Development of a Hypusine Reagent for Peptide Synthesis

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Received January 22, 1997[⊗]

The synthesis of a reagent that enables the incorporation of the unusual amino acid (2*S*,9*R*)-hypusine (Hpu) into peptide sequences is described. The reagent, (2*S*,9*R*)-11-[(benzyloxycarbonyl)-amino]-7-(carbobenzyloxy)-2-[(9-fluorenylmethoxycarbonyl)amino]-9-(tetrahydropyran-2-yloxy)-7-azaundecanoic acid, is utilized in the synthesis of a hexapeptide containing the primary pentapeptide sequence of the eukaryotic initiation factor eIF-5A, L-Cys-L-**Thr-Gly-Hpu**-L-**His-Gly**. The reagent is shown to be effective for both solution phase and Merrifield resin synthesis.

Introduction

(+)-Hypusine, **1**, an unusual naturally occurring amino acid, was first isolated from bovine brain extracts by T. Shiba *et al.* in 1971.[1] Since its discovery, the post-translational formation of hypusine has been shown to occur on a precursor protein of the eukaryotic initiation factor 5A (eIF-5A; formerly called eIF-4D).[2,3] This initiation factor 5A is unique in that it is the only known cellular protein that contains the amino acid hypusine (Hpu). In the mid-1970s, eIF-5A was shown to stimulate ribosomal subunit joining and to enhance 80 S-bound Met-t-RNA$_i$ reactivity with puromycin.[4,5] Later, in 1983, Folk and co-workers suggested that a hypusine-modified protein serves as an important initiation factor in all growing eukaryotic cells.[2] In 1986, Park *et al.* isolated the eIF-5A protein from human red blood cells and elucidated the amino acid sequence surrounding the single hypusine residue as Thr-Gly-**Hpu**-His-Gly-His-Ala-Lys.[6]

Furthermore, and most interesting, eIF-5A has been shown to play a critical role in the replication of human immunodeficiency virus-1 (HIV-1). The presence of Rev, an active gene product of the virus, is required for HIV-1 genome transcription events, nuclear export of HIV-1 mRNA, and subsequent translation. This phosphoprotein consists of 116 amino acid residues and binds to a fragment of viral mRNA located on the Stem-Loop IIB, called the Rev response element (RRE). However, Rev is not active in the absence of the hypusinated protein eIF-5A.[7a] In fact, antisense and missense DNA experiments have revealed that in the absence of eIF-5A there is no viral replication.[7a,b] Because of these observations, there is considerable interest in Rev-eIF-5A binding as a point of intervention in anti-HIV therapeutic strategies.[7a] The concept, at least, is simple: prevent Rev-eIF-5A binding. Thus, methodologies that would allow the assembly of hypusine-containing peptide fragments would be valuable in both unraveling the role of hypusine in Rev-eIF-5A complex formation and for the assembly of potential inhibitors.

We previously described a flexible synthetic approach to hypusine itself.[8] The key step in this method involved the *N*ϵ-alkylation of *N*ϵ-benzyl-*N*α-(carbobenzyloxy)-L-lysine benzyl ester with (*R*)- or (*S*)-epichlorohydrin to give the respective (2*S*,9*R*)- and (2*S*,9*S*)-chlorohydrins. Subsequent displacement of the respective chlorides by cyanide ion provided the protected hypusine skeletons. The final step, hydrogenation over PtO_2 in acetic acid followed by neutralization and reacidification, yielded the respective (2*S*,9*S*)- or (2*S*,9*R*)-hypusine dihydrochlorides in excess of 50% overall yield. A comparison of the reported hypusine optical rotation with that of our synthetic (2*S*,9*R*)-hypusine, **1**, confirmed the stereochemical integrity of both chiral centers throughout the synthesis.

Having developed synthetic methodology for accessing hypusine itself, we now focus on developing a selectively protected hypusine reagent that can be used to incorporate this unusual amino acid into peptides. The reagent is designed to be utilized both in solution and Merrifield resin-based synthesis.

Results and Discussion

Inspection of hypusine (**1**) (Figure 1) reveals five potentially reactive centers: two primary amino groups, a secondary amino group, a secondary hydroxyl, and a carboxyl group. In eIF-5A, the α-amino nitrogen and the carboxyl group are coupled to other amino acids. We envisioned the target hypusine reagent with all three nitrogens (N2, N7, and N12) protected while the carboxyl group remained free for peptide coupling. Additionally, the α-amino nitrogen (N2) required a protecting group orthogonal to those masking the other two potentially

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⊗ Abstract published in *Advance ACS Abstracts,* April 15, 1997.

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S0022-3263(97)00119-9 CCC: $14.00

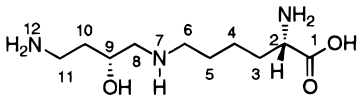

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Figure 1. (2*S*,9*R*)-Hypusine, **1**.

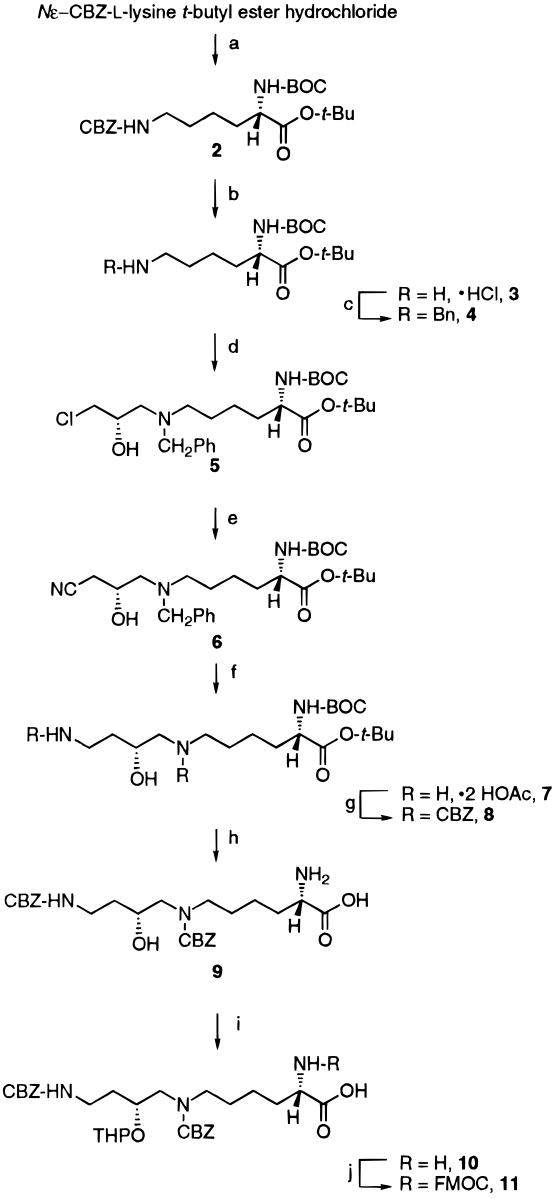
reactive amines (N7 and N12). Therefore, the N2 nitrogen was protected as the FMOC derivative, while the N7 and N12 amines were protected as carbobenzyloxy (CBZ) moieties. The 9-hydroxyl was masked as a tetrahydropyranyl ether (THP). This protection was necessary as the poorly reactive secondary hydroxyl was still expected to cause difficulty with the anticipated N-acylating agents used in solid phase synthesis.[9]

