Synthesis and Biological Evaluation of Potent, Selective, Hexapeptide CCK-A Agonist Anorectic Agents

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Cholecystokinin (CCK) is a 33-amino acid peptide with multiple functions in both the central nervous system (via CCK-B receptors) and the periphery (via CCK-A receptors). CCK mediation of satiety via the A-receptor subtype suggest a role for CCK in the management of obesity. The carboxy terminal octapeptide (CCK-8) is fully active in this regard, but is lacking in receptor selectivity, metabolic stability, and oral bioavailability. Inversion of the chirality of Asp^7 in conjunction with N-methylation of Phe^8 produces compound **5** which exhibits high affinity and 2100-fold selectivity for CCK-A receptors. Compound **6** (Hpa(SO_3H)-Nle-Gly-Trp-Nle-MeAsp-Phe-NH_2), derived from moving the *N*-methyl group from Phe to Asp, decreased CCK-B affinity substantially without affecting CCK-A affinity, giving a compound with 6600-fold selectivity for CCK-A receptors. These compounds inhibit food intake with nanomolar potency following intraperitoneal administration in fasted rats. In addition to greater potency, compound **6** produces weight loss in rats when administered over nine consecutive days. Intranasal administration of **6** potently inhibits feeding in beagle dogs. Compound **6** produces potent anorectic activity via the CCK-A receptor system.

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 (**1**, 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] CCK-B receptors are found mainly in the CNS where CCK-8 is proposed to be a neurotransmitter or neuromodulator involved in anxiety and pain modulation.[4] Peripheral CCK-A receptors mediate gall bladder contraction, pancreatic exocrine secretion, and satiety.[3] These actions are triggered by the release of CCK-8 from the small intestine following nutrient ingestion.[5,6] The satiety signal is relayed to the brain by CCK-8-mediated activation of CCK-A receptors on vagal afferents.[5,6]

Gibbs et al.[7] observed that intraperitoneally (ip) administered CCK-8 decreases meal size and produces classical postprandial behaviors in rats. The satiety effect of peripherally administered CCK-8 extends to a number of species, including humans.[8,9] Initial efforts to develop a metabolically more stable analogue of CCK-8 resulted in the identification of AR-R 14294 (**2**), which potently inhibits feeding in rats following ip administration and in dogs following intranasal administration.[10]

Using selective antagonists, it has been shown in rats that CCK-A, and not CCK-B, receptors mediate the anorectic effects of peripherally administered CCK-8.[11] However, the therapeutic potential of CCK-8 is limited by its short biological half-life and its poor bioavailability, by other than parenteral administration. Only a small number of compounds have been reported to be CCK-A selective agonists, most notably a series of tetrapeptides.[12−14] Structure−activity relationships of CCK-A agonists suggest that the anionic group of Asp^7 in CCK-8 is important for agonist activity.[15] We have previously reported that inversion of the stereochemistry at Asp^7 of **2** produced the CCK-A selective agonist **3**.[16] We report here further studies of CCK-A agonists leading to the identification of the highly potent CCK-A selective agonist AR-R 15849 (**6**).[17]

Methods

The unsulfated peptide amides were synthesized utilizing the Fmoc/tBu protection strategy on Pal Resin (Milligen/Biosearch).[18] Cleavage from the resin was accomplished using reagent K (TFA, 8.5; H_2O, 0.5; thioanisole, 0.5; phenol, 0.5; ethanedithiol, 0.2) for 2 h at room temperature.[19] The crude peptides were purified to homogeneity using preparative reverse phase HPLC. Sulfation was achieved using Pyr·SO_3, and the final products were purified to homogeneity using reverse phase HPLC and characterized by analytical reverse phase HPLC, mass spectrometry, and amino acid analysis.

A fragment condensation solution synthesis of **6** was developed to accommodate the larger quantity requirements for further biological investigation (Scheme 1). Boc-MeAsp(OBn)-OH was prepared using the method of Holladay.[14] The C-terminal tetrapeptide H-Trp-Nle-MeAsp(OBn)-Phe-NH_2·HCl and the N-terminal acyl dipeptide, Hpa-Nle-Gly-OH (Hpa is 4-hydroxyphenylacetyl), were assembled separately in a stepwise fashion and coupled to give Hpa-Nle-Gly-Trp-Nle-MeAsp(OBn)-Phe-NH_2. After removal of the benzyl protecting group by hydrogenation, the deprotected hexapeptide (Hpa-Nle-Gly-Trp-Nle-MeAsp-Phe-NH_2) was isolated by preparative reverse phase HPLC, thoroughly dried, and sulfated directly to give **6** which was isolated by preparative reverse phase HPLC.

