CCK Peptides with Combined Features of Hexa- and Tetrapeptide CCK-A Agonists

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Selective CCK-A agonist activity has been reported to induce satiety in a variety of animals, including man, and thereby suggests a therapeutic role for CCK in the management of obesity. To date, three general classes of CCK-A agonists have been reported, the full-length, sulfated hepta- and hexapeptides, a series of tetrapeptides, and most recently a series of benzodiazepines. The SAR of the hexa- and tetrapeptide classes suggests that the Hpa(SO_3H) and Tac groups may not interact at the CCK-A receptor in the same location. However, the C-terminal dipeptide part of the hexapeptides and tetrapeptides appear to interact at the CCK-A receptor in a similar manner. Compound **7** (Hpa-Nle-Gly-Trp-Lys(Tac)-Asp-MePhe-NH_2) derived from combining the features of the hexapeptides and the tetrapeptides has subnanomolar affinity and 3500-fold selectivity for CCK-A receptors. Compound **7** administered intraperitoneally produces potent, long-lasting reduction in food intake in rats and a corresponding weight loss when administered over nine consecutive days.

Introduction

Cholecystokinin (CCK), a peptide hormone found in the central nervous system and gastrointestinal tract, occurs in multiple biologically active forms (CCK-58, CCK-39, CCK-33, CCK-8, and CCK-4) with CCK-8 (see Table 1 for sequence) predominating.[1,2] CCK mediates many diverse hormonal and neuromodulatory functions through the action of two CCK receptor subtypes, CCK-A and CCK-B.[3,4] Peripheral CCK-A receptors mediate gall bladder contraction, pancreatic exocrine secretion, and satiety.[3,5]

Three general classes of selective CCK-A agonists have been identified; full-length, sulfated hepta- and hexapeptides,[6−8] tetrapeptides,[9,10] and recently benzodiazapines.[11] Very recently Bignon et al. reported a thiazole with CCK-A agonist activity, but limited SAR information is available.[12,13] A hypothesis has been proposed to describe how the related classes of peptide agonists bind to the G-protein-coupled CCK-A receptor.[14,15] In general, a sulfated tyrosine or tyrosine mimic at position 7 of CCK-8 is required for high affinity at CCK-A receptors because nonsulfated peptides exhibit 100−1000-fold less affinity than the corresponding sulfated peptides.

The suggestion that sulfated hexapeptides were required for high affinity at CCK-A receptors was brought into question with the discovery of potent, CCK-A selective, tetrapeptide agonists.[10] An important issue regarding this finding is, how do the tetrapeptides relate to the hexapeptides? To address this question, Shiosaki[14] and Holladay[15] generated sulfated and methylated tetrapeptide compounds. Shiosaki[14] found that the 4-hydroxyphenylpropionyl group attached to the ϵ-amine of lysine had twice the affinity of the unsubstituted phenylpropionyl or the 3-hydroxyphenylpropionyl group. This finding led them to speculate that the hydroxy group of 4-hydroxyphenylpropionyl interacted at the CCK-A receptor similarly to the hydroxy of Tyr[7] in CCK-8. However, sulfation of the 3- or 4-hydroxy groups only led to a doubling of CCK-A affinity, suggesting that these groups did not occupy the same space on the CCK-A receptor as the sulfated tyrosine in CCK-8.[14] Holladay methylated the amide nitrogen of each residue of the tetrapeptide one residue at a time, finding that the rank order of CCK-A affinities for the methylated tetrapeptides paralleled exactly the rank order of the CCK-A affinities of the corresponding methylated heptapeptides.[15] Here we report some of our findings on comparison of the hexa- and tetrapeptides and studies that led to hexapeptides incorporating the features of the tetrapeptides.