Synthesis of the Hypusine Reagent. As shown in Scheme 1, the synthesis began with the *tert*-butoxycarbonylation of *N*ϵ-CBZ-L-lysine *t*-Bu ester and gave **2** in 98% yield.[10] We had initially prepared **2** by esterification of *N*ϵ-CBZ-*N*α-BOC-L-lysine with *tert*-butyl alcohol, 1,3-dicyclohexylcarbodiimide, and 4-(dimethylamino)pyridine,[11,12] but this resulted in partial racemization as suggested by comparison of the optical rotations of samples of **2** prepared by the two methods. This was confirmed by analysis of the ^{1}H NMR spectra of two samples of L-lysyl-L-valine that were independently prepared from similarly protected lysines after each had been subjected to one of the above methods; 10% of the D-lysyl-L-valine diastereomer was observed in the dipeptide sample obtained from the esterification method while the sample that had been subjected to the *tert*-butoxycarbonylation method proved to be free of racemized compound (data not shown).

Having solved the initial racemization problem, the *N*ϵ-CBZ group of **2** (Scheme 1) was removed by hydrogenation over 10% Pd−C in ethanol and aqueous HCl to give **3** in 99% yield.[15] Conversion of **3** to the *N*ϵ-benzyl-*N*α-BOC-L-lysine *t*-Bu ester **4** was accomplished by reductive amination of the liberated *N*ϵ amine with benzaldehyde and sodium cyanoborohydride.[16]

Our earlier synthesis of (+)-hypusine[8] developed a chiral 4-amino-2-hydroxybutane synthon for accessing the parent molecule **1** from an L-lysine derivative. In particular, this fragment made it possible to elaborate the Nϵ benzyl group of a protected L-lysine into the N7−N12 structure of (+)-hypusine. In this report we further exploit this concept. As shown in Scheme 1, the subsequent *N*ϵ-alkylation of **4** with (*S*)-epichlorohydrin at rt gave the (2*S*,9*S*)-chlorohydrin **5** (77%). Displacement of the chloride in **5** by cyanide ion afforded the protected (2*S*,9*R*)-hypusine skeleton **6** in 70% yield. Simultaneous debenzylation at N7 and saturation of the terminal nitrile in **6** was accomplished by hydrogenation using a mixture

Scheme 1.[a] **Synthesis of the Hypusine Reagent**



[a] (a) $(BOC)_2O$, $NaHCO_3$; (b) H_2, Pd−C; (c) PhCHO, $NaBH_3CN$; (d) (*S*)-(+)-epichlorohydrin, $MgSO_4$; (e) KCN; (f) H_2, Pd−C, PtO_2; (g) CBZ-Cl, DIEA; (h) TFA, triethylsilane, CH_2Cl_2; (i) 3,4-dihydro-2*H*-pyran; (j) FMOC-ONSu, Na_2CO_3.

of 10% Pd−C and PtO_2 to give the amino alcohol **7** as a diacetate (99%). Protection of the amino functions of **7** at N7 and N12 using CBZ-groups provided **8** in 55% yield. Selective removal of the *tert*-butyl ester and *N*α-BOC protecting groups was accomplished with TFA and triethylsilane[13] to give the di-CBZ-derivative **9** (78%). The secondary 9-hydroxyl function was protected as tetrahydropyranyl ether[17] **10** (68%), and subsequent protection of the remaining Nα-amine function with 9-fluorenylmethyl *N*-succinimidyl carbonate (FMOC-ONSu) gave the hypusine reagent **11** with the desired protecting groups in 83% yield.[18]

Synthesis of a Hypusine-Containing Peptide. While the hypusine reagent **11** (Scheme 1) allows for the

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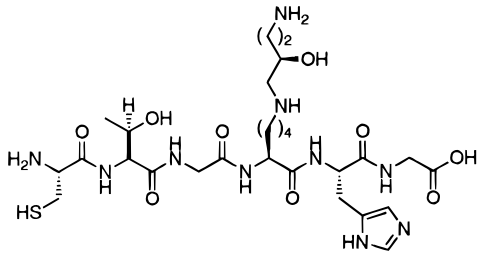


12, TFA salt

Figure 2. Hypusine-containing hexapeptide **12**.

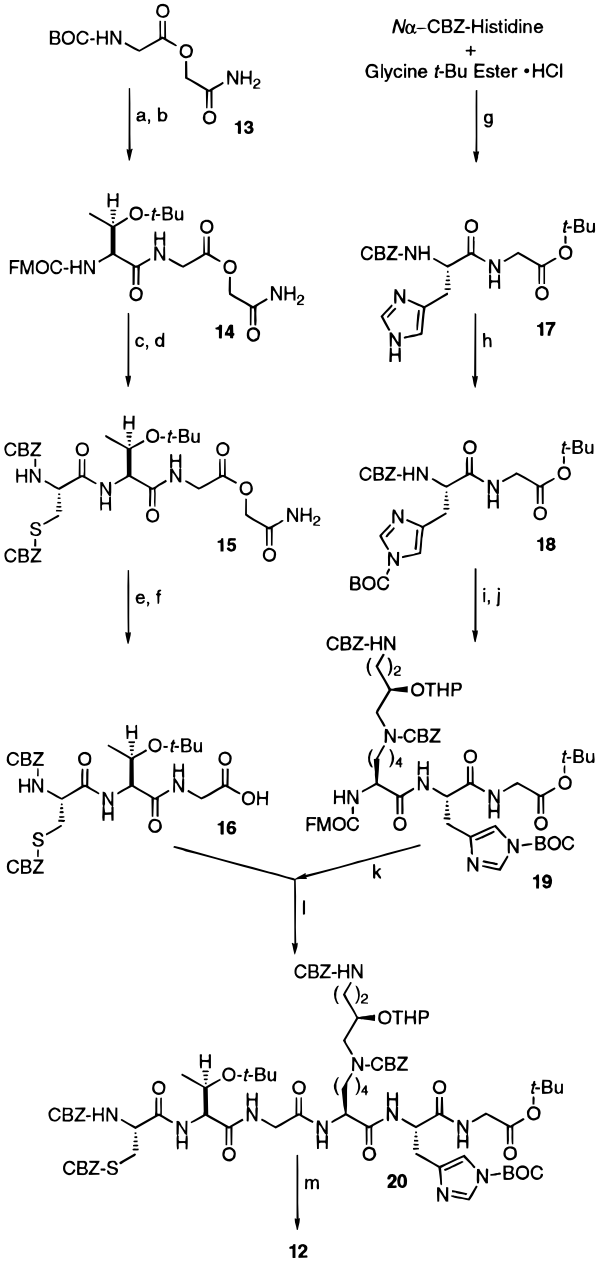
assembly of a variety of hypusine-containing peptides, our target was the hypusine-containing pentapeptide found in eIF-5A capped at its N-terminus with L-cysteine, i.e., Cys-**Thr-Gly-Hpu-His-Gly** (**12**, Figure 2).[6] The L-cysteine, which is not contained in the natural peptide, was added to the sequence as a means of covalently linking this peptidic hapten to a carrier protein for ultimate use in raising antibodies specific to hypusine-containing epitopes.[19] The solution synthesis, a convergent approach, was carried out by elaborating from the C to the N terminus, as shown in Scheme 2, and involved the coupling of three appropriately-protected pieces: Cys-Thr-Gly, Hpu, and His-Gly.