CCK-A receptor affinity was measured by displacement of [^{125}I]BH-CCK-8 from rat pancreatic tissue

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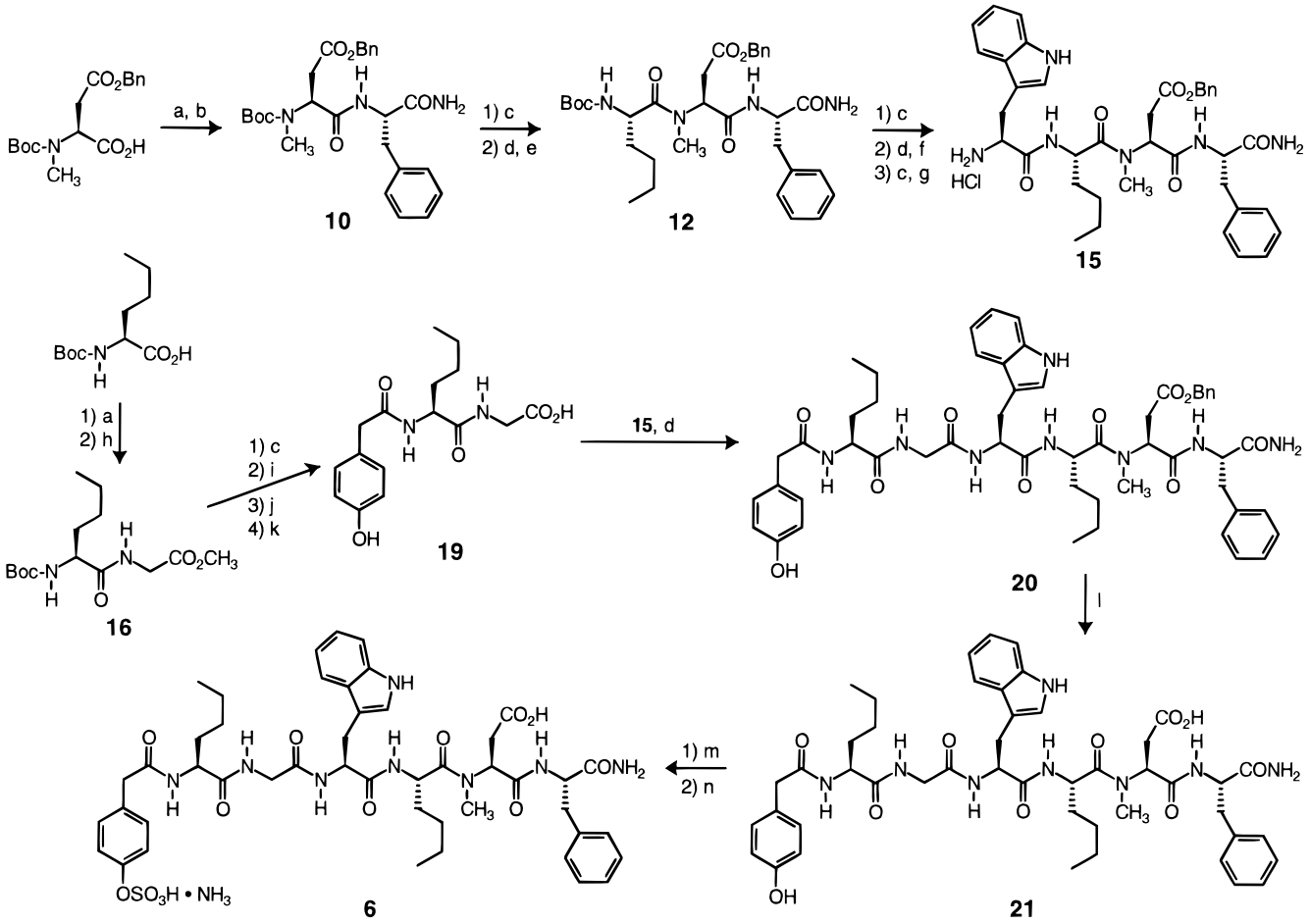
Table 1. Receptor Affinity and Selectivity