Methods

Tetrapeptides were prepared by standard solution phase mixed anhydride coupling procedures using isobutylchloroformate to form the mixed anhydride. Side chain protecting groups of lysine and aspartic acid were removed simultaneously with catalytic hydrogenation to yield the N-terminal Boc protected peptides. The ϵ-amino group of lysine was allowed to react with 2-methylphenyl isocyanate to generate the final urea-containing peptide. Unsulfated hexapeptide amides were synthesized utilizing the Fmoc/tBu protection strategy on RINKMBHA resin (Milligen/Biosearch).[16] Cleavage from the resin was accomplished using reagent K (TFA, H_2O, thioanisole, phenol, ethanedithiol, 185: 5:5:5:2) for 2 h at room temperature.[17] The crude peptides were purified to homogeneity using preparative reverse phase HPLC. The ϵ-amino group of lysine was allowed to react with either 2-methylphenyl isocyanate or 4-hydroxycinnamic acid *N*-hydroxysuccinimide ester to generate the final peptides. Sulfation was achieved using pyridine·SO_3, and the final products were purified to homogeneity using reverse phase HPLC and charac-

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Table 1. Receptor Affinity[a] and Selectivity[b]

no.	structure	K_i (nM)[c] CCK-A	K_i (nM)[c] CCK-B	CCK-A selectivity
1	Hpa(SO_3H)-Nle-Gly-Trp-Nle-MeAsp-Phe-NH_2	0.03 ± 0.005	224 ± 19	6590
2	Hpa-Nle-Gly-Trp-Nle-MeAsp-Phe-NH_2	4.2	960	228
3	Boc-Trp-Lys(Tac)-D-Asp-Phe-NH_2	8.1	580	72
4	Boc-Trp-Lys(Tac)-D-Asp-MePhe-NH_2	0.53	>1000	>2000
5	Hpa-Nle-Gly-Trp-Nle-Asp-MePhe-NH_2	51	ND	ND
6	Hpa-Nle-Gly-Trp-Lys(Hci)-Asp-MePhe-NH_2	3.4	36	11
7	Hpa-Nle-Gly-Trp-Lys(Tac)-Asp-MePhe-NH_2	0.05 ± 0.007	200	4000
8	Hpa(SO_3H)-Nle-Gly-Trp-Lys(Tac)-Asp-MePhe-NH_2	0.02	123	6150
9	Hpa(SO_3H)-Met-Gly-Trp-Met-D-Asp-MePhe-NH_2	0.98	84	86
10	Hpa(SO_3H)-Met-Gly-Trp-Met-D-Asp-Phe-NH_2	19	150	8
11	Boc-Trp-Lys(Tac)-Asp-MePhe-NH_2	0.30 ± 0.05	250	833
CCK-8	H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	0.09 ± 0.009	0.41 ± 0.06	5

[a] Assays and data analysis are summarized in the Experimental Section. [b] CCK-A selectivity is the ratio of CCK-B to CCK-A binding affinity. ND, not determined. [c] The values without standard errors are the results of one experiment performed in triplicate and the individual determinations varied by less than 20%.

terized by analytical reverse phase HPLC, mass spectrometry, and amino acid analysis.

CCK-A receptor affinity was measured by displacement of [^{125}I]BH-CCK-8 from rat pancreatic tissue according to the general procedure of Chang.[18] CCK-B receptor affinity was measured by displacement of [^{125}I]BH-CCK-8 from rat cortex synaptosomes according to the general procedure of Chang.[19] The ability of a compound to inhibit food intake and to promote weight loss in rats was measured by a modification of the method described by Cox and Maickel.[20]

Results and Discussion

AR-R 15849[6] (**1**) is a sulfated hexapeptide (see Table 1 for sequence) containing all of the elements classically described as required for CCK-A agonism. Generally a sulfated tyrosine or tyrosine mimic at position 7 of CCK-8 is required for high affinity at CCK-A receptors because nonsulfated peptides exhibit 100−1000-fold less affinity than the corresponding sulfated peptides. The selectivity of **1** results from N-methylation of Asp (see ref 6), but the sulfate group on the hydroxyphenylacetic acid (a tyrosine mimic) contributes 2 orders of magnitude to the CCK-A affinity (compare **1** with **2**). The nonsulfated compound **2**, while still having affinity for CCK-A receptors (K_i = 4.2 nM vs 0.03 nM), has much less than **1** and is much less selective for CCK-A receptors than **1** (230-fold vs 6590-fold). Correspondingly, compound **2** is also 52-fold less potent in inhibiting food intake following ip administration in rats (ED_{50} 3 h feeding = 13 μg/kg vs 0.25 μg/kg). These results show that sulfation is important for high receptor affinity and potency while N-methylation of Asp is important for CCK-A selectivity.