After several failed attempts with THP and silyl esters, the carboxamidomethyl (CAM) ester developed by Martinez[20] was employed as a carboxyl protecting group in generating the Cys-Thr-Gly fragment. This protecting group is orthogonal to the BOC, CBZ, and FMOC groups. Thus, the synthesis of the masked Cys-Thr-Gly fragment, **16**, began with the *N*-BOC-Gly-CAM ester **13**.[20a] Removal of the BOC group with trifluoroacetic acid (TFA) gave the amine salt (60%), which was immediately coupled with *N*α-FMOC-L-threonine *tert*-butyl ether to give the dipeptide **14** in 85% yield. Treatment of **14** with 10% diethylamine (DEA) in DMF followed by coupling with *N*,*S*-di-CBZ-L-cysteine using BOP and DIEA afforded the tripeptide **15** (50%). Removal of the CAM ester with aqueous Na_2CO_3 followed by acidification with aqueous citric acid generated the Cys-Thr-Gly building block **16** in 77% yield.

The design of the His-Gly fragment, **18**, was predicated on obtaining efficient coupling between the *N*α-His group and reagent **11**. Previous work by Yamashiro[21] suggested that *tert*-butoxycarbonylation of the imidazole side chain prior to the condensation step substantially increases coupling yields. For this reason the His-Gly fragment, **18**, was assembled in two steps. First, the condensation of glycine *tert*-butyl ester and *N*α-CBZ-L-histidine with diphenylphosphoryl azide (DPPA),[22] afforded **17** (72%). In the second step, the His side chain was masked by treatment with di-*tert*-butyl dicarbonate in THF to give the dipeptide **18** in 78% yield.

As shown in Scheme 2, the final hexapeptide **12** was constructed stepwise from the fragments **11**, **16**, and **18**. Hydrogenolysis of the *N*α-CBZ-group of **18** provided the amine hydrochloride salt (68%), which was condensed

Scheme 2.[a] **Hexapeptide Synthesis**



[a] (a) TFA; (b) FMOC-Thr(O-*t*-Bu)-OH, BOP, DIEA; (c) 10% DEA in DMF; (d) *N*,*S*-di-CBZ-cysteine, BOP, DIEA; (e) Na_2CO_3; (f) citric acid; (g) DPPA, TEA; (h) $(BOC)_2O$; (i) H_2, 10% Pd−C, EtOH, HCl; (j) Hpu reagent **11**, BOP, DIEA; (k) 4-AMP in $CHCl_3$; (l) BOP, DIEA; (m) TFA, phenol.

with hypusine reagent **11** to give the protected Hpu-His-Gly tripeptide **19** in 85% yield. The amine generated by treatment of **19** with 4-(aminomethyl)piperidine[23] (68%) was acylated by the tripeptide acid **16** with BOP to give the masked conjugate **20** in 81% yield. The final deprotection of **20** with TFA and phenol gave the hexapeptide Cys-Thr-Gly-**Hpu**-His-Gly as its trifluoroacetic acid salt **12** in 24% yield. The peptide was fully assigned by DQCOSY, TOCSY, and HMQC NMR (data not shown).

Polymer-bound synthesis of the hexapeptide **12** was performed on a *p*-(hydroxymethyl)phenoxymethyl polystyrene resin (Wang-resin) using an Applied Biosystems 432A Synthesizer and FMOC chemistry with HBTU as

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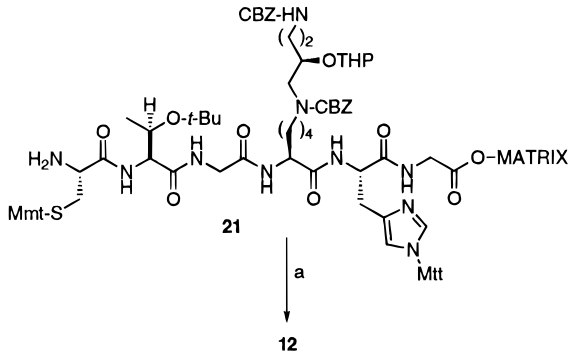
Scheme 3.[a] Deprotection of the SPPS Hexapeptide 21



[a] (a) HBr/acetic acid in TFA, phenol, pentamethylbenzene, triisopropylsilane, 1,2-ethanedithiol.

an activating agent.[24] The cysteine and histidine residues of the hexapeptide were initially protected with trityl groups, while the threonine was protected as its *tert*-butyl ether. Attempts to release the free hexapeptide by refluxing in TFA/phenol or with HBr/acetic acid in TFA with phenol and pentamethylbenzene, respectively, did not succeed. Use of the more labile 4-methoxytrityl-(Mmt)[25] and 4-methyltrityl-groups (Mtt)[26] to protect the cysteine and histidine derivatives, respectively, produced the protected hexapeptide **21** (Scheme 3). Final deprotection of **21** was then achieved with HBr/acetic acid in TFA and a "cocktail" of carbocation scavengers (phenol, pentamethylbenzene, triisopropylsilane, 1,2-ethanedithiol) at rt.[14] This method generated very few side products and provided **12**, after purification by HPLC, in 24% yield. All analytical data were consistent with hexapeptide **12** previously prepared by the solution-phase method.

In summary, the hypusine reagent described has been demonstrated to be a highly useful synthon in accessing the eIF-5A pentapeptide sequence. While the yields are generally excellent for these kinds of systems, the most notable feature is the flexibility that this methodology offers in synthesizing related eIF-5A mimics. We have now initiated the development of an antibody for the pentapeptide−protein coupled system and begun the synthesis of eIF-5A mimics.

