CCK-A Receptor Selective Antagonists Derived from the CCK-A Receptor Selective Tetrapeptide Agonist Boc-Trp-Lys(Tac)-Asp-MePhe-NH_2 (A-71623)†

Elizabeth E. Sugg,*,‡ Millard J. Kimery,‡ Jian Mei Ding,§ Deborah C. Kenakin,|| Laurence J. Miller,⊥ Kennedy L. Queen,|| and Thomas J. Rimele||

Departments of Medicinal Chemistry, Bioanalytical and Structural Chemistry, and Cellular Biochemistry, Glaxo Research Institute, 5 Moore Drive, Research Triangle Park, North Carolina 27709, and Center for Digestive Diseases, Mayo Clinic, Rochester, Minnesota 55905

Received August 24, 1994®

Analogs of the CCK-A receptor selective agonist Boc-Trp-Lys(Tac)-Asp-MePhe-NH_2 (A-71623) were prepared in which the lysine residue was replaced with L-4-aminophenylalanine and D- or L-3-aminophenylalanine. These new analogs were moderately potent antagonists of CCK-8 in the isolated guinea pig gallbladder with exceptional CCK-A receptor selectivity as evaluated in membrane preparations from CHO K1 cells stably transfected with human CCK-A and CCK-B receptors.

Cholecystokinin (CCK) is a gastrointestinal hormone and neurotransmitter first isolated from porcine intestine.[1] CCK is released from intestinal endocrine cells in response to nutrient ingestion.[2] While a variety of molecular forms of CCK have been identified, the C-terminal octapeptide (CCK-8, H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) retains full bioactivity, interacting with two receptor subtypes. CCK-A receptors predominate in the periphery (gallbladder, pancreas, pyloric sphincter, and vagal afferent fibers) but are also found in discrete regions of the brain.[3] CCK-B or gastrin receptors predominate in the brain and gastric glands.[4,5] The physiological role of CCK-8 is to aid digestion of nutrients through the induction of gallbladder contraction, pancreatic secretion, and delayed gastric emptying.[3,5] Exogenous CCK decreases meal size in a variety of species including lean[6] and obese[7] humans. This satiety effect of CCK requires an intact vagal afferent nerve and appears to be mediated by peripheral CCK-A receptors.[8]

The high molecular weight, the acid lability of the Tyr(SO_3H) residue, and the metabolic instability of CCK-8 provided the impetus for numerous chemical modifications.[9] In 1990, scientists at Abbott reported a series of unique CCK analogs derived by replacement of the methionine residue of Boc-CCK-4 (Boc-Trp-Met-Asp-Phe-NH_2) with side-chain-substituted Lys derivatives.[10,11] Boc-Trp-Lys(Tac)-Asp-MePhe-NH_2 (A-71623) was functionally equivalent to CCK-8 with remarkable CCK-A receptor selectivity (1200-fold), enhanced metabolic stability, and potent anorectic activity in rats,[12,13] mice, dogs, and monkeys.[14]

The tetrapeptide Boc-Trp-Lys(Tac)-Asp-Phe-NH_2 was approximately 3-fold less potent and less CCK-A receptor selective than the *N*-MePhe derivative.[11] In this series, the side-chain-truncated analog Boc-Trp-Orn-(Tac)-Asp-Phe-NH_2 had 170-fold reduced CCK-A receptor affinity, was a weak partial agonist in stimulating phophatidylinositide (PI) hydrolysis, and had 380-fold reduced efficacy in stimulating amylase release from isolated guinea pig pancreatic acini.[11] In contrast, the side-chain-extended analog Boc-Trp-hLys(Tac)-Asp-Phe-NH_2 had only 24-fold reduced CCK-A receptor affinity, was a full agonist in the PI hydrolysis assay, and had only 10-fold reduced efficacy in stimulating amylase release from isolated guinea pig pancreatic acini.[11]

This dependence of functional activity on the length of the methylene bridge between the urea moiety and the peptide backbone prompted our investigation of additional analogs of Boc-Trp-Lys(Tac)-Asp-MePhe-NH_2 in which the methylene bridge of the lysine residue was replaced by an aromatic ring. We now report the synthesis and biological activity of analogs of Boc-Trp-Lys(Tac)-Asp-MePhe-NH_2 in which the lysine residue was replaced by L-4-aminophenylalanine (L-4-Amf) and L- or D-3-aminophenylalanine (L-3-Amf or D-3-Amf). Side-chain-substituted (*o*-tolylamino)carbonyl (Tac) or [(*o*-chlorophenyl)amino]carbonyl (Cpac) urea derivatives of Lys and 4-Amf were prepared. Since we and others[15] have found that the N-terminal acetyl derivative was essentially equipotent to the N-terminal *tert*-butyloxycarbonyl derivative, all analogs were prepared as *N*-acetyl derivatives. The tetrapeptide Ac–Trp-Phe-Asp-MePhe-NH_2 was also prepared to evaluate the role of the urea substituent in these new analogs.