no.	structure[a]	K_i (nM) CCK-A	K_i (nM) CCK-B	CCK-A selectivity
1	H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	0.09	0.41	5
2	Hpa(SO_3H)-Met-Gly-Trp-Met-Asp-MePhe-NH_2	0.07	1.28	18
3	Hpa(SO_3H)-Met-Gly-Trp-Met-D-Asp-MePhe-NH_2	0.98	84	86
4	Hpa(SO_3H)-Met-Gly-Trp-Met-D-Asp-Phe-NH_2	19	150	8
5	Hpa(SO_3H)-Nle-Gly-Trp-Nle-D-Asp-MePhe-NH_2	0.20	430	2100
6	Hpa(SO_3H)-Nle-Gly-Trp-Nle-MeAsp-Phe-NH_2	0.03	224	6590
7	Hpa(SO_3H)-Nle-Gly-Trp-Ile-MeAsp-MePhe-NH_2	0.53	>1000	>2000
8	Hpa(SO_3H)-Ile-Gly-Trp-Ile-MeAsp-Phe-NH_2	0.24	2200	9200
9	Hpa(SO_3H)-Nle-Gly-Trp-Nle-D-MeAsp-Phe-NH_2	0.03	1600	48000

[a] Hpa(SO_3H) is sulfated 4-hydroxyphenylacetyl.

Scheme 1. Solution Phase Synthesis of AR-R 15849



[a] Reagents: (a) Piv-Cl, DIEA; (b) H-Phe-NH_2; (c) HCl in EtOAc; (d) TBTU, HOBt, DIEA; (e) Boc-Nle-OH; (f) Boc-Trp-OH; (g) anisole; (h) H-Gly-OMe·HCl, DIEA; (i) Hpa-OSu, DIEA; (j) methanolic NaOH; (k) HCl; (l) H_2 Pd/C; (m) Pyr·SO_3; (n) NH_4OH.

according to the general procedure of Chang.[20] 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.[21]

The ability of a compound to inhibit food intake in rats was measured by a modification of the method described by Cox and Maickel.[22] Individually caged male Sprague−Dawley rats (300−400 g) were maintained on a 12 h light/dark cycle and trained for at least 14 days to feed only during the initial 3 h period of the dark cycle. Inhibition of food intake was measured following intraperitoneal injection of saline or a solution of the test compound. A preweighed food jar containing powdered chow (Purina) was introduced 15 min after injection. Cumulative food intake for each individual animal was measured after 0.5 and 3 h by weighing the food jar. From this information an ED_{50}, i.e., the dose that inhibits feeding by 50%, was calculated for the 0.5 and 3 h feeding interval. This ED_{50} was generated by ALFIT analysis of pooled food consumption data from five doses (10 rats/dose) of each test compound.

Results and Discussion

We previously reported that replacement of Asp-Tyr-(SO_3H) of CCK-8 with Hpa(SO_3H) and N-methylation of Phe as in **2** does not diminish CCK-A or CCK-B affinity[16] (Table 1). However, **2** inhibits feeding in rats following ip administration much more potently than CCK-8 (3 h ED_{50} 0.56 and 112 μg/kg, respectively, Table 2).[10] Inversion of the stereochemistry of Asp in **2** to give **3** has a modest effect on CCK-A affinity (Table 1). However, the combination of D-Asp and MePhe in **3** appears to be more deleterious for CCK-B affinity, producing a compound that is 86-fold CCK-A selective

Table 2. Feeding Inhibition Potency in 21 h Fasted Rats (ip)

	feeding inhibition ED_{50} (μg/kg)	
no.	0.5 h period	3 h period
1	1.2 ± 0.1	112 ± 3.8
2	0.04 ± 0.01	0.56 ±0.11
5	0.45 ± 0.4	2.5 ± 0.1
6	0.11 ± 0.02	0.25 ± 0.01
7	2.0 ± 0.6	2.6 ± 0.4
8	0.56 ± 0.1	3.1 ± 0.3
9	0.06 ± 0.008	0.21 ± 0.04