Experimental Section

General. Reagents were purchased from the Aldrich, Fluka, or Sigma Chemical Co. and were used without further purification. *N*ε-CBZ-L-Lysine *tert*-butyl ester hydrochloride was obtained from BACHEM Bioscience Inc. Fisher Optima-grade solvents were routinely used, and organic extracts were dried with anhydrous sodium sulfate. THF was distilled from sodium metal and benzophenone. Acetonitrile was distilled from NaH. Silica gel 32−60 (40 μm "flash") from Selecto, Inc. (Kennesaw, GA) was used for column chromatography. ^{1}H NMR spectra were recorded at 300 MHz and ^{13}C NMR spectra at 75 MHz unless otherwise specified. Chemical shifts are given in parts per million downfield from an internal tetramethylsilane or 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt, standard. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA. Melting points are uncorrected. Samples for amino acid analysis were either hydrolyzed with 6 N HCl or with 12 N HCl plus propionic acid for resin samples and then run on an Applied Biosystems Amino Acid Analyzer using precolumn PTC-derivatization.

***N*α-BOC-*N*ε-CBZ-L-Lysine *tert*-Butyl Ester (2).** Sodium hydrogencarbonate (2.81 g, 33.47 mmol) in water (75 mL) was added to H-Lys(CBZ)-O-*t*-Bu hydrochloride (12.00 g, 32.18 mmol) in chloroform (100 mL), and the mixture was stirred at rt for 5 min under an N_2 atmosphere. Di-*tert*-butyl dicarbonate (7.02 g, 32.18 mmol) in chloroform (50 mL) was added, the mixture refluxed for 1.5 h and allowed to cool to rt. The layers were separated, the aqueous layer was extracted with chloroform (3 × 100 mL), and the combined organic layers were dried over magnesium sulfate. Concentration *in vacuo* followed by flash chromatography (3:1 hexane:ethyl acetate) gave **2** (13.82 g, 98%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 7.30 (s, 5 H), 5.10 (s, 2 H), 4.82 (m, 1 H), 4.18 (m, 1 H), 3.20 (m, 2 H), 1.90−1.30 (m, 6 H), 1.48 (s, 9 H), 1.46 (s, 9 H). ^{13}C NMR (CD_3OD) δ 173.8, 158.8, 158.1, 138.4, 129.4, 128.9, 128.7, 82.5, 80.4, 67.3, 55.7, 41.4, 32.4, 30.4, 28.7, 28.3, 24.0. HRMS *m*/*z* calcd for $C_{23}H_{37}N_2O_6$ 437.2652, found 437.2643. Anal. Calcd for $C_{23}H_{36}N_2O_6$: C, 63.28; H, 8.31; N, 6.42. Found: C, 63.13; H, 8.28; N, 6.47. $[\alpha]^{27}_D$ +5.0° (*c* 2.00, $CHCl_3$).

***N*α-BOC-L-Lysine *tert*-Butyl Ester Hydrochloride (3).** To a solution of **2** (34.51 g, 79.15 mmol) in 300 mL of ethanol and 1 N HCl (88 mL) was added 10% Pd−C (2.95 g) and H_2 gas was introduced. Additional catalyst (1.0 g) was added 7 h later. After 5 h the black suspension was filtered through Celite and washed with ethanol. The filtrate was concentrated and the residue dried *in vacuo* to give **3** as its hydrochloride salt (26.59 g, 99%). ^{1}H NMR (CD_3OD) δ 3.95 (dd, 1 H, J = 8.8, 5.0 Hz), 2.93 (t, 2 H, J = 7.7 Hz), 1.84−1.60 (m, 6 H), 1.45 (s, 9 H), 1.43 (s, 9 H). ^{13}C NMR (CD_3OD) δ 173.5, 158.2, 82.7, 80.5, 79.5, 55.5, 40.6, 32.1, 28.7, 28.3, 23.9. HRMS *m*/*z* calcd for $C_{15}H_{31}N_2O_4$ 303.2284, found 303.2272. $[\alpha]^{26}_D$ −10.1° (*c* 1.00, CH_3OH).

***N*ε-Benzyl-*N*α-BOC-L-Lysine *tert*-Butyl Ester (4).** A solution of **3** (25.97 g, 76.64 mmol) in $CHCl_3$ (300 mL) was washed with saturated Na_2CO_3 solution (2 × 100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated. The resultant oil was combined with benzaldehyde (10.42 g, 98.13 mmol), ethanol (150 mL), and activated 3 Å molecular sieves (46.0 g) and stirred under N_2 for 6 h. Sodium cyanoborohydride (2.41 g, 38.4 mmol) was added and the mixture stirred overnight at rt. The mixture was filtered, and the filtrate was acidified to pH 2 with 1 N HCl (110 mL). The solution was concentrated to dryness, dissolved in $CHCl_3$, and washed with saturated Na_2CO_3 solution and water. The organic layer was dried ($MgSO_4$) and concentrated. Flash column chromatography (10% ethanol/$CHCl_3$) afforded **4** (16.16 g, 54%) as a colorless oil. ^{1}H NMR (CD_3OD) δ 7.34−7.20 (m, 5 H), 3.91 (dd, 1 H, J = 9.0, 5.1 Hz), 3.72 (s, 2 H), 2.58 (t, 2 H, J = 7.2 Hz), 1.82−1.30 (m, 6 H), 1.45 (s, 9 H), 1.43 (s, 9 H). ^{13}C NMR ($CDCl_3$) δ 171.9, 155.3, 140.1, 128.3, 128.1, 126.9, 81.6, 79.5, 53.9, 48.9, 32.7, 29.5, 28.3, 27.9, 22.9. HRMS *m*/*z* calcd for $C_{22}H_{36}N_2O_4$ 392.2675, found 392.2676. Anal. Calcd for $C_{22}H_{36}N_2O_4$: C, 67.32; H, 9.24; N, 7.14. Found: C, 67.40; H, 9.28; N, 7.16. $[\alpha]^{25}_D$ +6.9° (*c* 1.00, $CDCl_3$).

(2*S*,9*S*)-7-Benzyl-2-[(*tert*-butoxycarbonyl)amino]-10-chloro-9-hydroxy-7-azadecanoic Acid *tert*-Butyl Ester (5). A mixture of **4** (16.0 g, 40.76 mmol), (*S*)-(+)-epichlorohydrin (4.17 g, 45.0 mmol), and anhydrous $MgSO_4$ (5.33 g, 44.28 mmol) in CH_3OH (40 mL) was stirred under N_2 for three days. The solids were filtered off and washed with CH_3OH. The filtrate was concentrated at rt to give a yellow oil which was purified by flash chromatography on silica gel (66% hexane/ethyl acetate) to provide **5** (13.23 g, 77%) as a colorless oil. ^{1}H NMR (C_6D_6) δ 7.18 (m, 5 H), 5.00 (br d, 1 H), 4.40 (m, 1 H), 3.62 (m, 1 H), 3.40−3.10 (m, 4 H), 2.20−2.00 (m, 4 H), 1.63 (m, 1 H), 1.40 (s, 9 H), 1.31 (s, 9 H), 1.20 (m, 2 H). ^{13}C NMR (C_6D_6) δ 171.7, 155.2, 138.8, 128.8, 127.9, 127.0, 80.8, 78.8, 67.7, 58.8, 57.2, 53.9, 53.7, 47.3, 32.5, 28.0, 27.5, 26.3, 22.8. HRMS *m*/*z* calcd for $C_{25}H_{42}ClN_2O_5$ 485.2782, found 485.2775. $[\alpha]^{25}_D$ +5.3° (*c* 1.00, $CHCl_3$).

