Synthesis of Reagents for the Construction of Hypusine and Deoxyhypusine Peptides and Their Application as Peptidic Antigens

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Two new synthetic methods which allow access to (2*S*)-deoxyhypusine, natural (2*S*,9*R*)-hypusine, (2*S*,9*S*)-hypusine, and deoxyhypusine- and hypusine-containing peptides are described. The methods involve both the construction of a deoxyhypusine reagent in which the α-nitrogen protecting group is orthogonal to the N-7 and N-12 protecting groups and an alternate synthesis of our previous hypusine reagent, a synthesis which provides for better stereochemical control at C-9. Synthetic hypusine and deoxyhypusine can be generated from these reagents. The hypusine-containing hexapeptide (Cys-Thr-Gly-Hpu-His-Gly) is conjugated to ovalbumin (OVA), keyhole limpet hemocyanin (KLH), and a bis-maleimide; KLH conjugates are also made with the deoxyhypusine- and lysine-containing hexapeptides. Monoclonal antibodies are generated to the hypusine-containing hexapeptide−OVA conjugate in mice. These are isolated and screened against the hypusine-containing hexapeptide−KLH and hypusine-containing hexapeptide−bis-maleimide conjugates, as well as against the deoxyhypusine-containing and lysine-containing hexapeptide−KLH conjugates. These antibodies may be useful in localizing intracellular hypusine-containing peptides as well as peptides containing hypusine analogues.

Introduction

While hypusine [(2*S*,9*R*)-2,11-diamino-9-hydroxy-7-azaundecanoic acid], an unusual amino acid, has been found in its free form and as α-(*β*-alanyl) and α-(*γ*-aminobutyryl) dipeptides in brain tissue,[1,2] it has gained most of its attention in association with eukaryotic initiation factor 5A (eIF-5A, formerly eIF-4D). This 17-kDa protein seems to be very highly conserved among many eukaryotic species including yeast and higher mammals, attesting to its importance from an evolutionary perspective.[3,4] In particular, the 12-amino acid region surrounding the hypusine residue, L-Ser-L-Thr-L-Ser-L-Lys-L-Thr-Gly-**Hpu**-L-His-Gly-L-His-L-Ala-L-Lys, is extremely well-conserved across species.[5] Hypusination of eIF-5A, or "maturation" of this protein, occurs as a posttranslational event.[6] An aminobutyl group is removed from spermidine; deoxyhypusine synthase catalyzes the attachment of the aminobutyl group to the side chain of Lys-50 of the human protein.[7,8] Next, deoxyhypusine hydroxylase, which appears to be an iron-dependent enzyme,[9] catalyzes the introduction of the hydroxyl group at C-9 in the (*R*)-configuration.[6]

There appears to be some confusion as to the primary intracellular localization of eIF-5A—the cytoplasm, the nucleus, the nuclear membrane, or some combination of these.[10,11] This controversy may derive from differences in the nature of the antibodies generated by two different research groups.[10,11] Because eIF-5A was shown to stimulate methionyl-puromycin synthesis, a model reaction for formation of the first peptide bond during protein assembly, it was initially thought that eIF-5A was a critical initiation factor in protein synthesis.[12] However, workers have since shown (e.g., in *Saccharomyces cerevisiae*)[13] that when the eIF-5A protein is rendered unavailable via genetic manipulation, overall protein synthesis, rather than ceasing completely, is reduced to about 70% of control. This suggests that eIF-5A is not responsible for protein synthesis in a global sense. Instead, eIF-5A may be associated with a subgroup of mRNAs; the specific class still remains elusive.[13] However, two issues are quite clear: eIF-5A is critical to mitotic processes,[14] and the hypusine fragment of eIF-5A is required for the protein's function.[15]

The inhibitor of deoxyhypusine synthase, N^1-guanyl-1,7-diaminoheptane (CG_7), diminishes the growth of CHO cells without affecting polyamine metabolism.[16] Site-directed mutagenesis experiments[15] in which Lys-50 was replaced with arginine resulted in a nonfunctional protein in yeast cells; the arginine could not be modified to form hypusine. Furthermore, yeast cells that had the wild-type copy of the gene replaced with the mutant copy failed to grow. The precise role of the hypusine residue in eIF-5A activity remains elusive. While it is clear that the N-terminal methionine of the protein is replaced with an acetyl group and that acetylation occurs at Lys-47, neither event seems critical to the protein's function;[17,18] modification (e.g., phosphorylation) of the C-9(*R*)-hydroxyl of the hypusine residue has not yet been demonstrated.

