Communications to the Editor

Analogs of CCK Incorporating Conformationally Constrained Replacements for Asp[32]

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Cholecystokinin (CCK) is a family of brain–gut peptides which are released from gut endocrine cells in response to nutrient ingestion. Peripheral activities of CCK are primarily mediated through CCK-A receptors[1] and include stimulation of enzyme secretion from pancreatic acinar cells,[2] inhibition of gastric emptying,[3] and stimulation of gall bladder contraction.[4] Both peripheral type (CCK-A) and brain (CCK-B) CCK receptors are found in the central nervous system and among other activities, may modulate dopaminergic and opiate mediated neural transmission.[5] Exogenous administration of CCK decreases meal size in a number of species including rats[6] and humans[7] and recent evidence suggests that this effect is mediated by CCK-A receptors.[8-11] As part of our overall program to develop orally active CCK mimetics for use as appetite suppressants, we require detailed knowledge of the role played by each structural element of CCK at its A-type receptor.

$$\text{Ac-Tyr(SO}_3\text{H)}^{27}\text{-Met}^{28}\text{-Gly}^{29}\text{-Trp}^{30}\text{-Met}^{31}\text{-Asp}^{32}\text{-Phe}^{33}\text{-NH}_2$$

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Structure–activity work with Ac-CCK-7 (1) suggests that requirements for potent agonist activity at these receptors include an intact C-terminal carboxamide,[12] an aryl- or cycloalkylalanine in position 33,[13,14] tryptophan or a close analog in position 30,[15,16] and a sulfated tyrosine or an isosteric replacement separated by an appropriate spacer[17] at the N-terminus.[18-20] Surprisingly, only scant attention has been paid to the potential contribution of Asp[32] to activity at CCK receptors. Previous workers have shown that in CCK analogs, substitution of Asp[32] by alanine,[21,22] β-alanine,[23] β-aspartic acid,[24] or glutamic acid[23] leads to a marked decrease in potency in assays based on CCK-A receptor responses whereas substitution by a sulfated serine, threonine, or hydroxyproline gave fully active analogs.[25] In the later case, the authors suggested that the hydroxyproline moiety may act to stabilize a preferred conformation. While these results suggest that the β-carboxyl group of Asp[32] or a suitably charged substitute

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Table I. SAR of Asp Substituted Ac-CCK-7 Analogs
Ac-Tyr(SO_3H)-Met-Gly-Trp-Met-**Y**-Phe-NH_2

compd	Y	CCK binding IC_{50} (nM) pancr	CCK binding IC_{50} (nM) striat	feeding assay dose (μg/kg ip)	feeding assay % control food intake[c]	feeding assay ED_{50} (μg/kg)
1	Asp	0.60	4.4	32[a]	6 ± 1**	7
				10	36 ± 2**	
				3	81 ± 8	
2	D-Asp	5.5	22	320[a]	8 ± 5**	
				32	22 ± 7**	
				10	59 ± 11*	13
				3	62 ± 15	
3	Asn	1.4	200	32[a]	8 ± 2**	
				10	17 ± 3**	
				3	43 ± 15**	2.5
				1	84 ± 4	
4	Pro	8.3	3900	320[a]	12 ± 2**	59
				100	27 ± 5**	
				32	75 ± 9	
5	Aib	2.4	170	240[a]	9 ± 4**	~70
				80	43 ± 16**	
6	*R*-Dtc	0.05	2310	320[b]	0 ± 0**	4.4
				32	17 ± 12**	
				3	57 ± 13**	
7	*S*-Dtc	314	6160	320[b]	66 ± 7*	>320

[a] "1-2-1 protocol", first meal results shown. [b] "Overnight fasted" protocol. [c] $^{**}p < 0.01$, $^{*}p < 0.05$.

plays an important role in CCK-A receptor activation, consideration of a possible exclusively conformational role for this moiety prompted us to carry out the work reported here in which we demonstrate that Asp[32] may be replaced by nonacidic and highly constrained amino acids with retention of potent binding to pancreatic acinar receptors and suppression of food intake in rats.