In another hexapeptide series, inversion of the stereochemistry of Asp, to give D-Asp, in conjunction with methylation of the amide nitrogen of Phe, to give MePhe, leads to hexapeptides with similar CCK-A affinity but reduced CCK-B affinity, resulting in increased CCK-A selectivity.[6] Thus, in the hexapeptide series the combination of D-Asp, MePhe leads to potent selective agonists, whereas the D-Asp, Phe analogue has less affinity for both CCK-A and CCK-B receptors (compounds **9** and **10** of Table 1 from ref 6). In the tetrapeptide series, we also found that the D-Asp, Phe analogue **3** had reduced affinity for CCK-A receptors, when compared to the D-Asp, MePhe analogue **4** (K_i = 8.1 nM vs 0.53 nM). Combining these results with the methylation study conducted by Holladay suggests that the C-terminal portion of the hexa- and tetrapeptides may occupy similar locations at the CCK-A receptor. It may be that the CCK-A receptor requirements are less stringent for the C-terminal portion of the peptides than the CCK-B receptor.

The studies described above led us to consider replacing the Nle corresponding to Nle^3 of **5** with the Lys(Tac) (i.e., Lys(2-tolylaminocarbonyl)) group found in the tetrapeptides. This change generates the hybrid peptides **6**−**8**. Generally, nonsulfated hexapeptides such as **5** have low affinity for CCK-A receptors; **2** is a notable exception but still has 100-fold less affinity than **1**, its sulfated derivative. The hybrid hexapeptides **6** and **7** have very good affinity and selectivity for the CCK-A receptor, even without a sulfate group. Sulfation of the Hpa phenol of **7** to give **8** did not change the CCK-A affinity; usually sulfation in the hexapeptides increase affinity for CCK-A receptors 100−1000-fold. This is similar to what Shiosaki et al.[14] found when sulfating a phenolic group attached to the side chain amine of Lys in the tetrapeptide series. It is not completely clear why sulfation of the hybrid compound **7** did not result in an increase in affinity.

All of the peptides inhibited food intake in a dose dependent manner. Figure 1 shows the inhibition of the food intake dose−response curve for compound **7** in rats following ip administration. Compound **7** more potently inhibited food intake following intraperitoneal administration than the sulfated analogue **8** or the tetrapeptide **11**[21] (Table 2). To examine the weight loss producing effects of **7**, the compound was administered to rats for 9 consecutive days (Figure 2). Acutely, a 0.8 μg/kg dose of **7** was found to inhibit food intake by 65%, while a 0.3 μg/kg dose was found to inhibit feeding by 30%. Over the 9-day study, **7** dose dependently induced weight loss. At the higher dose (0.8 μg/kg) **7** produced a 3% weight loss after the first dose that was maintained over the course of the study. The lower dose (0.3 μg/kg) produced a slight decease in weight for the first 6 days of the study, but as the study progressed, the animals gained a small amount of weight, as compared to their predose weights. However, even at the low dose the rats weighed significantly less on day 9 than control animals that had received saline injections for 9 days. It should

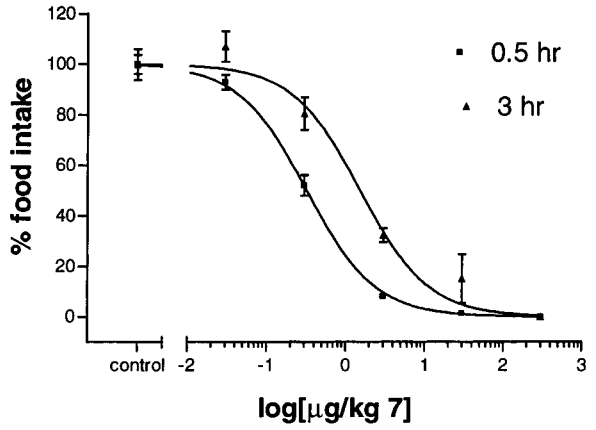

Figure 1. Percent inhibition of food intake of **7** in rats 0.5 and 3 h following ip administration. Ten rats were tested per dose (0.03, 0.3, 3.0, 30, 300 μg/kg). Half-hour and 3 h data were taken from the same experiment. Assay and data analysis summarized in the Experimental Section.