(Table 1). The nonmethylated analogue of **3**, compound **4**, has less affinity for CCK-A and CCK-B receptors, suggesting that MePhe contributes to the CCK-A affinity and therefore selectivity of **3** (Table 1). Replacing Met with Nle as in **5** has little effect on CCK-A affinity but removes sites of oxidation and presumably increases chemical stability of the peptides. Compound **5** (Hpa-(SO_3H)-Nle-Gly-Trp-Nle-D-Asp-MePhe-NH_2), the Nle analogue of **3**, is a selective CCK-A agonist that inhibits feeding in rats after ip administration more potently than CCK-8, especially for the 3 h feeding period (2.5 vs 112 μg/kg, Table 2). The finding that D-Asp-MePhe-containing peptides were selective for CCK-A receptors led to further manipulation of Asp.

Methylation of the amide nitrogen of Asp in the hexapeptides, regardless of the methylation status of Phe, leads to a dramatic increase in CCK-A selectivity as a result of substantial decrease in CCK-B affinity (**6**−**8**). Compounds **6**−**8** all have subnanomolar affinity for CCK-A receptors and are greater than 2000-fold selective for CCK-A over CCK-B receptors. The fact that CCK-A receptors are highly tolerant of CCK analogues containing MeAsp in the penultimate position correlates well with a tetrapeptide series reported by Holladay.[23] On the other hand, CCK-B receptors are far less tolerant of this MeAsp modification, accounting for the high CCK-A selectivity observed for compounds **6**−**8**. While **6**−**8** are all potent inhibitors of food intake following ip administration, **6** is the most potent at both the 0.5 and 3 h periods (Table 2). Replacing Asp[7] with N-methylated D-Asp gives **9**, the most CCK-A selective agonist in the series. Compound **9** also potently inhibits food intake in rats following ip administration (Table 2).

Relative to CCK-8, all of the Hpa(SO_3H) analogues (**2**−**9**) showed greater feeding inhibition potency following ip administration (Table 2). This increased potency was particularly evident for the 3 h feeding period. The increased feeding inhibition of these CCK analogues may result from improved metabolic stability of these compounds relative to CCK-8. Significantly, the highly selective CCK-A ligands **6** and **9** are basically equipotent in feeding inhibition at the 0.5 and 3 h time periods. The apparent long-lasting effect on food intake by compounds **6** and **9** is possibly due to an increased metabolic stability resulting from methylation of Asp. On the basis of their high affinity and selectivity for the CCK-A receptor and potent, long-lasting feeding inhibition effects following ip administration, compounds **6** and **9** were chosen for further pharmacological profiling.

Both **6** and **9** decrease food intake following intranasal administration to dogs. Fasted dogs were allowed to consume 100 g of food over 15 min followed by a 20 min delay. Each dog was then dosed intranasally with test compound 10 min prior to the presentation of 400 g of food. After 15 min the food containers were removed

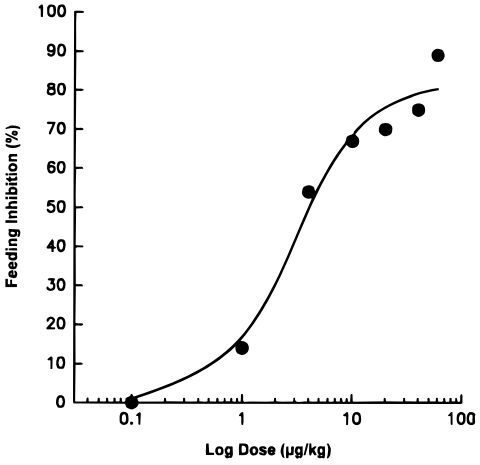


Figure 1. Intranasal dose response of AR-R 15849 to inhibit feeding in male beagles.