(2*S*,9*R*)-7-Benzyl-2-[(*tert*-butoxycarbonyl)amino]-10-cyano-9-hydroxy-7-azadecanoic Acid *tert*-Butyl Ester (6).

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A mixture of **5** (6.99 g, 14.4 mmol), dry KCN (9.38 g, 144 mmol), and 18-crown-6 (0.76 g, 2.88 mmol) in dry acetonitrile (275 mL) was stirred at 45 °C for 5 days (it should be noted that heating to reflux causes significant decomposition). The reaction mixture was cooled, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (25% ethyl acetate/hexane) to give **6** as a colorless oil (4.82 g, 70%). ^{1}H NMR (CD_3OD) δ 7.34−7.18 (m, 5 H), 3.97−3.83 (m, 2 H), 3.67 (dd, 1 H, J = 13.4, 2.6 Hz), 3.54 (dd, 1 H, J = 13.4, 4.0 Hz), 2.72−2.40 (m, 6 H), 1.80−1.50 (m, 4 H), 1.45 (s, 9 H), 1.44 (s, 9 H), 1.40−1.30 (m, 2 H). ^{13}C NMR ($CDCl_3$) δ 171.8, 155.3, 138.1, 128.8, 128.4, 127.3, 117.1, 81.6, 79.5, 63.6, 58.8, 54.0, 32.6, 28.2, 27.9, 22.1. HRMS m/z calcd for $C_{26}H_{42}N_3O_5$ 476.3124, found 476.3121. Anal. Calcd for $C_{26}H_{41}N_3O_5$: C, 65.66; H, 8.69; N, 8.83. Found: C, 65.71; H, 8.67; N, 8.80. $[\alpha]^{25}_D$ +4.7° (*c* 1.00, $CHCl_3$).

(2*S*,9*R*)-2-[(*tert*-Butoxycarbonyl)amino]-11-amino-9-hydroxy-7-azaundecanoic Acid *tert*-Butyl Ester, Diacetate Salt (7). To a solution of **6** (4.80 g, 10.7 mmol) in glacial acetic acid (100 mL) were added 10% Pd−C (0.50 g) and PtO_2 (1.00 g), and H_2 gas was introduced. The catalyst was removed after 6 h by filtration through Celite and washed with acetic acid. The filtrate was concentrated *in vacuo*. Azeotropic removal of acetic acid with toluene provided **7** as a colorless oil (5.10 g, 99%). ^{1}H NMR (500 MHz) (CD_3OD) δ 4.02−3.94 (m, 2 H), 3.14−2.86 (m, 6 H), 1.94 (s, 6 H), 1.87−1.58 (m, 8 H), 1.46 (s, 9 H), 1.44 (s, 9 H). ^{13}C NMR (CD_3OD) δ 169.6, 156.54, 85.3, 70.2, 62.6, 56.6, 54.2, 53.9, 34.0, 31.1, 28.2, 26.3, 23.2. HRMS m/z calcd for $C_{19}H_{40}N_3O_5$ 390.2968, found 390.2977. $[\alpha]^{25}_D$ +0.6° (*c* 1.00, CH_3OH).

(2*S*,9*R*)-11-[(Benzyloxycarbonyl)amino]-2-[(*tert*-butoxycarbonyl)amino]-9-hydroxy-7-(carbobenzyloxy)-7-azaundecanoic Acid *tert*-Butyl Ester (8). A solution of **7** (1.17 g, 2.30 mmol) in $CHCl_3$ (100 mL) was washed with saturated Na_2CO_3 solution. The aqueous layer was extracted with $CHCl_3$ (3 × 100 mL), and the combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. A solution of the resultant oil (the free amine, 0.85 g, 2.18 mmol) in CH_2Cl_2 (60 mL) was cooled to 0 °C and treated with diisopropylethylamine (0.59 g, 4.57 mmol) and benzyl chloroformate (0.79 g, 4.60 mmol). The reaction mixture was stirred overnight at rt, concentrated to dryness, and purified by flash chromatography (50% ethyl acetate/hexane) to give **8** (790 mg, 55%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 7.23 (m, 10 H), 5.45 (m, 1 H), 5.08 (s, 2 H), 5.04 (s, 2 H), 4.10 (m, 1 H), 3.80 (m, 1 H), 3.40 (m, 1 H), 3.23 (m, 5 H), 1.80−1.43 (m, 6 H), 1.41 (s, 18 H), 1.23 (m, 2 H). ^{13}C NMR ($CDCl_3$) δ 171.8, 157.5, 156.9, 155.3, 136.4, 128.4, 128.3, 127.9, 127.7, 81.6, 79.5, 69.2, 67.2, 66.5, 53.7, 48.5, 37.7, 34.8, 32.5, 28.2, 27.9, 22.3. HRMS m/z calcd for $C_{35}H_{52}N_3O_9$ 658.3703, found 658.3774. $[\alpha]^{24}_D$ +4.6° (*c* 0.50, $CHCl_3$).

(2*S*,9*R*)-2-Amino-11-[(benzyloxycarbonyl)amino]-7-(carbobenzyloxy)-9-hydroxy-7-azaundecanoic Acid (9). The ester **8** (500 mg, 0.76 mmol) was added to a mixture of trifluoroacetic acid (1.12 g, 9.90 mmol), CH_2Cl_2 (2.05 g, 24.0 mmol), and triethylsilane (220 mg, 1.9 mmol) and stirred at rt for 20 h. The reaction mixure was concentrated to dryness and stirred again in the mixture as described above for an additional 6 h. The reaction mixture was concentrated and the resultant oil dissolved in 1.0 mL water and adjusted to pH 8 with saturated $NaHCO_3$ solution. The solution was concentrated and the residue purified by chromatography on a C-18 column (55% acetone/water) to give **9** (300 mg, 78%) as a colorless oil. ^{1}H NMR (CD_3OD) δ 7.40−7.25 (m, 10 H), 5.11 (s, 2 H), 5.06 (s, 2 H), 3.82 (m, 1 H), 3.55 (m, 1 H), 3.40−3.10 (m, 6 H), 1.95−1.30 (m, 8 H). HRMS m/z calcd for $C_{26}H_{36}N_3O_7$ 502.2553, found 502.2531. $[\alpha]^{24}_D$ +4.2° (*c* 1.00, CH_3OH).