Table 2. Feeding Inhibition Potency[a]

	feeding inhibition (ED_{50}, μg/kg)	
no.	0.5 h	3 h
1	0.11 ± 0.02	0.25 ± 0.01
2	3.8 ± 0.7	13 ± 2.7
7	0.27 ± 0.04	0.81 ± 0.2
8	0.85 ± 0.07	1.2 ± 0.2
11	0.9 ± 0.2	3.2 ± 0.2
CCK-8	1.2 ± 0.1	112 ± 3.8

[a] Assays and data analysis are summarized in the Experimental Section.

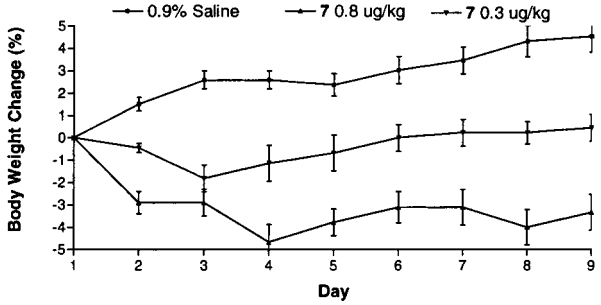

Figure 2. Body weight change effect of **7** in rats following daily ip dosing over 9 days. Values for day 2−9 for both doses of **7** are significantly different from the control ($P < 0.05$, ANOVA, Newman−Keuls). Assay and data analysis are summarized in the Experimental Section.

be noted that the control group of rats continued to gain weight in the expected manner throughout the study.

Previously, we have discussed our efforts to identify and evaluate sulfated, hexapeptide-based, selective CCK-A agonists. Compound **1** is such a compound that produces weight loss following ip administration in a 9-day study. Abbott[9,10] scientists have disclosed the CCK-A agonist activity of a series of tetrapeptides. Here we report that combining the features of the hexapeptides and the tetrapeptides successfully generates hexapeptides that no longer require sulfation for high affinity and good in vivo potency. The SAR of the hexa- and tetrapeptides classes of molecules suggests that the Hpa(SO_3H) and Tac groups may not interact at the CCK-A receptor in the same location, but the C-terminal dipeptide part of the hexapeptides and tetrapeptides interact at the CCK-A receptor in a similar manner. The hybrid peptide **7** was found to be a potent, selective, fully efficacious CCK-A agonist even without sulfation. This compound produced dose dependent weight loss over a 9-day study period, suggesting that CCK-A agonists may be useful anorectic agents.

Experimental Section

Abbreviations. Peptide and amino acid abbreviations used follow the guidelines of the IUPAC−IUB Joint Commission on Biochemical Nomenclature[22] and are of the L-configuration unless otherwise noted. AAA, amino acid analysis; Boc, *tert*-butyloxycarbonyl; Gly, glycine; Lys, lysine; MeAsp, *N*-methylaspartic acid; MePhe, *N*-methylphenylalanine; Nle, norleucine (2-aminohexanoic acid); Phe, phenylalanine; Trp, tryptophan. Other abbreviations are as follows: CMA, chloroform−methanol−acetic acid; DCM, methylene chloride; DIEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; EtOAc, ethyl acetate; Hci, 4-hydroxycinnamoyl; HOBt, 1-hydroxybenzotriazole; Hpa, 4-hydroxylphenylacetyl; Hpa(SO_3H), sulfated 4-hydroxyphenylacetyl; ip, intraperitoneal; OSu, succinimidyloxy ester; Piv-Cl, pivaloyl chloride; Pyr, pyridine; Pyr·SO_3, pyridine−sulfur trioxide complex; Tac, 2-tolylaminocarbonyl; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; t_R, HPLC retention time.