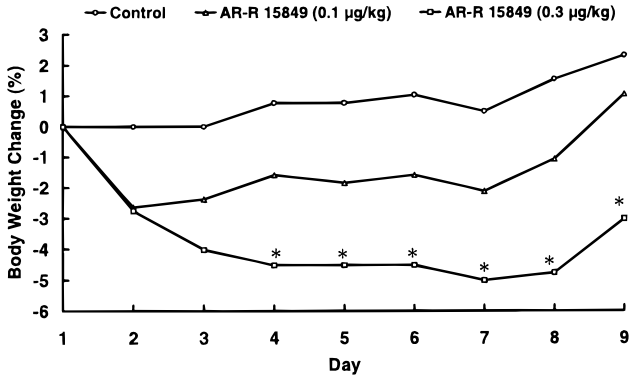


Figure 2. Body weight change effect of AR-R 15849 in rats following daily ip dosing over 9 days. *Values are significantly different from controls ($P < 0.05$, ANOVA, Newman-Keuls).

and weighed. Comparison of food intake data with that of vehicle-dosed controls gave the percent feeding inhibition response. Compound **6** inhibits food intake with an ED_{50} of 5.0 μg/kg following intranasal administration (Figure 1). However, compound **9** inhibits food intake less potently with an ED_{50} of 20 μg/kg following intranasal administration. Because of its enhanced potency, compound **6** was chosen for further evaluation.

To examine the weight loss producing effects of **6**, the compound was administered to rats for nine consecutive days (Figure 2). Twenty 1 h fasted rats, trained to feed at the beginning of a 12 h dark cycle, were weighed and dosed on a daily basis 15 min prior to initiation of a 3 h feeding period. Food intakes were measured hourly during each feeding period. Acutely, a 0.3 μg/kg dose of **6** was found to inhibit food intake by 50%, while a 0.1 μg/kg dose was found to inhibit feeding by 30% (data not shown). Over the 9 day study **6** dose dependently induced weight loss. At the higher dose **6** continued to decrease body weight over days 2−4; thereafter, a 4−5% reduction in body weight was maintained (days 4−8) which differed significantly from control's weight gain even on day 9. The upturn (Figure 2) in the low dose (0.1 μg/kg) and high dose (0.3 μg/kg) curves at days 8 and 9, respectively, is most likely due to pharmacological and behavioral adaptation to prolonged severe caloric deprivation.[24] Further pharmacological profiling of compound **6** is reported elsewhere.[17] Thus, **6** not only reduces food intake but also significantly reduces body weight in growing rats over a 9 day period.

Table 3. Analytical Data

	amino acid analysis									MS		HPLC[a] t_R (min)	
compd	Met	Nle	Ile	Gly	Trp	Asp	MeAsp	Phe	MePhe	*m/z*	adduct	Ia	IIb
3	1.81			1.03	1.04	1.06			1.11	1012	$(M-H)^-$	38.1	4.93
4	1.83			1.11	0.59	1.03		1.04		997	$(M-H)^-$	37.6	4.95
5		1.91		1.04	1.00	1.00			1.05	975	$(M-H)^-$	41.7	6.76
6		1.90		1.04	0.61		1.07	0.98		975	$(M-H)^-$	41.8	7.11
7		0.77	1.02	1.00	ND[b]		0.92		1.29	989	$(M-H)^-$	42.4	7.42
										909	$(M-SO_3H)^-$		
8			1.83	1.00	0.73		1.11	1.06		975	$(M-H)^-$	40.5	6.41
										897	$(M-SO_3H)^-$		
9		1.91		1.02	0.86		1.00	1.06		975	$(M-H)^-$	40.9	6.40
										897	$(M-SO_3H)^-$		

[a] See Experimental Section for description of HPLC Ia, IIb. [b] Not determined.

Several other compounds have been reported to be CCK-A selective agonists that inhibit food intake following ip administration. Most notable are a hexapeptide (A-71378) and a tetrapeptide (A-71623) reported by the Abbott group.[12−14,25] However, in our hands neither compound shows any feeding inhibitory activity at 5.0 μg/kg following intranasal administration in dogs (data not shown). In contrast, compound **6** following intranasal administration in beagle dogs potently inhibits food intake via a natural satiety mechanism.