Probably the most intriguing aspect regarding eIF-5A is its role in the replication of human immunodeficiency virus (HIV); eIF-5A is a transactivating factor during replication of HIV.[10] The eIF-5A molecule binds to a complex formed between the Rev response element

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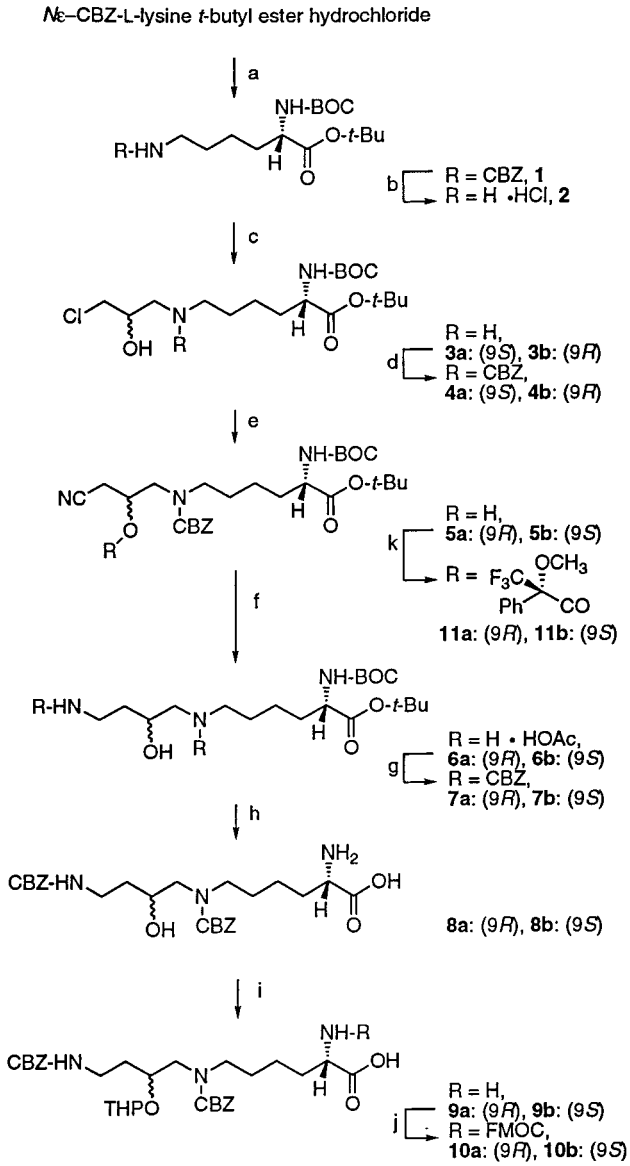
(RRE) in the stem−loop IIB of the viral mRNA and Rev, a viral protein that serves as a nuclear export signal.[19] Once eIF-5A binds to Rev-RRE, the now active eIF-5A−Rev-RRE complex is able to be exported from the nucleus; viral replication ensues. In experiments in which antisense nucleotides were used to prevent eIF-5A synthesis, viral replication was inhibited.[10] It has also been demonstrated in gel shift experiments that the hypusine- or deoxyhypusine-containing fragments were required for this binding of eIF-5A to Rev-RRE.[20] Rev has domains which direct both nuclear import and nuclear export.[20,21] Certain eIF-5A mutants, while capable of being transported into the nucleus and binding to Rev-RRE, actually prevent nuclear export and, thus, viral replication.[20,21]

The observations that eIF-5A is required for both mitotic events and HIV viral replication and that immature eIF-5A must be deoxyhypusinated or hypusinated for activity render inhibition of eIF-5A deoxyhypusination or hypusination an interesting target in therapeutic strategies for anticancer and antiviral drug development. Another potential antiviral strategy involves identifying the basic platform within eIF-5A responsible for nuclear import that will permit Rev-RRE binding but not nuclear export of viral message. The current study develops a number of tools, including new synthetic methods for accessing hypusine and deoxyhypusine as well as the corresponding peptides and murine monoclonal antibodies to a hypusine-containing hexapeptide. These antibodies should be useful in monitoring potential migration of hypusine-containing peptides into and out of the nucleus.

Synthesis

In an earlier paper[22] we described the synthesis of a hypusine reagent (**11** in that work; **10a**, Scheme 1, in the present paper) useful for accessing hypusine-containing peptides. The two key steps in assembling this reagent in the earlier study involved reaction of *N*ε-benzyl-*N*α-BOC-L-lysine *tert*-butyl ester with (*S*)-(+)-epichlorohydrin to generate (2*S*,9*S*)-7-benzyl-2-[(*tert*-butoxycarbonyl)amino]-10-chloro-9-hydroxy-7-azadecanoic acid *tert*-butyl ester. This chlorohydrin was next treated with potassium cyanide in the presence of 18-crown-6 to produce the corresponding nitrile, (2*S*,9*R*)-7-benzyl-2-[(*tert*-butoxycarbonyl)amino]-10-cyano-9-hydroxy-7-azadecanoic acid *tert*-butyl ester. A series of reductions and functional group protections ensued, culminating in the final reagent **10a**. The orthogonally protected reagent was shown to be of utility in accessing hypusine-containing peptides by either matrix synthesis or standard solution methods. However, in attempting scaleup of this reagent, we observed that the stereochemistry of the cyanation product at C-9 is very sensitive to reaction conditions, particularly to temperature and the ratio of 18-crown-6 to potassium cyanide. This problem may revolve around potential azetidinium salt formation;[23] this intermediate, on cyanide opening, would result in racemization at C-9.[24] Thus, the extent of the racemization depends on the ratio of azetidinium formation and cyanide opening to direct displacement of the chloride by cyanide, a relationship affected by the reaction conditions, which can be difficult to control. Azetidinium formation likely occurred during cyanation rather than spontaneously from the intermediate chlorohydrin on standing. Upon standing for 1 week at room temperature, the optical rotation of the chlorohydrin did not change.