Peptide Purification. Preparative reverse phase HPLC (Prep-LC) was conducted using an Amicon C18 column (500 × 50 mm, 20 μm, 100 A). Peptides were eluted using a 110 min gradient of 40−60% solvent B [0.025% TFA (solvent A) and 0.025% TFA in CH_3CN (solvent B)] at 25 mL/min. Pure fractions were freeze-dried from H_2O/CH_3CN.

Analytical HPLC. Analytical reverse phase HPLC was performed on a Hewlett-Packard 1050 system using the following columns and mobile phases. Columns: (I) Phenomenex Nucleosil C18 (150 × 3.2 mm, 5 μm, 100 Å) eluted at 0.5 mL/min, (II)) Phenomenex Columbus C18 (150 × 3.2 mm, 5 μm, 100 Å) eluted at 0.5 mL/min, or (III) Keystone Nucleosil C18 (150 × 4.6 mm, 5 μm) heated at 60 °C and eluted at 1.0 mL/min. Mobile phases: (a) 20 min gradient of 20−80% solvent B [0.05% TFA (solvent A) and 0.05% TFA in CH_3CN (solvent B)], (b) 30 min gradient of 0−100% solvent B [0.1% TFA (solvent A) and 0.1% TFA in CH_3CN (solvent B)], (c) 15 min gradient of 35−60% solvent B (solvents same as in mobile phase a), (d) 15 min gradient of 25−50% solvent B (solvents same as in mobile phase a) By example, the term "HPLC (Ia)" is used below to describe HPLC column I with mobile phase a. All analytes were determined to be >95% pure when monitored at 220 nm.

General Method for Solid-Phase Peptide Synthesis. The nonsulfated peptide amides were prepared on a MilliGen/Biosearch 9600 synthesizer using standard solid-phase synthesis techniques and the Fmoc/tBu protection strategy.[16] Syntheses were performed starting with the RINKMBHA resin (1.0 g, 0.30−0.43 mmol). Fmoc group removals were carried out by treatment with piperidine/DMF. Double couplings were performed using 6.67 equiv of Fmoc-amino acid with equivalent amounts of *N,N*-diisopropylcarbodiimide and HOBt in DMF (1−2 h). NH_2-Terminal acylation was conducted using 6.67 equiv of Hpa-OSu in DMF (1−2 h). Completed peptidyl resins were cleaved and deprotected using reagent K (TFA, H_2O, thioanisole, phenol, ethanedithiol, 185:5:5:5:2) for 2 h at room temperature.[17] The crude unsulfated peptide amides were purified to homogeneity by Prep-LC. Fractions shown by HPLC to be >95% pure were pooled and lyophilized to provide unsulfated peptide amide products.

All peptides were found to have the expected composition as determined by acid hydrolysis and amino acid analysis. Peptide identity was further confirmed by mass spectral analysis (MS, electrospray) with the expected (M − H) anionic molecular ion peak observed for all peptides. Peptide purity was determined by HPLC to be >98% when analyzed on two different columns (Table 3).

Method A: Boc-D-Asp(OBn)-MePhe-NH_2 (11). General Piv-Cl Method. To a stirred, ice-cold solution of Boc-D-

Table 3. Analytical Data

	amino acid analysis							MS ES *m/z*	HPLC t_R	
no.	Nle	Gly	Trp	Lys	Asp	Phe	MePhe	(M − H)	Ia	IIb
3			0.74	0.94	1.03	1.03		827	14.08	20.35
4			0.98	0.97	1.08	0.95		826	14.22	20.44
5	2.11	1.01	0.53		1.00		0.89	880	12.86	18.92
6	0.91	1.07			1.10		0.91	1056	10.88	17.74
7	1.04	1.04	0.96	0.94	1.01		0.98	1084	13.20	19.18
8	1.10	1.04	0.84	0.93	1.01		0.92	1122		

Asp(OBn)-OH (10.87 g, 33.6 mmol.) in 300 mL of THF were added Piv-Cl (4.14 mL, 33.6 mmol) and DIEA (16.7 mL, 96 mmol). After 30 min, a cold solution of H-MePhe-NH_2·HCl (6.87 g, 32 mmol) was added. While the pH was maintained at 7 using DIEA (2.8 mL, 16 mmol), the reaction was stirred at room temperature for 3.5 h. The solvent was removed in vacuo, and the resulting oil was dissolved in EtOAc; washed with 1 M $KHSO_4$, H_2O, saturated $NaHCO_3$, and saturated NaCl; dried ($MgSO_4$); and evaporated to a foam: TLC (CM, 9:1, NH_3 plate) R_f 0.53.