Experimental Section

Abbreviations. Peptide and amino acid abbreviations used follow the guidelines of the IUPAC−IUB Joint Commission on Biochemical Nomenclature.[26] AAA, amino acid analysis; Boc, *tert*-butyloxycarbonyl; Gly, glycine; MeAsp, *N*-methylaspartic acid; 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; HOBt, 1-hydroxybenzotriazole; Hpa, 4-hydroxylphenylacetyl; OSu, succinimidyloxy ester; Piv-Cl, pivaloyl chloride; Pyr, pyridine; Pyr·SO_3, pyridine sulfur trioxide complex; 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 Å). Peptides were eluted with one of two mobile phases. Prep-LC (**A**): 30 min gradient of 0−60% solvent B (10 mM NH_4OAc (solvent A) and 10 mM NH_4OAc in MeOH (solvent B)) at 75 mL/min. or Prep-LC (**B**): 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.

Analytical reverse phase HPLC was performed on a Hewlett-Packard 1050 system using the following columns and mobile phases. Columns: (I) Keystone Nucleosil C18 (150 × 3.2 mm, 5 μm) eluted at 0.5 mL/min, (II) Waters Symmetry C8 (50 × 3.9 mm, 5 μm, 100 Å) eluted at 3.0 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) 60 min gradient of 0−60% solvent B (0.05% TFA (solvent A) and 0.05% TFA in MeOH (solvent B)); (b) 15 min gradient of 35−100% solvent B (solvents same as in mobile phase a); (c) 30 min gradient of 25−75% solvent B (0.1% TFA (solvent A) and 0.1% TFA in MeOH (solvent B)); (d) 30 min gradient of 20−75% solvent B (solvents same as in mobile phase c); (e) 20 min gradient of 50−90% solvent B (solvents same as in mobile phase c); or (f) 55% MeOH in 0.1% TFA. 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 unsulfated peptide amides were prepared on a MilliGen/Biosearch 9600 synthesizer using standard solid-phase synthesis techniques and the Fmoc/tBu protection strategy. Syntheses were performed starting with Pal Resin[18] (1 g, 0.33 mmol). Fmoc group removals were carried out by treatment with piperidine/DMF. 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 with 6.67 equiv of Hpa-OSu in DMF (1−2 h). Completed protected peptidyl resins were cleaved and deprotected using reagent K[19] (TFA, H_2O, thioanisole, phenol, ethanedithiol, 185:5:5:5:2) for 2 h at room temperature. 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.

The peptide amide products (dried overnight under high vacuum) were sulfated with Pyr·SO_3 according to method D.[27] The crude sulfated products were purified to homogeneity by Prep-LC. Fractions shown by HPLC to be >95% pure were pooled and lyophilized to provide sulfated peptide amide products **3**−**9**. 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) with the expected $(M-H)^-$ molecular ion peaks observed for all peptides. The peptides were determined by HPLC to be >98% pure when analyzed on two different columns (Table 3).

Method A. Boc-MeAsp(OBn)-Phe-NH_2 (10). General Piv-Cl Coupling Method. To a stirred solution of Boc-MeAsp(OBn)-OH (1.00 g, 2.97 mmol; prepared by the method of Holladay[14] from Fmoc-MeAsp(OBn)-OH supplied by Peptides International) in DMF (15 mL) was added DIEA (0.52 mL, 2.97 mmol) and Piv-Cl (0.37 mL, 2.97 mmol). After 15 min a solution of H−Phe-NH_2·HCl (0.490 g, 2.97 mmol) and DIEA (0.52 mL, 2.97 mmol) in DMF (10 mL) was added, and the mixture was stirred at room temperature for 6 h. The solvent was removed in vacuo, and the resulting oil was dissolved in EtOAc; washed with 10% citric acid, 1 N $NaHCO_3$, and saturated NaCl; dried ($MgSO_4$); and evaporated to an oil: TLC (CMA, 9:1:1) R_f 0.69.

Method B. H-MeAsp(OBn)-Phe-NH_2·HCl (11). General Boc Deprotection Method. To a stirred, −10 °C solution of **10** (1.44 g, 2.97 mmol) in EtOAc (100 mL) was added HCl gas to saturation (10 min). The solution allowed to warm to room temperature over 1 h. The solution was reduced in vacuo to a small volume, and hexanes were added with cooled to −20 °C. The white precipitated product was collected by filtration: 1.13 g (99% from Boc-MeAsp(OBn)-OH); TLC (CMA 9:1:1) R_f 0.38; mp 155−158 °C; MS (CI) *m/z* 384 $(M+H)^+$; HPLC (IIIc) t_R 12.7 min. Anal. ($C_{21}H_{25}N_3O_4$·HCl) C, H, N.