(2*S*,9*R*)-2-Amino-11-[(benzyloxycarbonyl)amino]-7-(carbobenzyloxy)-9-(tetrahydropyran-2-yloxy)-7-azaundecanoic Acid (10). Trifluoroacetic acid (115 mg, 1.01 mmol) was added to a solution of **9** (265 mg, 0.53 mmol) in $CHCl_3$ (5 mL). The solution was concentrated *in vacuo*. The resultant oil was dissolved in CH_2Cl_2 (15 mL), and 3,4-dihydro-2*H*-pyran (51 mg, 55 μL, 0.61 mmol) was added at rt. The reaction progress was monitored by TLC, and three additional portions of 3,4-dihydro-2*H*-pyran (51 mg each) were added over the next 7 h. The reaction mixture was stirred for an additional 12 h and concentrated *in vacuo*. The oil was dissolved in water and methanol (1:1; 4 mL) and adjusted to pH 7 with saturated $NaHCO_3$ solution. The solution was concentrated and the crude oil purified by chromatography on a C-18 column (55% acetone/water) to give **10** (210 mg, 68%) as a colorless oil and recovered starting material **9** (20 mg, 8%). ^{1}H NMR (CD_3OD) δ 7.40−7.22 (m, 10 H), 5.10 (m, 2 H), 5.04 (s, 2 H), 4.62−4.32 (m, 1 H), 4.02−3.68 (m, 2 H), 3.50 (m, 1 H), 3.44−3.06 (m, 7 H), 1.98−1.28 (m, 14 H); ^{13}C NMR (CD_3OD) δ 174.3, 158.7, 158.1, 138.5, 129.7, 129.6, 129.5, 129.3, 129.0, 128.8, 101.7, 100.1, 74.9, 68.3, 67.4, 65.3, 56.2, 48.4, 38.2, 34.3, 32.6, 32.1, 28.5, 26.4, 23.6, 21.8, 21.2. HRMS m/z calcd for $C_{31}H_{43}N_3O_8$ 586.3128, found 586.3118. $[\alpha]^{24}_D$ +4.0° (*c* 0.25, CH_3OH).

(2*S*,9*R*)-11-[(Benzyloxycarbonyl)amino]-7-(carbobenzyloxy)-2-[(9-fluorenylmethoxycarbonyl)amino)-9-(tetrahydropyran-2-yloxy)-7-azaundecanoic Acid (11). A solution of 9-fluorenylmethyl *N*-succinimidyl carbonate (181 mg, 0.53 mmol) in DMF (2.5 mL) was added to a solution of **10** (210 mg, 0.36 mmol) in 9% Na_2CO_3 (0.836 mL, 0.72 mmol) at 0 °C and stirred overnight at rt. The pH was adjusted to 7 with 0.1 N HCl. The mixture was concentrated to an oil and purified by flash chromatography (90% $CHCl_3$/MeOH) to give **11** (239 mg, 83%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 7.78 (m, 2 H), 7.60 (m, 2 H), 7.30 (m, 14 H), 5.72 (m, 2 H), 5.18 (s, 2 H), 5.16 (s, 2 H), 4.60 (m, 1 H), 4.52−4.22 (m, 3 H), 4.20 (m, 1 H), 4.00−3.72 (m, 3 H), 3.50−3.10 (m, 6 H), 2.00−1.22 (m, 14 H); ^{13}C NMR (150 MHz) ($CDCl_3$) δ 174.6, 156.7, 156.4, 143.9, 143.8, 141.3, 136.8, 136.5, 128.5, 128.5, 128.1, 128.0, 127.9, 127.7, 127.1, 125.1, 124.9, 120.0, 100.7, 73.5, 67.4, 67.2, 66.6, 53.5, 48.4, 47.2, 32.9, 32.0, 31.5, 30.8, 28.0, 27.3, 25.2, 22.0, 21.1, 19.8. HRMS m/z calcd for $C_{46}H_{53}N_3O_{10}Na$ 830.3629, found 830.3661. Anal. Calcd for $C_{46}H_{53}N_3O_{10}$: C, 68.38; H, 6.61; N, 5.20. Found: C, 68.55; H, 6.63; N, 5.26. $[\alpha]^{26}_D$ +3.4° (*c* 1.00, $CHCl_3$).

FMOC-Thr(*O*-*t*-Bu)-Gly Carboxamidomethyl Ester (14). A solution of **13**[20a] (1.16 g, 5.0 mmol) in TFA (10 mL) was stirred for 10 min at 0 °C. The amine TFA salt was precipitated with diethyl ether (130 mL), filtered, dried (0.75 g, 60%), and dissolved in dry DMF (10 mL). FMOC-Thr(*O*-*t*-Bu)-OH (1.20 g, 3.0 mmol) and BOP (1.33 g, 3.0 mmol) were added. The solution was cooled to 0 °C, and DIEA (1.15 mL, 6.6 mmol) was added dropwise. The solution was warmed to rt and stirred overnight. The volatiles were removed *in vacuo*; the resulting oil was dissolved in ethyl acetate (200 mL) and washed with 1 M citric acid, water, 5% aqueous $NaHCO_3$ solution, and water. The organic layer was dried over $MgSO_4$ and concentrated. Flash chromatography (90% ethyl acetate/hexane) gave **14** as a colorless solid (1.30 g, 85%). ^{1}H NMR ($CDCl_3$) δ 7.77 (d, 2 H, J = 7.5 Hz), 7.65 (m, 1 H), 7.60 (d, 2 H, 7.5 Hz), 7.45−7.27 (m, 4 H), 6.60 (s br, 1 H), 5.87 (d br, 1 H), 5.54 (s br, 1 H), 4.67 (m, 2 H), 4.51−4.35 (m, 2 H), 4.28−4.12 (m, 4 H), 4.05 (m, 1 H), 1.29 (s, 9 H), 1.08 (d, 3 H, J = 6.4 Hz). Anal. Calcd for $C_{27}H_{33}N_3O_7$: C, 63.39; H, 6.50; N, 8.21. Found: C, 63.14; H, 6.60; N, 8.10. $[\alpha]^{22}_D$ −0.8° (*c* 1.00, CH_3OH).