Methods

The urea-substituted amino acids were prepared by reaction of the corresponding *N*-α-Boc derivatives of L-Lys or L-4-Amf with *o*-tolyl isocyanate or *o*-chlorophenyl isocyanate in water or aqueous acetonitrile. *N*-α-Boc-D,L-3-aminophenylalanine was prepared by catalytic reduction of *N*-α-Boc-D,L-3-nitrophenylalanine[16,17] and converted to the urea derivative in a similar manner. Analogs **1**–**4** and **7** were prepared by automated solid-phase peptide synthesis and acetylated prior to cleavage and deprotection.

The peptides containing D- or L-3-Amf were prepared as a mixture of diastereomers and cleaved from the resin prior to acetylation. The diastereomeric amino terminal peptides were separated by preparative RP-HPLC. A

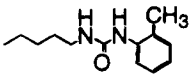
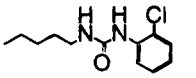
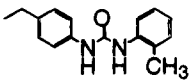
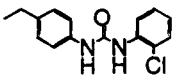
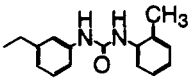
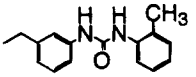
† Abbreviations: 4-Amf, 4-aminophenylalanine; 3-Amf, 3-aminophenylalanine; Boc, *tert*-butyloxycarbonyl; Cpac, [(*o*-chlorophenyl)amino]-carbonyl; DCC, dicyclohexylcarbodiimide; HOBt, *N*-hydroxybenzotriazole; Tac, (*o*-tolylamino)carbonyl; hLys, homolysine;

* To whom correspondence should be addressed.
‡ Department of Medicinal Chemistry.
§ Department of Bioanalytical and Structural Chemistry.
|| Department of Cellular Biochemistry.
⊥ Center for Digestive Diseases.

® Abstract published in *Advance ACS Abstracts,* December 1, 1994.

0022-2623/95/1838-0207$09.00/0

Table 1. Functional Activity of Tetrapeptides Ac-Trp-NH-CHR-CO-Asp-MePhe-NH_2

Analog	R	Stereo	GPGB pEC50[a]	GPGB %mCCK-8[b]	GPGB pA2[c]	GPGB Slope[d]
1		L	8.8 ± 0.2 (4)	105%		
2		L	9.3 + 0.6 (4)	117%		
3		L		i.a.[e]	7.4 (8.5 - 6.9)	1.2 (1.6 - 0.8)
4		L		i.a.[e]	7.1 (7.9 - 6.6)	1.4 (1.9 - 0.9)
5		L		i.a.[e]	7.2 (8.4 - 6.6)	0.9 (1.3 - 0.6)
6		D		i.a.[e]	7.4 (7.7 - 7.1)	1.5 (1.2 - 1)
7		L		i.a.[e]		

[a] −log of the EC_{50} ± SD (number of determinations). In the same assay, CCK-8 had a pEC_{50} of 9.41 ± 0.15 (4). [b] % mCCK-8, percent contraction induced by test compound at 30 μM, normalized to the percent contraction induced by 1 μM CCK-8. [c] pA_2, −log of the concentration required to shift the CCK-8 concentration−response curve 2-fold; 95% confidence limits in parentheses. [d] Slope of the Schild plot; 95% confidence limits in parentheses. [e] i.a., inactive at 30 μM.

portion of each amino terminal peptide was treated with leucine aminopeptidase (16 h at 37 °C), and the resultant mixtures were monitored by LC-MS. The diastereomer completely degraded by enzyme treatment was assigned L-stereochemistry. The diastereomer which was resistant to enzymatic degradation was assigned D-stereochemistry. The remaining samples of the purified amino terminal diastereomeric peptides were treated with acetic anhydride/pyridine to obtain the *N*-acetyl derivatives, **5** and **6**.

Analogs **1**−**7** were purified to homogeneity (>98%) by preparative RP-HPLC, and the lyophiles were characterized by analytical RP-HPLC, ^{1}H-NMR spectroscopy, and high-resolution fast-atom bombardment (FAB) mass spectrometry. Analogs were evaluated for functional activity in the isolated guinea pig gallbladder (GPGB, Table 1). Receptor binding affinities were measured on membrane preparations from CHO K1 cell lines stably transfected with cDNA for human CCK-A[18] or CCK-B[19] receptors (Table 2).

Results and Discussion

While the lysine-substituted derivatives **1** and **2** were full agonists in the isolated GPGB (Table 1), the Amf derivatives **3**−**6** were moderately potent antagonists of CCK-8 in the GPGB, with pA_2 values ranging from 7.1 to 7.4. The D-3-Amf analog **6** was slightly more potent than the L-3-Amf analog **5**. Analog **6** also blocked the agonist activity of **1** in the GPGB with a pA_2 of 6.9 (6.5−7.7), suggesting that these analogs were interacting with the same CCK receptor as CCK-8 in the isolated GPGB preparation. The L-Phe analog, **7**, was completely inactive in the GPGB, confirming that the antagonist activity observed with **3**−**6** was related to the urea side-chain substituent.

The receptor affinities and subtype selectivities of these new analogs for human CCK-A and CCK-B receptors are reported in Table 2. It should be noted that all of the standards reported in Table 2 show slightly better CCK-B affinity and receptor selectivity on the human receptors than has been reported[10,21,22] for comparisons with membrane preparations from rat pancreatic acinar cell (CCK-A) and guinea pig cerebral cortical (CCK-B) membranes.