Method C. Boc-Nle-MeAsp(OBn)-Phe-NH_2 (12). General TBTU Coupling Method. To a stirred solution of Boc-Nle-OH (0.414 g, 1.79 mmol) in DMF (10 mL) were added TBTU (0.570 g, 1.79 mmol), HOBt (0.270 g, 1.79 mmol), and DIEA (0.62 mL, 3.58 mmol). After 5 min a solution of **11** (0.500 g, 1.19 mmol) and DIEA (0.207 mL, 1.19 mmol) in DMF (5 mL) was added, and the mixture was stirred at room temperature for 2 h. The reaction was found to be incomplete by TLC. Additional Boc-Nle-OH (0.414 g, 1.79 mmol) in DMF (10 mL) with TBTU (0.570 g, 1.79 mmol), HOBt (0.270 g, 1.79 mmol), and DIEA (0.62 mL, 3.58 mmol) was added, and stirring was continued for 2 days. After removal of the solvent in vacuo,

the residue was dissolved in EtOAc; washed with 10% citric acid, 1 N $NaHCO_3$, and saturated NaCl; dried ($MgSO_4$); and evaporated to an oil (0.71 g). Further purification can be performed using silica gel chromatography with EtOAc: TLC (CMA 9:1:1) R_f 0.64; HPLC (IIId) t_R 33.4 min.

H-Nle-MeAsp(OBn)-Phe-NH_2·HCl (13). Compound **12** (0.71 g, 1.28 mmol) was subjected to deprotection using method B: yield 0.502 g (79% from **11**); TLC (CMA 9:1:1) R_f 0.13; MS (TS) *m/z* 497 $(M + H)^+$; HPLC (IIId) t_R 20.9 min.

Boc-Trp-Nle-MeAsp(OBn)-Phe-NH_2 (14). Using method C, Boc-Trp-OH (0.960 g, 3.17 mmol) was coupled to **13** (1.21 g, 2.44 mmol). Following solvent removal, EtOAc (75 mL) and hexane (100 mL) were added to the residue, precipitating a white solid which was collected by filtration, 1.72 g (90% yield). Further purification can be performed using silica gel chromatography with EtOAc or $CHCl_3$: TLC (CMA 9:1:1) R_f 0.67; mp 150−153 °C; HPLC (IIIe) t_R 17.2 min. Anal. ($C_{43}H_{54}N_6O_8$·0.4EtOAc) C, H, N.

H-Trp-Nle-MeAsp(OBn)-Phe-NH_2·HCl (15). Compound **14** (2.93 g, 3.75 mmol) with anisole (1.63 mL, 15 mmol) was deprotected using method B and lyophilized from glacial acetic acid: yield 2.37 g (88%); TLC (CMA 9:1:1) R_f 0.2; MS (TS) *m/z* 783 $(M + H)^+$; HPLC (IIIe) t_R 8.6 min. Anal. ($C_{38}H_{46}N_6O_6$·0.12HCl·0.02HOAc·0.8H_2O) C, H, N.

Boc-Nle-Gly-OMe (16). Using coupling method A, Boc-Nle-OH (1.61 g, 11.16 mmol) was coupled with H-Gly-OMe·HCl (0.900 g, 10.1 mmol): yield 1.425 g (47%); TLC (CMA 9:1:1) R_f 0.88; mp 97−99 °C; MS (CI) *m/z* 303 $(M + H)^+$. Anal. ($C_{14}H_{26}N_2O_5$) C, H, N.

H-Nle-Gly-OMe·HCl (17). Compound **16** (3.77 g, 12.5 mmol) was deprotected using method B: yield 2.88 g (97%); TLC (CMA, 9:1:1) R_f 0.16; mp 147−148 °C. Anal. ($C_9H_{18}N_2O_3$·HCl) C, H, N.