Method B: H-D-Asp(OBn)-MePhe-NH_2·HCl (12). General Boc Deprotection Method. To a stirred, −10 °C solution of **11** (1.5 g, 3.1 mmol) in EtOAc (100 mL) was added HCl gas to saturation (10 min). The solution was allowed to warm to room temperature over 1 h. The solution was reduced in vacuo to a small volume, and hexanes were added with cooling to −20 °C. The white precipitated product was collected by filtration to yield 1.30 g (3.1 mmol). TLC: (CMA 9:1:1) R_f 0.2. The product was used as is.

Method C: BocLys(*Z*)-D-Asp(OBn)-MePhe-NH_2 (13). General IBCF Method. To a stirred, iced solution of Boc-Lys(*Z*)-OH (1.29 g, 3.4 mmol) in DMF (25 mL) were added DIEA (0.59 mL, 3.4 mmol) and isobutylchloroformate (0.44 mL, 3.4 mmol). After 10 min, a cooled solution of H−D-Asp(OBn)-MePhe-NH (0.54 mL, 3.1 mmol) in 25 mL of DMF was added. After 2 h the reaction was filtered, the solvent was removed in vacuo. The resulting oil was dissolved in EtOAc; washed with 1 N $NaHCO_3$, cold 1 N HCl, and saturated NaCl; dried ($MgSO_4$); and evaporated to an oil. The crude was purified on a flash SiO_2 column using $CHCl_3$. Evaporation in vacuo of the product fractions gave 2.3 g of **13** that was used as is: TLC (CMA 9:1:1) R_f 0.72.

H-Lys(*Z*)-D-Asp(OBn)-N-Me-Phe-NH_2·HCl (14). Compound **13** (2.36 g, 3.16 mmol) was subjected to deprotection using method B: yield 1.54 g (2.26 mmol); TLC (CMA 9:1:1) R_f 0.2. The product was used as is.

Boc-Trp-Lys(*Z*)-D-Asp(OBn)-N-Me-Phe-NH_2 (15). Using Method C, Boc-Trp-OH (0.83 g, 2.71 mmol) was coupled to **14**; yield 1.83 g (1.96 mmol, 87% yield); TLC (CMA 9:1:1) R_f 0.64.

Method D: Boc-Trp-Lys-D-Asp-N-MePhe-NH_2(16). General Side Chain Deprotection Method. To a solution of **15** (1.83 g, 1.96 mmol) in 50 mL of glacial acetic acid was added 0.5 g of 10% Pd/C. The reaction was shaken under hydrogen pressure on a hydrogenator for 16 h. The Pd/C was removed by filtration through a layer of Celite and the solvent removed in vacuo. The resulting oil was dissolved in MeOH and the product precipitated with ether to yield 1.14 g (1.61 mmol, 82% yield) of **16** as a white solid that was used directly in the next reaction.

Method E. Boc-Trp-Lys(Tac)-D-Asp-MePhe-NH_2 (4). General Tac Addition Method. To a solution of **16** (0.4 g, 0.56 mmol) in 40 mL of DMF was added DIEA (0.25 mL, 1.4 mmol) and 2-tolyl isocyanate (0.07 mL, 0.56 mmol). After 1 h the reaction was complete as measured by HPLC and the solvent was removed in vacuo. The peptide was purified by Prep-LC, to yield 195 mg of compound **4**. See Table 3 for analytical data.