Table 2. Receptor Binding Affinities of Tetrapeptides for Human CCK-A and CCK-B Receptors

analog	pIC_{50}[a] CCK-A	pIC_{50}[a] CCK-B	B/A[b]
1	7.7 ± 0.4 (3)	5.3 ± 0.2 (5)	251
2	8.3 ± 0.1 (3)	6 ± 0.05 (3)	200
3	7.5 ± 0.1 (3)	4.0 ± 0.3 (3)	3162
4	7.0 ± 0.02 (3)	4.2 ± 0.1 (3)	631
5	7 ± 0.1 (5)	5 ± 0.1 (3)	100
6	7.2 ± 0.2 (5)	4.3 ± 0.2 (3)	794
7	<4 (3)	5.4 ± 0.01 (3)	<0.04
CCK-8	9.4 ± 0.1 (3)	9.5 ± 0.4 (9)	0.8
CCK-8NS	5.7 ± 0.8 (4)	8.3 ± 0.4 (3)	0.003
MK-329	9.6 ± 0.5 (9)	7 ± 0.2 (3)	398
L 365,260	6.8 ± 0.4 (4)	8.2 ± 0.2 (2)	0.04

[a] −log of the concentration displacing 50% of [^{125}I]Bolton Hunter CCK-8 from membrane preparations isolated from CHO K1 cells stably transfected with cDNA of human CCK-A and CCK-B receptors. ±SD. Number of determinations are in parentheses. [b] CCK-A receptor selectivity. IC_{50}(CCK-B)/IC_{50}(CCK-A).

The rank order of human CCK-A receptor affinities for **1**−**6** paralleled the rank order of potencies (pED_{50} or pA_2) seen in the isolated GPGB. Lys derivatives **1** and **2** were 200-fold CCK-A receptor selective, with the Cpac derivative **2** having slightly higher CCK-A receptor affinity than the Tac derivative **1**. The L-4-Amf derivatives **3** and **4** were even more CCK-A receptor selective (3100- and 630-fold, respectively), with the Tac derivative **3** having slightly higher CCK-A receptor affinity than the Cpac derivative **4**. The D-3-Amf analog **6** had similar CCK-A receptor affinity but better CCK-A

receptor selectivity than the corresponding L-3-Amf analog **5**. Despite being equipotent to **3** in the GPGB assay, the D-3-Amf analog **6** had lower CCK-A receptor affinity and selectivity than the L-4-Amf analog **3**. The control tetrapeptide **7** had weak affinity for the human CCK-B receptor but did not bind the human CCK-A receptor (up to 10 μM), demonstrating the critical role of the urea substituent for CCK-A receptor recognition.

The ability of analogs **1** or **6** to influence intracellular calcium was evaluated with the stably transfected CHO K1 cells loaded with FURA2-AM (n = 1). Lysine derivative **1** was a full agonist on both the hCCK-A (ED_{50} = 4.2 nM) and hCCK-B (ED_{50} = 530 nM) receptor-containing cell lines, while the D-3-Amf analog **6** was inactive as an agonist. However, as predicted from the human receptor binding affinities, a single 1 μM concentration of analog **6** blocked the agonist activity of **1** in the human CCK-A receptor-containing cell line (15.6-fold shift; pK_B = 7.2) but not in the human CCK-B receptor-containing cell line.

In summary, analogs of Boc-Trp-Lys(Tac)-Asp-Me-Phe-NH_2 were prepared in which the methylene bridge between the urea substituent and the peptide backbone was replaced with a four-carbon (L-4-Amf) or three-carbon (L- and D-3-Amf) aromatic linker. To our surprise, these modifications produced moderately potent antagonists, with CCK-A receptor selectivity equal to or better than the lysine-substituted derivatives. In addition to altering the bridge length between the urea substituent and the peptide backbone, substitution of an aromatic ring would be expected to increase the steric bulk and hydrophobicity and decrease the conformational mobility of the urea-substituted amino acid. Moreover, substitution of an aniline for an alkylamine could alter the electronics and preferred conformation of the urea. The few compounds reported here do not provide a sufficient database to define which, if any, of these steric and/or electronic factors contribute to this remarkable conversion of functional activity.

Experimental Section

All chemicals and solvents were reagent grade unless otherwise specified. CCK-8 and nonsulfated CCK-8 (CCK-8NS) were purchased from Sigma (St. Louis, MO). MK-329[20] and L 365,260[21] were obtained from Merck & Co. (Rathway, NJ).

The ^{1}H-NMR spectra were recorded on either a Varian VXR-300 or a Varian Unity-300 instrument. Chemical shifts are expressed in parts per million (ppm, δ units). Coupling constants are in units of hertz (Hz). Splitting patterns are designated as s, singlet; d, doublet; q, quartet; m, multiplet; br, broad. Low-resolution mass spectra (MS) were recorded on a JOEL JMS-AX505HA, JOEL SX-102, or SCIEX-APIII spectrometer. All mass spectra were taken in the positive ion mode under electrospray ionization (ESI) or fast-atom bombardment (FAB) methods. Reactions were monitored by thin-layer chromatography on 0.25 μm silica gel plates (E. Merck, 60F-254), visualized with UV light, 7% ethanolic phosphomolybdic acid, or p-anisaldehyde solution.