The unsulfated peptides **1**–**5** were prepared by solid-phase methodology utilizing the Boc/Bzl strategy, employing benzhydrylamine (BHA) resin and hydrogen fluoride (HF) cleavage.[26] Coupling reactions were mediated by diisopropylcarbodiimide/hydroxybenzotriazole and monitored by the ninhydrin test.[27] Compounds **6** and **7** were prepared by solid-phase synthesis using the Boc/OFm protection strategy employing [[4-[(acyloxy)methyl]-phenyl]acetamido]methyl (PAM) resin and ammonolysis for cleavage.[28] In each case, the crude products were purified to homogeneity by preparative HPLC on a micro bondapack C-18 column in a 5–65% 0.022% trifluoroacetic acid (TFA)–acetonitrile system and their purity was verified by analytical HPLC prior to characterization by FAB MS and amino acid analysis. Sulfation to give the peptides shown in the Table I was accomplished using pyridinium acetyl sulfate.[23] The products were purified by preparative HPLC on a micro bondapack C-18 column in a 10–40% 0.01 M ammonium acetate–acetonitrile system. The IR of each gave a 1050 cm^{-1} peak characteristic of a tyrosine sulfate ester.[29]

Receptor binding activity for the CCK-A and CCK-B receptor subtypes was determined using solubilized membranes prepared from fresh pancreatic tissue, obtained from fasted rats or bovine striatum, respectively, as previously described by Van Dijk[30] and detailed in previous publications from our labs.[14,18] Nonspecific binding was determined in the presence of 1 μM native CCK-8 and subtracted from all samples to determine specific binding. The concentration of the peptides necessary to inhibit 50% of total specific [^{3}H]propanoyl-CCK-8 binding (IC_{50} value) was determined by log-probit analysis and data for active compounds were confirmed by duplicate experiments.

Test peptides were evaluated for their ability to suppress food intake in one of two meal-fed-rat models. In the "1-2-1" protocol, male Sprague–Dawley rats (200–250 g) were trained to take their daily meals during two 1-h periods separated by a 2-h interval for 4–5 days prior to test peptide administration. On the test day, peptides were given by intraperitoneal injection 15 min before the first meal to groups of five or six rats and the amount of food eaten during each meal was determined. Since the compounds prepared during the course of this work were not optimized to resist proteolysis, the simpler "overnight-fasted protocol" was also employed. In this assay, groups of five to seven rats were fasted overnight, administered vehicle or drug by ip injection, and presented with food cups for 1 h. The average food consumed during the test period was compared with that of vehicle-treated controls. In both models, the treated groups were compared to the control groups using the *t*-test. Data are expressed as percent of saline-treated control food intake during the first hour of the experiment, and as the dose which caused 50% inhibition of the control intake (ED_{50}) as determined from log-probit analysis. We have previously demonstrated that the ED_{50} values for CCK-8 and several analogs at the 1-h time point are equivalent using either protocol.[14,17]

As the data in the table indicate, substitution of Asp[32] with D-Asp to give **2** led to only a 10-fold loss of potency in both binding assays and a 2-fold loss of potency on inhibition of food intake in comparison with Ac-CCK-7 (**1**). Replacement by the nonacidic amino acids asparagine, proline, and aminoisobutyric acid (Aib) gave **3**, **4**, and **5**, respectively, which have similar potency to **2** in binding to CCK receptors on rat pancreas (CCK-A) and effectively suppress food intake, but have decreased affinity to bovine striatal receptors (CCK-B). Given the relatively potent activity of the proline analog **4**, it was of interest to substitute the more hindered proline analog 5,5-dimethylthiazolidine-4-carboxylic acid (Dtc), which like proline, introduces a local Φ-angle constraint and also markedly limits accessible ψ-angles in the resulting peptide.[31] The analog **6** incorporating the *R*-enantiomer of Dtc, which corresponds to L-proline, was highly potent in the rat pancreas binding assay (IC_{50} = 0.05 nM) and only weakly active in the bovine striatal binding assay (IC_{50} = 2300 nM), indicating a high selectivity for binding to the CCK-A receptor subtype. This compound also inhibited food intake somewhat more effectively than Ac-CCK-7 (**1**). The corresponding *S*-Dtc derivative **7** was only weakly active