Hpa-Nle-Gly-OMe (18). To a stirred solution of Hpa-OSu (3.32 g, 13.3 mmol; prepared by the method of Hankovszky[27]) and DIEA (2.32 mL, 13.3 mmol) in DMF (10 mL) was added a solution of **17** (2.88 g, 12.1 mmol) and DIEA (2.11 mL, 12.1 mmol) in DMF (40 mL). After 18 h the solvent was removed in vacuo; the residue was dissolved in EtOAc; washed with 10% citric acid, 1 N $NaHCO_3$, and saturated NaCl; dried ($MgSO_4$); and evaporated to a small volume. Addition of hexanes precipitated the product as a white solid, 3.02 g (77%): TLC (CMA, 9:1:1) R_f 0.53; mp 146−147.5 °C; MS (CI) *m/z* 337 $(M + H)^+$. Anal. ($C_{17}H_{24}N_2O_5$) C, H, N.

Hpa-Nle-Gly-OH (19). To a stirred, −10 °C solution of Hpa-Nle-Gly-OMe (3.00 g, 8.92 mmol) in MeOH (30 mL) was added slowly 1 N NaOH (17.8 mL, 17.8 mmol). After the solution was warmed to warm to room temperature over 1 h, water (50 mL) was added. The aqueous solution was washed with ether and acidified (1 N HCl) to pH 3. The product was extracted into EtOAc, washed with saturated NaCl, dried ($MgSO_4$), and evaporated under reduced pressure. The residue was recrystallized from EtOAc/hexanes, 1.47 g (51%): TLC (CMA, 9:1:1) R_f 0.19; mp 136−138 °C; MS (CI) *m/z* 322 $(M + H)^+$. Anal. ($C_{16}H_{22}N_2O_3$) C, H, N.

Hpa-Nle-Gly-Trp-Nle-MeAsp(OBn)-Phe-NH_2 (20). Using coupling method C, **19** (0.538 g, 1.67 mmol) was coupled to **15** (1.00 g, 1.39 mmol). To the product in EtOAc (50 mL) was added ether, and the precipitate was collected by filtration, 1.04 g (76%): HPLC (IIId) t_R 32.8 min.

Hpa-Nle-Gly-Trp-Nle-MeAsp-Phe-NH_2 (21). To a solution of **20** (1.07 g, 1.08 mmol) in MeOH (150 mL) was added 10% Pd/C (300 mg), and the suspension was shaken under a hydrogen atmosphere for 6 h. The catalyst was removed by filtration and the solvent evaporated to an oil. The product was purified by preparative reverse-phase chromatography, Prep-LC (A), t_R 39 min (loading dependent). Product-containing fractions were evaporated to dryness, and the residue was dissolved in EtOAc and precipitated with hexanes, 0.408 g (42%): HPLC (IIIf) t_R 9.8 min; MS (FAB) *m/z* 897 $(M + H)^+$; AAA, Gly 1.07, MeAsp 1.06, Nle 1.93, Phe 0.94. Anal. ($C_{47}H_{60}N_8O_{10}$·1.8H_2O) C, H, N.

Method D. Hpa(SO_3H)-Nle-Gly-Trp-Nle-MeAsp-Phe-NH_2·NH_3 (6). Sulfation Method. To a stirred solution of **21** (242 mg, 0.27 mmol, dried overnight under high vacuum) in anhydrous pyridine (4 mL) under nitrogen was added Pyr·SO_3 (60 mg, 0.38 mmol).[24] After 1 h the reaction was found to be incomplete by HPLC, and more Pyr·SO_3 (60 mg, 0.38 mmol) was added followed in 1 h with additional Pyr·SO_3 (360 mg, 2.26 mmol). After 2.5 h the reaction mixture was diluted with 5% NH_4OH (100 mL), stirred for 0.5 h, and evaporated in vacuo. The residue was purified by preparative reverse-phase chromatography, Prep-LC (B), 0.347 g (74%): HPLC (Ia) t_R 41.8 min; HPLC (Ia) t_R 7.11 min; MS (TS) *m/z* 975 $(M - H)^-$; $[\alpha]^{23}_D$ −68.4° (*c* 0.1, MeOH). Anal. ($C_{47}H_{63}N_9O_{13}S$·NH_3·1.8H_2O) C, H, N.

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