Hpa-Nle-Gly-Trp-Lys-Asp-MePhe-NH_2 (18). Hpa-Nle-Gly-Trp-Lys-Asp-MePhe-NH_2 was prepared by standard solid-phase synthesis techniques using the Fmoc/tBu protection strategy, with the lysine side chain amine protected with a Boc group. Each amino acid was added sequentially to the RINKMBHA resin (1 g, 0.3 mmol/g, lot A09655) using a Biosearch 9600 peptide synthesizer.[15] The synthesis was performed using the program CNDIPCDI (Biosearch). Fmoc group removals were carried out by treatment with piperidine/DMF. Double couplings were performed using 6.67 equiv of Fmoc-amino acid with equivalent amounts of *N*,*N*-diisopropylcarbodiimide and HOBt in DMF (1−2 h). NH_2-Terminal acylation was conducted using 6.67 equiv of Hpa-OSu in DMF (1−2 h). Completed peptidyl resins were cleaved and deprotected using reagent K for 2 h at room temperature.[16] The resin was removed by filtration, the filtrate evaporated, and the fully deprotected free peptide precipitated with ether. Collection by filtration and drying under high vacuum gave **18** as a white solid (234 mg, 65% yield, t_R = 8.87 min, 95% pure at 270 nm and 75% pure at 220 nm by analytical HPLC (IIId).

Hpa-Nle-Gly-Trp-Lys(Hci)-Asp-MePhe-NH_2 (6). Compound **18** (246 mg, 0.29 mmol) was combined with 1 equiv of DIEA (0.050 mL, 0.29 mmol) and 1.2 equiv of HciOSu (52 mg, 0.35 mmol) in 5 mL of DMF with stirring for 18 h, at which time an additional 1 equiv of DIEA (0.050 mL) was added. The reaction was then directly isolated by Prep-LC with a 110 min gradient of 40−60% solvent B [10 mM NH_4OAc (solvent A) and 10 mM NH_4OAc in MeOH (solvent B)] at 25 mL/min. The pure factions were combined and 26 mg of product was obtained. Analysis was by analytical HPLC (IIIc), t_R = 13.74 min.

Hpa-Nle-Gly-Trp-Lys(Tac)-Asp-MePhe-NH_2 (7). Compound **18** (364 mg, 0.43 mmol) was combined with 1.25 equiv of DIEA (0.1 mL, 0.58 mmol) and 1.0 equiv of 2-methylphenyl isocyanate (0.53 mL, 0.43 mmol) in 10 mL of DMF with stirring for 1 h at room temperature. The reaction was then directly isolated by Prep-LC with a 180 min gradient of 50−100% solvent B [10 mM NH_4OAc (solvent A) and 10 mM NH_4OAc in MeOH (solvent B)] at 25 mL/min. The pure factions were combined and lyophilized.

Hpa(SE)-Nle-Gly-Trp-Lys(Tac)-Asp-MePhe-NH_2 (8). Pyridine−sulfate complex (330 mg, 2.07 mmol, 23 equiv) was added to thoroughly dried Hpa-Nle-Gly-Trp-Lys(Tac)-Asp-MePhe-NH_2 (**7**) (96 mg, 0.088 mmol) in 2 mL of pyridine with stirring. After 6 h at room temperature the solution was poured into 50 mL of 5% NH_4OH, allowed to stir for 30 min, and then evaporated to dryness. MeOH (50 mL) was added to the resulting solid, the suspension filtered, and the filtrate evaporated to give crude peptide.

The product was then isolated by Prep-LC with a 180 min gradient of 50−90% solvent B [10 mM NH_4OAc (solvent A) and 10 mM NH_4OAc in MeOH (solvent B)] at 25 mL/min. The product fractions were combined and lyophilized to give 41 mg of **8**. Due to the acid sensitivity of this compound, analytical HPLC was performed with the same solvent system used to isolate the compound: HPLC (I) 35−100% solvent B in 30 min, t_R = 8.9 min; HPLC (II) 40−100% solvent B in 30 min, t_R = 13.8 min [10 mM NH_4OAc (solvent A) and 10 mM NH_4OAc in MeOH (solvent B)] at 0.5 mL/min.