CBZ-Cys(CBZ)-Thr(*O*-*t*-Bu)-Gly Carboxamidomethyl Ester (15). A solution of **14** (0.46 g, 0.90 mmol) and diethylamine (0.8 mL) in dry DMF (8 mL) was stirred overnight at rt. The volatiles were removed *in vacuo*, and the residue was dissolved in dry DMF (15 mL). *N*,*S*-Di-CBZ-L-cysteine (0.35 g, 0.90 mmol) and BOP (0.40 g, 0.90 mmol) were added; the solution was cooled to 0 °C. DIEA (0.25g, 1.89 mmol) was added, and the solution was stirred at rt for 2 days. The reaction mixture was concentrated *in vacuo*, diluted with ethyl acetate (100 mL), and washed with 10% citric acid, water, 5% aqueous $NaHCO_3$ solution, and water. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The resulting oil was purified by flash column chromatography (80% ethyl acetate/10% hexane/10% $CHCl_3$) to obtain **15** (0.29 g, 50%). Mp 95−97 °C. ^{1}H NMR ($CDCl_3$) δ 7.70 (m, 1 H), 7.28 (s, 10 H), 6.78 (s, 1 H), 6.00 (s, 1 H), 5.50 (s, 1 H), 5.23 (s, 2 H), 5.17 (s, 2 H), 4.62 (m, 2 H), 4.41 (m, 1 H), 4.30 (m, 1 H), 4.25 (m, 1 H), 4.15 (m, 1 H), 3.94 (m, 1 H), 3.30 (m, 2 H), 1.65 (s, 1 H), 1.20 (s, 9 H), 1.07 (d, 3 H). Anal. Calcd for $C_{31}H_{40}N_4O_{10}S$: C,

56.35; H, 6.10; N, 8.48. Found: C, 56.62; H, 6.14; N, 8.38. $[\alpha]^{25}_D$ −14.0° (*c* 1.10, CH_3OH).

CBZ-Cys(CBZ)-Thr(*O*-*t*-Bu)-Gly-OH (16). To a solution of **15** (0.90 g, 1.36 mmol) in DMF (14 mL) was added aqueous Na_2CO_3 solution (2.72 mmol, 8 mL) at rt. The solution warmed on addition, and water was added until the solution was clear. After 5 min, the pH was adjusted to 6 with citric acid (0.5 N, 9 mL). The reaction mixture was concentrated *in vacuo*; the residue was dissolved in ethyl acetate and washed with 0.5 N citric acid. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography using LH-20 (lipophilic Sephadex; 50 g; 1% ethanol/toluene) to give **16** as a white solid (0.63 g, 77%). Mp 124−125 °C. ^{1}H NMR (CD_3OD) δ 7.39−7.25 (m, 10 H), 5.24 (s, 2 H), 5.12 (s, 2 H), 4.46 (dd, 1 H, J = 8.6, 5.0 Hz), 4.33 (d, 1 H, J = 3.0 Hz), 4.16 (m, 1 H), 3.92 (m, 2 H), 3.45 (dd, 1 H, J = 14.3, 5.0 Hz), 3.16 (dd, 1 H, J = 14.3, 8.6 Hz), 1.20 (s, 9 H), 1.09 (d, 3 H, J = 6.4 Hz). Anal. Calcd for $C_{29}H_{37}N_3O_9S$: C, 57.70; H, 6.18; N, 6.96. Found: C, 57.76; H, 6.24; N, 7.02. $[\alpha]^{22}_D$ +8.6° (*c* 0.50, $CHCl_3$).

CBZ-His-Gly-O-*t*-Bu (17). A solution of glycine *t*-Bu ester hydrochloride (2.20 g, 13.0 mmol) and *N*α-CBZ-L-histidine (3.44 g, 11.9 mmol) in dry DMF (40 mL) was cooled to 0 °C under an N_2 atmosphere, and diphenylphosphoryl azide (DPPA, 3.58 g, 13.0 mmol) was added dropwise over 5 min. The solution was stirred for 10 min, and triethylamine (2.63 g, 26.0 mmol) was added. The solution was stirred at rt overnight and concentrated *in vacuo*, and the residue purified by flash chromatography (8% ethanol/$CHCl_3$) to give **17** as a white solid (3.45 g, 72%). Mp 64−66 °C. ^{1}H NMR (CD_3OD) δ 7.56 (d, 1 H, J = 1.1 Hz), 7.30 (m, 5 H), 6.88 (s, 1 H), 5.04 (m, 2 H), 4.42 (m, 1 H), 3.80 (m, 2 H), 3.12 (m, 1 H), 2.91 (m, 1 H), 1.46 (s, 9 H). Anal. Calcd for $C_{20}H_{26}N_4O_5$: C, 59.69; H, 6.51; N, 13.92. Found: C, 59.43; H, 6.46; N, 13.81.

CBZ-His(BOC)-Gly-O-*t*-Bu (18). A solution of di-*tert*-butyl dicarbonate (1.33 g, 6.0 mmol) in THF (3 mL) was added dropwise at 0 °C to a solution of **17** (2.0 g, 5.0 mmol) in THF (15 mL). The reaction mixture was stirred overnight at rt and concentrated *in vacuo*. The residue was purified by flash chromatography (60% ethyl acetate/hexane) to give **18** (1.96 g, 78%). ^{1}H NMR ($CDCl_3$) δ 8.00 (s, 1 H), 7.30 (m, 6 H), 6.60 (m, 1 H), 5.10 (s, 2 H), 4.58 (m, 1 H), 3.85 (m, 2 H), 3.03 (m, 2 H), 2.38 (s, 1 H), 1.60 (s, 9 H), 1.40 (s, 9 H). Anal. Calcd for $C_{20}H_{26}N_4O_5$: C, 59.75; H, 6.82; N, 11.15. Found: C, 59.69; H, 6.87; N, 11.09. $[\alpha]^{22}_D$ −4.0° (*c* 1.00, CH_3OH).

FMOC-Hpu(*N*7,*N*12-di-CBZ,*O*9-THP)-His(BOC)-Gly-O-*t*-Bu (19). To a solution of **18** (185 mg, 0.37 mmol) and 1 N HCl (370 μL) in ethanol (15 mL) was added 10% Pd−C (18 mg), and the reaction mixture was hydrogenated for 2.5 h at rt. The reaction mixture was filtered through Celite, concentrated *in vacuo*, and purified by flash chromatography (9:1 $CHCl_3$:ethanol) to give H-His(BOC)-Gly-O-*t*-Bu hydrochloride (101 mg, 68%). A portion of this ammonium salt (69 mg, 0.17 mmol) and **11** (134 mg, 0.17 mmol) were dissolved in dry DMF (12 mL). The solution was cooled to 0 °C. BOP (87 mg, 0.20 mmol) was added and stirred for 20 min. DIEA (47.5 mg, 0.37 mmol) was added dropwise at 0 °C, and the solution was warmed to rt and stirred overnight. The volatiles were removed under reduced pressure, and the residue was dissolved in ethyl acetate and washed with 5% aqueous $NaHCO_3$ solution and water. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* and the residue purified by flash chromatography (4% ethanol/$CHCl_3$) to give **19** as a colorless oil (165 mg, 85%). ^{1}H NMR (600 MHz) (CD_3OD) δ 7.86 (m, 1 H), 7.70 (d, 2 H, J = 7.5 Hz), 7.58 (m, 2 H), 7.31−7.11 (m, 15 H), 5.00 (m, 2 H), 4.92 (s, 2 H), 4.60 (m, 1 H), 4.50−4.32 (m, 1 H), 4.30−4.26 (m, 2 H), 4.08 (t, 1 H, J = 6.8 Hz), 3.90 (m, 1 H), 3.86−3.52 (m, 5 H), 3.38−2.76 (m, 8 H), 1.70−1.12 (m, 14 H), 1.40 (s, 9 H), 1.28 (s, 9 H). HRMS *m*/*z* calcd for $C_{63}H_{80}N_7O_{14}$ 1158.5763, found 1158.5739. $[\alpha]^{26}_D$ −1.5° (*c* 1.00, $CHCl_3$).