***N*-α-Boc-L-lysine[(N^ϵ-*o*-tolylamino)carbonyl].** o-Tolyl isocyanate (6.7 g, 50 mmol) was added to a solution of *N*-α-Boc-L-lysine (12.3 g, 50 mmol) dissolved in 1 N aqeuous sodium hydroxide (50 mmol). The reaction mixture was stirred at room temperature for 3 h, acidified with 1 N aqueous HCl, and extracted into ethyl acetate (6 × 50 mL). The combined organic extracts were washed with brine (1 × 30 mL), dried ($MgSO_4$), and concentrated in vacuo to give the crude urea (18 g, 94%) which was used without further purification: ^{1}H-NMR (300 MHz, DMSO-d_6) δ 1.36 (s, 13H), 3.83 (m, 1H), 2.15 (s, 3H), 3.05 (d, J = 5.13 Hz, 2H), 6.51 (t, J = 4.88 Hz, 1H), 6.83 (t, J = 7.33 Hz, 1H), 7.08 (m, 3H), 7.58 (s, 1H), 7.81 (d, 1H); MS (FAB) m/z 380 (MH^+); TLC (9:1 $CHCl_3$:CH_3OH) R_f = 0.06. Anal. ($C_{19}H_{29}N_3O_5$), C, H, N.

***N*-α-Boc-L-lysine[(N^ϵ-*o*-chlorophenyl)amino]carbonyl].** Via *N*-α-Boc-L-lysine (5.0 g, 20.5 mmol) and o-chlorophenyl isocyanate (3.69 g, 24 mmol), the title compound (4.41 g, 54%) was prepared as previously described: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.4 (s, 9H), 1.55 (m, 2H), 1.75 (m, 2H), 1.85 (m, 2H), 3.2 (br s, 2H), 4.23 (m, 1H), 5.3 (br d, 1H), 6.83 (br t, 1H), 7.1–7.3 (m, 3H), 8.05 (d, 1H); MS (FAB) m/z 400 (MH^+); TLC (9:1 $CHCl_3$:CH_3OH) R_f = 0.08. Anal. ($C_{18}H_{26}N_3O_5Cl$) C, H, N.

***N*-α-Boc-4-L-aminophenylalanine[(N^ϕ-*o*-tolylamino)carbonyl].** Via *N*-α-Boc-4-L-aminophenylalanine (5.6 g, 20 mmol) and o-tolyl isocyanate (3.2 g, 24 mmol), the title compound (4.1 g, 49%) was prepared as previously described and used without further purification: ^{1}H-NMR (300 MHz, CD_3OD) δ 1.4 (s, 9H), 2.25 (s, 3H), 2.88 (dd, 1H), 3.1 (dd, 1H), 4.3 (dd, 1H), 7.03 (m, 1H), 7.18 (m, 4H), 7.38 (d, 2H), 7.63 (d, 1H); MS (FAB) m/z 414 (MH^+); TLC (9:1 $CHCl_3$:CH_3OH) R_f = 0.52. Anal. ($C_{22}H_{27}N_3O_5$) C, H, N.

***N*-α-Boc-4-L-aminophenylalanine[(N^ϕ-*o*-chlorophenyl)amino]carbonyl].** Via *N*-α-Boc-4-L-aminophenylalanine (5.6 g, 20 mmol) and o-chlorophenyl isocyanate (3.69 g, 24 mmol), the title compound (6.2 g, 72%) was prepared as previously described and used without further purification: ^{1}H-NMR (300 MHz, CD_3OD) δ 1.4 (s, 9H), 2.88 (dd, 1H), 3.13 (dd, 1H), 4.33 (dd, 1H), 6.95–7.45 (m, 7H), 8.1 (dd, 1H); MS (FAB) m/z 434 (MH^+); TLC (9:1 $CHCl_3$:CH_3OH) R_f = 0.8. Anal. ($C_{21}H_{24}N_3O_5Cl$) C, H, N.

***N*-α-Boc-3-D/L-aminophenylalanine.** *N*-α-Boc-3-D/L-nitrophenylalanine[16,17] (8.9 g, 18 mmol) and 10% Pd/C (0.8 g) in methanol (100 mL) were shaken under hydrogen (45 psi) overnight. The mixture was filtered through Celite, and the filtrate was evaporated in vacuo to give the title compound (8.7 g, 18 mmol) which was used without further purification: ^{1}H-NMR (300 MHz, CD_3OD) δ 1.4 (s, 9H), 2.83 (dd, 1H), 3.05 (dd, 1H), 4.33 (dd, 1H), 6.65 (m, 3H), 7.05 (m, 1H); MS (FAB) m/z 281 (MH^+); TLC (9:1 $CHCl_3$:CH_3OH) R_f = 0.14. Anal. ($C_{14}H_{20}N_2O_4$) C, H, N.