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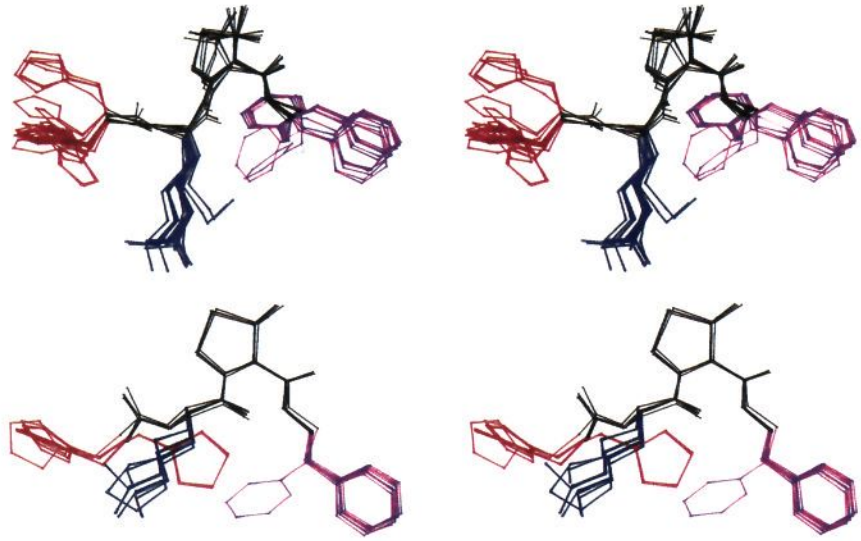
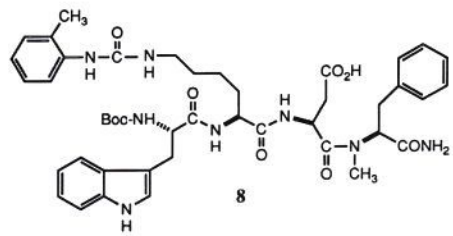


Figure 1. Stereoviews of optimized conformers for the tetrapeptide **9**. For clarity, the peptide backbone extends only from Trp[1] Cα to Phe[4] Cα, the Trp side chain has been truncated to include only the pyrrole ring, and the ε-[(phenylamino)carbonyl] group has been removed from the Lys[2] side chain. The color code is as follows: backbone and Dtc, black; Trp, red; Lys, blue; Phe, purple. (a, top) Family 1, 21 members and (b, bottom) family 2, 9 members.

in the binding assays and as an appetite suppressant. The selectivity in the binding assays noted for the compounds incorporating a nonacidic substitution for Asp[32] suggests that the carboxyl moiety of Asp[32] may play a direct role in mediating the interaction of CCK analogs with CCK-B receptors.

The lack of a more rigorous correlation between pancreatic binding affinity and ID_{50} in the feeding assay for these compounds may be a reflection of subtle differences in transport and metabolism or more intriguingly, to the existence of subclasses of CCK-A like receptors which vary in their ability to mediate food intake and which are sensitive to modifications at the C-terminus of CCK. We have previously noted that certain analogs of Ac-CCK-7 with large hydrophobic groups substituting for the C-terminal phenylalanine aromatic ring also bind to pancreatic receptors more potently than predicted based on their effects on food intake in rats.[14] Since the anorectic activity of CCK has been postulated to involve stimulation of pyloric[32] and vagal[33,34] receptors, candidate receptor subpopulations could reside on either of these tissues. Further research to characterize the interaction of compounds of this class with the CCK receptors residing on various peripheral tissues is clearly in order.