CBZ-Cys(CBZ)-Thr(*O*-*t*-Bu)-Gly-Hpu(*N*7,*N*12-di-CBZ,*O*9-THP)-His(BOC)-Gly-O-*t*-Bu (20). A solution of 4-(aminomethyl)piperidine (1.0 mL) and **19** (156 mg, 0.14 mmol) in $CHCl_3$ (10 mL) was stirred for 2 h at rt. An additional portion of 4-(aminomethyl)piperidine (1.0 mL) was added, and stirring was continued for another 40 min. The reaction mixture was taken up in $CHCl_3$ (50 mL) and extracted with phosphate buffer (pH 5.5; 3 × 75 mL). The organic layer was dried over Na_2SO_4 and concentrated; the residue was purified by flash chromatography (10% methanol/$CHCl_3$) to give H-Hpu(*N*7,*N*12-di-CBZ,*O*9-THP)-His(BOC)-Gly-O-*t*-Bu as a colorless oil (88 mg, 68%). A portion of this oil (10 mg, 11 μmol) and **16** (7 mg, 11 μmol) in dry DMF (2 mL) was cooled to 0 °C. BOP (6 mg, 12 μmol) was added, and the solution was stirred for 30 min. DIEA (4.5 μL, 26 μmol) was added dropwise. The solution was warmed to rt and stirred overnight. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in ethyl acetate (15 mL) and extracted with 5% aqueous $NaHCO_3$ solution (10 mL) and water (10 mL). The organic layer was concentrated under reduced pressure and the residue purified by flash chromatography (10% ethanol/$CHCl_3$) to give **20** as a colorless oil (13 mg, 81%). ^{1}H NMR (600 MHz) (CD_3OD) δ 7.99 (m, 1 H), 7.28−7.14 (m, 21 H), 5.17−5.07 (m, 2 H), 5.06−4.88 (m, 6 H), 4.58 (dd, 1 H, J = 9.0, 4.2 Hz), 4.51−4.32 (m, 2 H), 4.26 (m, 1 H), 4.14 (m, 1 H), 4.10 (m, 1 H), 4.08−3.98 (m, 2 H), 3.86−3.58 (m, 6 H), 3.40−2.94 (m, 8 H), 2.88 (m, 1 H), 1.70−0.94 (m, 17 H), 1.48 (s, 9 H), 1.34 (s, 9 H), 1.08 (s, 9 H). HRMS *m*/*z* calcd for $C_{77}H_{105}N_{10}O_{20}S$ 1521.7227, found 1521.736. $[\alpha]^{26}_D$ −1.2° (*c* 1.00, $CHCl_3$).

Cys-Thr-Gly-Hpu-His-Gly, Trifluoroacetic Acid Salt (12). Method a (Scheme 2): A solution of **20** (13 mg, 7.2 μmol) and phenol (5 mg, 50 μmol) was heated to reflux for 90 min in degassed TFA (5.0 mL) under an argon atmosphere. The reaction mixture was concentrated *in vacuo* and the residue applied to a C-18 plug (Supelco; water/acetonitrile = 88/22 + 0.1% TFA). Further purification was performed by preparative HPLC (solvent systems A, aqueous 0.1% TFA; and B, 0.1% TFA in CH_3CN; linear gradient of 0−20% B in 50 min; flow rate 4.0 mL/min; detection at 214 nm; retention time = 8.4 min) using a C-18 reverse phase column (Dynamax 300 A C_{18}) to give **12** as a colorless oil (2 mg, 24%). ^{1}H NMR (D_2O) (600 MHz) δ 8.69 (d, 1 H, J = 1.2 Hz), 7.38 (s, 1 H), 4.80 (m, 1 H), 4.49 (d, 1 H, J = 4.9 Hz), 4.41 (t, 1 H, J = 5.6 Hz), 4.36 (dd, 1 H, J = 8.5, 6.0 Hz), 4.29 (m, 1 H), 4.11 (m, 1 H), 4.09−4.00 (m, 4 H), 3.37 (dd, 1 H, J = 15.5, 7.2 Hz), 3.29−3.06 (m, 9 H), 1.99 (m, 1 H), 1.86 (m, 2 H), 1.76 (m, 3 H), 1.45 (m, 2 H), 1.32 (d, 3 H, J = 6.4 Hz). MS (MALDI-Tof) *m*/*z* calcd for $C_{27}H_{48}N_{10}O_9S$ 688.33 (M^+), found 688.96.

Method b (Scheme 3): The polymer-bound peptide **21** was synthesized using an Applied Biosystems 432A Synthesizer. Amino acid analysis for **21**: Gly 2.09, His 1.03, Thr 0.88. An aliquot of **21** (49 mg, 19.3 μmol), phenol (250 mg), and pentamethylbenzene (250 mg) were dissolved in degassed TFA (5.0 mL) at 0 °C. Saturated HBr in acetic acid solution (0.2 mL), triisopropylsilane (0.1 mL), and 1,2-ethanedithiol (0.1 mL) were added under an argon atmosphere. The solution was stirred at rt for 1 h and concentrated under reduced pressure. The residue was dissolved in 10% acetic acid (10 mL) and extracted with methyl *tert*-butyl ether (3 × 25 mL). The aqueous layer was concentrated *in vacuo*, and the residue was purified by preparative HPLC as in method a using a C-18 reverse phase column (Dynamax 300 A C_{18}) to give **12** as a colorless oil (5.2 mg, 24%). ^{1}H NMR and MS analytical data were identical with those for **12** prepared by method a. $[\alpha]^{26}_D$ −16.7° (*c* 0.30, H_2O). Amino acid analysis: Gly 1.94, His 1.02, Thr 1.04.

Acknowledgment. Financial support was provided by SunPharm Corp., Jacksonville, FL. The authors thank A.Y.K. Chung of the Dept. of Biochemistry and Protein Core ICBR, University of Florida, Gainesville, FL, for performing the SPPS and HPLC purifications; H. D. Plant and J. R. Rocca of the Center for Structural Biology—Brain Institute, University of Florida, Gainesville, FL, for high-field and 2D NMR spectral analyses.

JO970119Z