***N*-α-Boc-3-D/L-aminophenylalanine[(N^ϵ-*o*-tolylamino)carbonyl].** Via *N*-α-Boc-3-D/L-aminophenylalanine (4.7 g, 16.7 mmol) and o-tolyl isocyanate (2.7 g, 20 mmol), the title compound (5.1 g, 74%) was prepared as previously described and used without further purification: ^{1}H-NMR (300 MHz, CD_3OD) δ 1.4 (s, 9H), 2.3 (s, 3H), 2.9 (dd, 1H), 3.15 (dd, 1H), 4.23 (m, 1H), 6.85–7.25 (m, 6H), 7.43 (d, 1H), 7.63 (d, 1H); MS (FAB) m/z 414 (MH^+); TLC (9:1 $CHCl_3$:CH_3OH) R_f = 0.54. Anal. ($C_{22}H_{27}N_3O_5$) C, H, N.

Peptide Synthesis. Analogs were prepared by automated solid-phase peptide synthesis (ABI 430) on p-methylbenzhydrylamine resin[22] (Peptides International, Louisville, KY). *N*-α-*tert*-Butyloxycarbonyl (Boc) amino acid derivatives were purchased from Bachem Biosciences (Philadelphia, PA) or Applied Biosystems Inc. (Foster City, CA). DCC/HOBt single coupling protocols were utilized for the N-α-Boc-MePhe-OH, N-α-Boc-Lys(N^ϵ-Tac)-OH, N-α-Boc-Lys(N^ϵ-Cpac)-OH, N-α-Boc-4-Amf(N^ϕ-Tac)-OH, N-α-Boc-4-Amf(N^ϕ-Cpac)-OH, N-α-Boc-D/L-3-Amf(N^ϵ-Tac)-OH, and N-α-Boc-Trp-OH. DCC/HOBt double coupling protocols were utilized for N-α-Boc-Asp(benzyl). Peptides were cleaved from the resin, and all protecting groups were removed by treatment at 0 °C for 1 h with anhydrous liquid HF (10 mL/g of resin) containing dithioethane (1 mL/g of resin) and anisole (1 mL/g of resin). The HF was removed in vacuo, and the resin was washed with cold diethyl ether and filtered. Peptides were extracted from the resin with aqueous acetic acid (30%). The crude peptides obtained by lyophilization were purified to homogeneity by preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) using a Waters Model 3000 Delta Prep column equipped with a Delta-pak radial compression cartridge (G18, 300 A, 15 μm, 47 mm × 300 mm) as the stationary phase. The mobile phase employed 0.1% aqueous TFA with acetonitrile (Burdick and Jackson) as the organic modifier. Linear gradients were used in all cases, and the flow rate was 100 mL/min (t_0 − 5 min). Appropriate

Table 3. Physical Data for Analogs 1–7

analog	empirical formula	HRMS[a] calculated	HRMS[a] found	t_R, min (% CH_3CN/30 min)[b]	purity[c]
1	$C_{41}H_{50}N_8O_8$	783.3830	783.3833	19.4 (30–48%)	98.7
2	$C_{40}H_{47}N_8O_8Cl$	803.3284	803.3297	15.4 (33–51%)	98.1
3	$C_{44}H_{48}N_8O_8$	817.3673	817.3663	18.1 (33–51%)	98.2
4	$C_{43}H_{45}N_8O_8Cl$	837.3127	837.3135	21.6 (33–51%)	98.5
5	$C_{44}H_{48}N_8O_8$	817.3673	817.3679	16 (36–54%)	99.3
6	$C_{44}H_{48}N_8O_8$	817.3673	817.3687	15.8 (36–54%)	99.0
7	$C_{36}H_{41}N_6O_7$	669.3037	669.3039	21.6 (24–42%)	98.4

[a] MH^+, obtained in positive ion mode, utilizing FAB ionization on an AMD 604 double focusing magnetic sector mass spectrometer. [b] Analytical retention time on a Vydac C18 column (5 μm, 4.6 mm × 200 mm), utilizing a linear gradient of acetonitrile and a flow rate of 1.5 mL/min. [c] Purity assessed by integration at λ = 214 nm.

fractions were combined and lyophilized to obtain the target peptide analogs. Analytical purity was assessed by RP-HPLC using a Waters 600E system equipped with a Waters 990 diode array spectrometer (λ range 200–400 nm). The stationary phase was a Vydac C18 column (5 μm, 4.6 mm × 200 mm). The mobile phases were the same as above, linear gradients were again utilized, and the flow rate was 1.5 mL/min (t_0 = 2.8 min). Analytical data (Table 3) is reported as retention time, t_R, in minutes (% acetonitrile over time). High-resolution mass spectra (Analytical Instrument Group, Raleigh, NC) were recorded in the positive ion mode on a AMD-604 (AMD Intectra GmbH) high-resolution double focusing mass spectrometer under FAB conditions (Table 3).