Biological Evaluations. Affinity for CCK-A receptors in rat pancreatic membranes was measured according to the methods described by Chang et al.[18] A pancreas from a Sprague−Dawley rat was homogenized for 20 s with a Brinkman Polytron (setting 5) in 50 vol of a 50 mM Tris buffer, at pH 8.0 and 4 °C. The homogenate was centrifuged at 50000*g* for 10 min and then the pellet was washed twice, recentrifuged, and resuspended in 2000 vol of cold 5 mM Tris buffer (pH 7.4 at 37 °C), containing 5 mM $MgCl_2$, 5 mM dithiothreitol, 2 mg/mL BSA, and 0.14 mg/mL bacitracin. Affinity for CCK-A

receptors was measured using [^{125}I]BH-CCK-8 (DuPont, 2000 Ci/mmol). One milliliter (0.5 mg original wet weight of tissue) of membrane suspension, and 15 pmol of [^{125}I]BH-CCK-8 was incubated for 40 min at 37 °C. Bound ligand was collected onto Whatman GF/B filters, and the filters were washed twice with 8 mL of ice-cold 50 mM Tris buffer, pH 7.4, and analyzed using a Beckman liquid scintillation counter. Binding constants (K_i) were determined ($n = 3$) using ALLFIT, an iterative logistic curve fitting program, as described by Cheng and Prusoff.[23] The calculated K_d value for [^{125}I]BH-CCK-8 was 0.12 nM.

Affinity for CCK-B receptors was measured in rat cerebral cortex.[19] Tissue was homogenized in 50 vol (vol/g of wet weight of tissue) of 50 mM Tris (pH 7.5 at 25 °C). The homogenate was centrifuged at 50000*g* for 10 min and the resulting pellet was resuspended and recentrifuged. The final pellet was resuspended in 80 vol of assay buffer (10 mM Hepes, 5 mM $MgCl_2$, 1 mM EGTA, bacitracin (0.25 mg/mL), and 130 mM NaCl, pH 6.5). Then, 0.45 mL of resuspended membranes, unlabeled compound, and 1 nmol of [^{125}I]BH-CCK-8 in a final volume of 0.5 mL was incubated at 25 °C for 2 h. Membrane-bound ^{125}I was collected by vacuum filtration on Whatman GF/B filters presoaked in 50 mM Tris buffer, pH 7.7, containing 1 mg/mL bovine serum albumin (Sigma). Radioactivity was determined with a Beckman γ-counter (efficiency of 45%). K_i values ($n = 3$) were determined using the Lundon AccuFit Competition nonlinear curve fitting software program, which is base in part on models described by Linden[24] and Feldmen.[25]

Food intake was measured by a modification of the method described by Cox and Maickel.[20] Individually caged male Sprague−Dawley rats (300−400 g) were maintained on a 12 h light/dark cycle and were trained for at least 14 days to feed (powdered Purina rat chow) during the initial 3 h period of the dark cycle. To assess feeding inhibition potency, saline control and peptide doses were administered intraperitoneally (in 0.5 mL of 0.9% saline, pH 7.0−8.0) 10 min prior to food availability, and cumulative food intake was then measured after 0.5 and 3 h of feeding. Both 0.5 and 3 h time points were determined in the same experiment. Dose−response curves were constructed of pooled consumption data from five doses (10 rats/dose) for each peptide, and ED_{50} (i.e., the dose that inhibits feeding by 50%) values were determined using ALLFIT. A repeated-measures ANOVA followed by a Newman−Keuls analysis was used to determine significant differences in food intake between groups.

Weight loss was measured by a modification of the method described by Cox and Maickel.[20] To examine the weight loss producing effects of **7**, the compound was intraperitoneally administered to rats for nine consecutive days (Figure 2). Male Sprague−Dawley rats (300−400 g) trained to feed during the initial 3 h of a 12 h dark cycle were weighed and dosed on a daily basis 10 min prior to the 3 h feeding period. Cumulative food intake was measured hourly during each feeding period. Day 1 predosing body weights served as starting body weights for each group of 10 animals. Each group was composed of animals with similar body weight and similar feeding behavior. Vehicle control and treatment groups were composed of the following: (1) 0.9% saline, (2) 0.8 μg/kg of **7**, and (3) 0.3 μg/kg of **7**. Vehicle and **7** were administered in a volume of 0.5 mL of 0.9% saline. Mean and standard error (SEM) values for each treatment group were calculated for food intake and body weight each day. A repeated-measures ANOVA followed by a Newman−Keuls analysis was used to determine significant differences in food intake and body weight values across treatment groups both within days and between days.

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