} (nM) cortex	IC_{50} (nM) pancreas	% Max PI
10	Boc-Trp-ψ(CH_2NH)-Lys(Tac)-Asp-Phe-NH_2	2500 ± 240 (3)	470 ± 130 (3)	8.9 ± 2.6 (3)
11	Boc-Trp-Lys(Tac)-ψ(CH_2NH)-Asp-Phe-NH_2	2400 ± 100 (3)	29 ± 7.7 (4)	64 ± 6.2 (4)
12	Boc-Trp-Lys(Tac)-Asp-ψ(CH_2NH)-Phe-NH_2	>10,000 (3)	300 ± 20 (3)	6.3 ± 1.7 (3)
13	Boc-Trp-Lys(Tac)-ψ(CH_2NH)-Asp-(NMe)Phe-NH_2	>10,000 (3)	1600 ± 370 (3)	0 (3)

The results from both series of pseudopeptides indicate that all three of the amide bonds in the peptide backbone of both **1a** and **2a** can be replaced, with varying degrees of success, with the methylene amino isostere and maintain affinity (IC_{50} < 1 uM) for the pancreatic CCK-A receptor. However, functional activity as measured by PI hydrolysis was much more sensitive to such substitutions. For example, pseudopeptide **8**, which possesses appreciable affinity for the pancreatic receptor with an IC_{50} of 130 nM, was devoid of any activity in the PI assay. Replacement of the Trp-Lys bond with the methylene amino isostere was the least tolerated by the pancreatic CCK receptor for maintaining binding affinity and functional efficacy. However, the Lys-Asp bond in both series could be replaced by the surrogate with minimal reduction in binding potency at the pancreatic receptor while maintaining a significant portion of PI efficacy relative to the parent compound. These results suggest that the amide bond between the Lys-Asp residues does not appear to be an important factor for maintaining affinity to the pancreatic receptor; the methylene amino group is able to position the side chain groups of the Lys and Asp at the correct distance relative to each other in order to interact with the appropriate sites on the CCK-A receptor that are important for binding. However, the reduced functional efficacies of the pseudopeptides indicate the methylene amino isostere lacks a feature of the amide that is important for maintaining high functional efficacy by the peptides.

In addition to the conformational elements that are no longer available upon replacement of the amide bond with the methylene amino isostere, the general chemical nature of the surrogate bonds are altered in the pseudopeptides with respect to the parent compound. The hydrogen bond accepting capabilities of the carbonyl group is now absent in the isostere and thereby suggests that such bonding, either in an inter- or intramolecular manner, is not important for high binding affinity to the pancreatic receptor by pseudopeptides such as **7** or **11**. Whether this interaction was important in the parent amide is not understood at this time. Furthermore, the methylene amino isostere now contains a basic nitrogen, which may now play a key role in binding of the pseudopeptides to the receptor. Further studies with other amide bond surrogates, e.g. ethylene isostere, may provide additional information regarding the role of the nitrogen for binding in these compounds. Whether the pseudopeptides **7** and **11** are binding in a similar or different manner as **1a** or **2a**, respectively, is not understood at this time and this information would be critical to fully appreciate the implications surrounding the reasons for the biological results observed with the pseudopeptides.

A series of CCK heptapeptide derivatives containing the methylene amino surrogate has been prepared and tested by Martinez and co-workers[11] including analogues that directly correspond to our series: Z-Tyr(SO_3^-)-Nle-Gly-Trp-$\psi(CH_2NH)$-Nle-Asp-Phe-NH_2, Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-$\psi(CH_2NH)$-Asp-Phe-NH_2, and Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-Asp-$\psi(CH_2NH)$-Phe-NH_2. These pseudoheptapeptides possess affinity for the pancreatic receptor with IC_{50} values of 300, 30 and 800 nM, respectively, and behave as full agonists relative to CCK-8 in eliciting amylase release from rat pancreatic acini. Interestingly, the pseudoheptapeptide (Z-Tyr(SO_3^-)-Nle-Gly-Trp-Nle-$\psi(CH_2NH)$-Asp-Phe-NH_2) possessing the highest binding affinity for the pancreatic receptor contains the methylene amino isostere between the Nle-Asp bond, directly corresponding to the Lys-Asp bond in our tetrapeptides that yielded the most potent pseudopeptides **7** and **11**.

In conclusion, pseudopeptides **7** and **11** have been identified that maintain high binding affinity and functional efficacy at the CCK-A type receptor. These findings will aid in identifying the pharmacophores that are important for binding and function at the CCK-A receptor by this unique class of tetrapeptide CCK agonists. In

addition, this information will contribute to our current modeling studies underway to establish structural correlations of these tetrapeptides with other classes of CCK ligands and toward development of peptidomimetics.

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