H-Trp-3-D,L-Amf[(N^{ϵ}-tolylamino)carbonyl]-Asp-MePhe-NH_2. This compound was prepared as a mixture of diastereomers containing D,L-3-Amf(Tac) and cleaved from the resin prior to N-terminal acetylation. Separation by preparative RP-HPLC afforded the individual diastereomeric analogs in greater than 98% purity. H-Trp-L-3-Amf[(N^{ϵ}-*o*-tolylamino)-carbonyl]-Asp-MePhe-NH_2: $C_{42}H_{46}N_8O_7$; MS (FAB) *m*/*z* 775 (MH^+); t_R = 20.3 min (27–45%/30 min); H-Trp-D-3-Amf[(N^{ϵ}-*o*-tolylamino)carbonyl]-Asp-MePhe-NH_2: $C_{42}H_{46}N_8O_7$; MS (FAB) *m*/*z* 775 (MH^+); t_R = 22.3 min (27–45%/30 min). The individual diastereomeric peptides were converted to the *N*-acetyl derivatives **5** and **6** by treatment with acetic anhydride (0.01 mL) and pyridine (0.02 mL) in DMF (3 mL) at ambient temperature for 30 min. The reaction mixture was diluted with aqueous TFA (10%, 50 mL) and lyophilized to a solid. Purification by RP-HPLC afforded the *N*-acetyl derivatives in >99% purity.

Assignment of Stereochemistry. Each diastereomeric peptide were incubated at 37 °C for 16 h with leucine aminopeptidase (Sigma; 1:50) in 100 μL of 50 mM NH_4HCO_3 (pH 8.5) solution. The hydrolysis reactions were terminated by the addition of TFA (1 μL), and the reaction mixtures were reduced almost to dryness by lyophilization and then reconstituted with 0.05% aqueous TFA (100 μL) to a final concentration of 10 μM. About 100 pmol of peptide hydrolysate was used for each LC/MS experiment. Samples were analyzed on a Vydac C18 column (5 μm, 320 μm × 30 cm) conditioned to a flow rate of 7 μL/min. The mobile phase was 0.1% aqueous TFA (10% B to 60% B) with acetonitrile as the organic modifier. Masses were detected with a Sciex API-III triple quadrupole electrospray mass spectrometer. The collision gas used for MS/MS was Ar with 10% N_2. Digested peptides were analyzed from *m*/*z* 300 to 1000 using repetitive scans at a rate of 3.5 s/scan.

Guinea Pig Gallbladder Tissue Preparation. Gallbladders were removed from male Hartley guinea pig sacrificed with CO_2 atmosphere. The isolated gallbladders were cleaned of adherent connective tissue and cut into two rings from each animal (2–4 mm in length). The rings were suspended in organ chambers containing a physiological salt solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_3, 25 mM $NaHCO_3$, 11.1 mM dextrose). The bathing solution was maintained at 37 °C and aerated with 95% O_2/5% CO_2 to maintain pH 7.4. Tissues were connected via gold chains and stainless steel mounting wires to isometric force displacement transducers (Grass, Model FT03 D). Responses were then recorded on a polygraph (Grass, Model 7E). One tissue from each animal served as a time/solvent control and did not receive test compound. Rings were gradually stretched (over a 120 min/period) to a basal resting tension of 1 g which was maintained throughout the experiment. During the basal tension adjustment period, the rings were exposed to acetylcholine (10^{-6} M) four times to verify tissue contractility. The tissues were then exposed to a submaximal dose of sulfated CCK-8 (Sigma; 3×10^{-9} M). After obtaining a stable response, the tissues were washed out three times rapidly and every 5–10 min for 1 h to reestablish a stable base line.

Agonist ED_{50}'s. Compounds were dissolved in dimethyl sulfoxide (DMSO) and then diluted with water and assayed via a cumulative concentration–response curve to test compound (10^{-11}–3×10^{-5} M) followed by a concentration–response curve to sulfated CCK-8 (10^{-10}–10^{-6} M) in the presence of the highest concentration of the test compound. As a final test, acetylcholine (1 mM) was added to induce maximal contraction. A minimum of three determinations of activity was made for each test compound.

Antagonist pA_2's. Gallbladder tissues from at least three different animals were incubated for 60 min with a given concentration (three to five concentration per analog) of the antagonist followed by a cumulative concentration–response curve with CCK-8 or compound **1**. One paired tissue from each animal did not receive the antagonist and served as the time/solvent control used to calculate the concentration ratio for the rightward shift in the CCK-8 concentration response. pA_2's were determined by Schild analysis for a given number of observations.[23] Upper and lower 95% confidence limits and slopes of the Schild plot are given for each pA_2.

Establishment of Stable CCK Receptor-Bearing Cell Lines. The cDNA clones for the human CCK-A[18] or CCK-B[19] receptors were ligated into cDNA1-Neo vector from Invitrogen Corp. (San Diego, CA) for direct transfection. DNA was prepared by the alkaline lysis method and transfected into CHO K1 cells (ATCC, Rockville, MD) using the Lipofectin reagent[24] (Gibco BRL, Gaithersberg, MD). Stable transfectants were initially selected by the use of Geneticin (Gibco BRL), and receptor-bearing resistant cells were enriched by fluorescence-activated cell sorting based on binding of Fluorescein-Gly-[Nle28,31]-CCK-8. Clonal lines were subsequently established by the limiting dilution method.