Researchers from Abbott have recently described a series of potent CCK-A receptor agonists based on the tetrapeptide Boc-Trp-ε-[(phenylamino)carbonyl]Lys-Asp-(N-methyl)Phe-NH_2 of which the urea derivative **8** was among the most potent.[35,36] We have synthesized **8** and several analogs using solid-phase methodology as described above. Consonant with the literature reports, in our hands, **8** had



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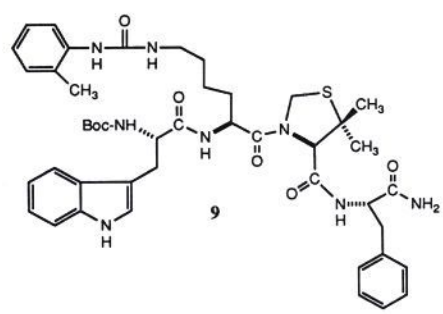

9

IC_{50}s of 0.05 and 1900 nM in the rat pancreas and bovine striatal binding assays, respectively, and inhibited food intake in rats more potently than **1** (ED_{50} = 1.3 μg/kg, ip). Preliminary structure–activity studies carried out on analogs of **8** indicated that, with the exception of the substituted lysine, requirements for activity are similar to those previously observed for the corresponding elements of the C-terminal portion of CCK-7. Thus, we chose to prepare the *R*-Dtc analog **9** and were pleased to find that it retains significant binding affinity for the rat pancreatic receptor (IC_{50} of 59 nM) and inhibited food intake in the overnight-fasted protocol with an ED_{50} of 85 μg/kg ip. In the binding assay employing bovine striatal receptors, **9** had an IC_{50} of 7400 nM. The lower potency of **9** relative **8** and the *R*-Dtc-containing heptapeptide **6** observed in vivo may be due to a slight unfavorable conformational bias on the lysine side chain induced by the Dtc moiety.

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Despite its somewhat lower potency, **9** retains substantial anorectic activity and is relatively compact compared to the heptapeptides **1–6**. Thus we were encouraged to determine the conformational consequences of the *R*-Dtc moiety in **9** by NMR as prelude to the design of potential orally active mimetics.

For **9** in DMSO solution, NMR spectra were obtained, assignments made, and nuclear Overhauser effects (NOEs) measured. Interproton distances were derived from the NOEs and used as constraints in an optimization procedure based on molecular dynamics and energy minimization within the CHARMM program package.[37] As has been described, this procedure readily folds a peptide from its fully-extended conformation to an ensemble of 30 low-energy conformers which satisfy the experimental distance constraints.[38,39] For **9**, the 45 distance constraints are well fit with no significant deviations; the root mean square deviation is ca. 0.1 Å beyond the estimated error range of ±0.5 Å. The tetrapeptide has well-defined backbone conformations with a number of possible side-chain rotamers. There are two backbone conformational families which differ in the position of lysine relative to the two aromatic residues as shown in Figure 1. It is thus apparent that incorporation of the Dtc residue has significantly constrained the tetrapeptide.

In conclusion, the aspartic acid present in CCK-related peptides plays a conformational role rather than mediating binding to the CCK-A receptor subtype through a charge–charge interaction. This finding has important consequences for the design of selective, nonpeptidic mimetics and permits us to propose that critical elements required for CCK-A agonist activity and anorectic activity are limited to the C-terminal carboxamide, a hydrophobic moiety represented in CCK itself by the phenylalanine aromatic ring, and the tryptophan in a suitable three-dimensional array. In addition, high potency requires occupancy of one of two auxiliary binding sites: either that occupied by $Tyr(SO_3H)^{27}$ in naturally occurring CCK derivatives or that occupied by the ϵ-(arylamino)carbonyl moiety in **8** and related compounds.[35,36] As illustrated by the results of NMR studies with **9**, the constraint introduced by inclusion of *R*-Dtc for Asp^{32} should provide important clues as to the spatial orientation of these pharmacophores and constitutes a significant advance toward the design of orally active CCK-A receptor agonists.

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