Cell Membrane Preparation. CHO K1 cells stably transfected with human CCK-A or CCK-B receptor cDNA were grown at 37 °C under a humidified atmosphere (95% O_2/5% CO_2) in Ham's F12 medium supplemented with 5% heat-inactivated fetal bovine serum. The cells were passaged twice weekly and grown to a density of 2–4 million cells/mL. The cells were collected by centrifugation (600*g*, 15 min, 4 °C) and resuspended in buffer (20 mL, pH 7.4) containing Tris-HCl (25 mM), EDTA (5 mM), EGTA (5 mM), phenylsulfonyl fluoride (0.1 mM), and soybean trypsin inhibitor (100 μg/mL). Cells were disrupted with a motorized glass teflon homogenizer (25 strokes), and the homogenate was centrifuged at low speed (600*g*, 10 min, 4 °C). The supernatant was collected and centrifuged at high speed (500000*g*, 15 min 4 °C) to pellet the particulate fraction. The low-speed pellet was processed three additional times. High-speed particulate fractions were combined and resuspended in buffer (1–5 mg of protein/mL) and frozen at −80 °C. Protein concentration was determined according to manufacturer's directions using BioRad reagent and bovine serum albumin as standard.

Receptor Binding Assays. [^{125}I]Bolton Hunter CCK-8 (Amersham; 2000 Ci/mmol) was dissolved in binding buffer (pH 7.4, 100 000 cpm/25 μL) containing HEPES (20 mM), NaCl (118 mM), KCl (5 mM), $MgCl_2$ (5 mM), and EGTA (1 mM). Nonspecific binding was determined with MK-329[20] (10 μM, CCK-A) or L 365,260[21] (10 μM, CCK-B). Test compounds were dissolved in DMSO at a stock concentration of 100 times the final assay concentration and diluted to appropriate concentrations with binding buffer. Binding assays were performed in triplicate using 96-well plates to which the following were added sequentially: test compound (25 μL), [^{125}I]Bolton Hunter CCK-8 (25 μL), buffer (pH 7.4, 150 μL), and receptor preparation (50 μL). The final concentration of DMSO was 1% in all assay wells. After 3 h at 30 °C, the incubation was terminated by rapid filtration of the mixture onto glass filters (Whatman GF/B) with subsequent washing to remove unbound ligand. Bound radioactivity was quantified by γ counting.

Intracellular Calcium Measurements. Cells grown on glass coverslips to 75–90% confluency were loaded for 50 min in serum-free culture medium containing 5 μM FURA2-AM and 2.5 mM probenecid. A JASCO CAF-102 calcium analyzer was used to measure changes in intracellular calcium concentration by standard ratiometric techniques using excitation wavelengths of 340 and 380 nm. Cells were perfused with increasing concentrations of compound **1** ($n = 1$) until a plateau in the 340/380 ratio was achieved. A washout/recovery period of at least 10 min was allowed between successive stimulations. Following collections of control agonist responses, cells were perfused for 60 min with analog **6** (1 μM, $n = 1$) and a second set of responses to compound **1** was collected. The single concentration K_B was calculated according to the formula,

$$K_B = \frac{[\text{test compound}]}{(\text{CR-1})}$$

where CR is the fold-shift of the concentration response curve to **1** in the presence of the antagonist, **6**.

Acknowledgment. The authors would like to thank Simon Tate (Glaxo Research and Development, Greenford, U.K.) for providing the human CCK-B receptor cDNA clone and acknowledge NIH Grant DK-32878 (L.J.M.) for partial support of this work.

References

(1) Jorpes, J. E.; Mutt, V. Cholecystokinin and Pancreozymin, One Single Hormone? *Acta Physiol. Scand.* **1966**, *66*, 196–202.
(2) Liddle, R. A.; Green, G. M.; Conrad, C. K.; Williams, J. A. Proteins But Not Amino Acids, Carbohydrates, or Fats Stimulate Cholecystokinin Secretion in the Rat. *Am. J. Physiol.* **1986**, *251*, G243–G248.
(3) Silvente-Poirot, S.; Dufresne, M.; Vaysse, N.; Fourmy, D. The Peripheral Cholecystokinin Receptors. *Eur. J. Biochem.* **1993**, *215*, 513–529.
(4) Makovec, F. CCK-B/Gastrin Receptor Antagonists. *Drugs Future* **1993**, *18*, 919–931.
(5) McHugh, P. R.; Moran, T. H. The Stomach, Cholecystokinin, and Satiety. *Fed. Proc.* **1986**, *45*, 1384–1390.
(6) Kissileff, H. R.; Pi-Sunyer, F. X.; Thornton, J.; Smith, G. P. C-Terminal Octapeptide of Cholecystokinin Decreases Food Intake in Man. *Am. J. Clin. Nutr.* **1981**, *34*, 154–160.
(7) Pi-Sunyer, X.; Kissileff, H. R.; Thornton, J.; Smith, G. P. C-Terminal Octapeptide of Cholecystokinin Decreases Food Intake in Obese Men. *Physiol. Behav.* **1982**, *29*, 627–630.
(8) Dourish, C. T.; Ruckert, A. C.; Tattersall, F. D.; Iversen, S. D. Evidence That Decreased Feeding Induced by Systemic Injection of Cholecystokinin is Mediated by CCK-A Receptors. *Eur. J. Pharmacol.* **1989**, *173*, 233–234.
(9) Hermkens, P. H. H.; Ottenheijm, H. C. J.; van der Werf-Pieters, J. M. L.; Broekkamp, C. L. E.; de Boer, T.; van Nispen, J. W. CCK-A Agonists: Endeavours Involving Structure-Activity Relationship Studies. *Recl. Trav. Chim. Pays-Bas* **1993**, *112*, 95–106.
(10) Shiosaki, K.; Lin, C. W.; Kopecka, H.; Craig, R.; Wagenaar, F. L.; Bianchi, B.; Miller, T.; Witte, D.; Nadzan, A. M. Development of CCK-Tetrapeptide Analogues as Potent and Selective CCK-A Receptor Agonists. *J. Med. Chem.* **1990**, *33*, 2952–2956.
(11) Shiosaki, K.; Lin, C. W.; Kopecka, H.; Tufano, M. D.; Bianchi, B. R.; Miller, T. R.; Witte, D. G.; Nadzan, A. M. Boc-CCK-4 Derivatives Containing Side-Chain Ureas as Potent and Selective CCK-A Receptor Agonists. *J. Med. Chem.* **1991**, *34*, 2837–2842.
(12) Asin, K. E.; Gore, P. A., Jr.; Bednarz, L.; Holladay, M.; Nadzan, A. M. Effects of Selective CCK Receptor Agonists on Food Intake After Central or Peripheral Administration in Rats. *Brain Res.* **1992**, *571*, 169–174.
(13) Asin, K. E.; Bednarz, A. L.; Nikkel, A. L.; Gore, P. A., Jr.; Montana, W. E.; Cullen, M. J.; Shiosaki, K.; Craig, R.; Nadzan, A. M. Behavorial Effects of A-71623, a highly selective CCK-A Agonist Tetrapeptide. *Am. J. Physiol.* **1992**, *263*, R125–R135.
(14) Asin, K. E.; Bednarz, L.; Nikkel, A. L.; Gore, P. A.; Nadzan, A. M. A-71623, A Selective CCK-A Receptor Agonist, Suppresses Food Intake in the Mouse, Dog and Monkey. *Pharmacol. Biochem. Behav.* **1992**, *42*, 699–704.
(15) Elliott, R. L.; Kopecka, H.; Bennett, M. J.; Shue, Y.-K.; Craig, R.; Lin, C.-W.; Bianchi, B. R.; Miller, T. R.; Witte, D. G.; Stashko, M. A.; Asin, K. E.; Nikkel, A.; Bednarz, L.; Nadzan, A. M. Tetrapeptide CCK Agonists: Structure Activity Studies on Modifications at the N-terminus. *J. Med. Chem.* **1994**, *37*, 309–313.
(16) Chung, J. Y. L.; Tufano, M. D.; May, P. D.; Shiosaki, K.; Nadzan, A. M.; Garvey, D. S.; Shue, Y. K.; Brodie, M. S.; Holladay, M. W. Preparation of Tetrapeptide Type-B CCK Receptor Ligands. Eur. Pat. Appl. 405506, 1991, 38–39.
(17) Knittel, J. J.; He, X. Q. Synthesis and Resolution of Novel 3'-Substituted Phenylalanine Amides. *Pept. Res.* **1990**, *3*, 176–181.
(18) Ulrick, C. D.; Ferber, I.; Holicky, E.; Hadac, E.; Buell, G.; Miller, L. J. Molecular Cloning and Functional Expression of the Human Gallbladder Cholecystokinin A Receptor. *Biochem. Res. Commun.* **1993**, *193*, 204–211.
(19) Lee, Y.-M.; Beinborn, M.; McBride, E. W.; Lu, M.; Kolakowski, L. F., Jr.; Kopin, A. S. The Human Brain Cholecystokinin B/Gastrin Receptor. *J. Biol. Chem.* **1993**, *268*, 8164–8169.
(20) Evans, B. E.; Bock, M. G.; Rittle, K. E.; Dipardo, R. M.; Whitter, W. L.; Veber, D. L.; Anderson, P. S.; Freidinger, R. M. Design of Potent, Orally Effective, Nonpeptidyl Antagonists of the Peptide Hormone Cholecystokinin. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4918–4922.
(21) Bock, M. G.; Dipardo, R. M.; Evans, B. E.; Rittle, K. E.; Whitter, W. L.; Veber, D. F.; Anderson, P. S.; Freidinger, R. M. Benzodiazepine Gastrin and Brain CCK Receptor Ligands, L365,260. *J. Med. Chem.* **1989**, *32*, 16–23.
(22) Orlowski, R. C.; Walter, R.; Winkler, D. Study of Benzhydrylamine-Type Polymers. Synthesis and Use of p-Methoxybenzhydrylamine Resin in the Solid-Phase Synthesis Preparation of Peptides. *J. Org. Chem.* **1976**, *41*, 3702–3706.
(23) Arunlakshana, O.; Schild, H. O. Some Quantitative Uses of Drug Antagonists. *Br. J. Pharmacol.* **1959**, *14*, 48–58.
(24) Felgner, P. L.; Gadek, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Northrup, J. P.; Ringold, G.; Danielsen, M. Lipofection: a Highly Efficient, Lipid-Mediated DNA-Transfection Procedure. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7413–7417.

JM940558F