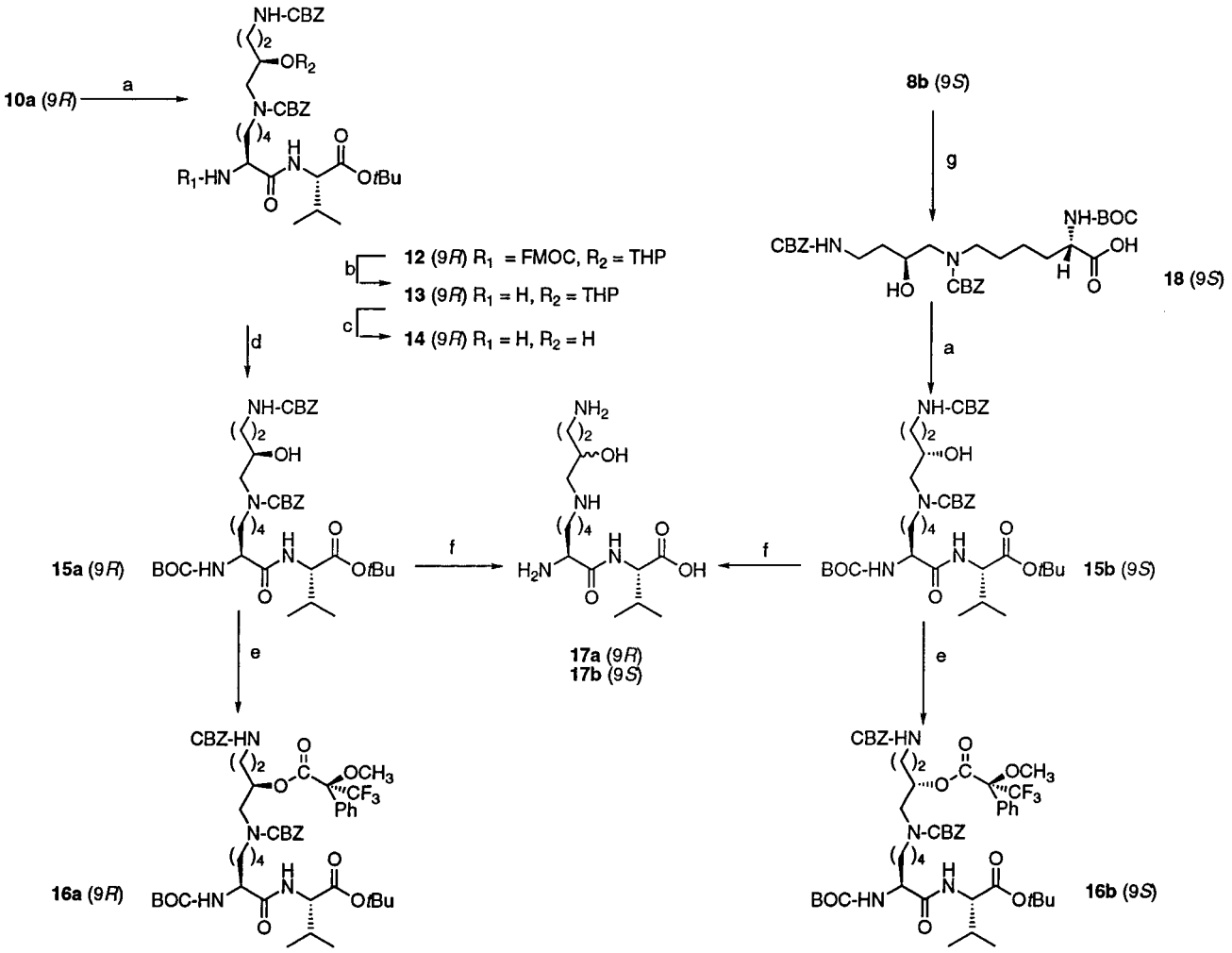
Scheme 1. Synthesis of the Hypusine Reagent[a]



[a] Reagents: (a) $(BOC)_2O$, $NaHCO_3$; (b) H_2, Pd−C; (c) (*S*)-(+)- or (*R*)-(−)-epichlorohydrin, respectively; (d) benzyl chloroformate, triethylamine; (e) KCN, 18-crown-6; (f) H_2, Pd−C, PtO_2; (g) CBZ-ONSu, $KHCO_3$; (h) TFA, triethylsilane, CH_2Cl_2; (i) 3,4-dihydro-2*H*-pyran; (j) FMOC-ONSu, Na_2CO_3; (k) (*R*)-(−)-Mosher's acid chloride, pyridine.

Assuming that azetidinium formation was the origin of the problem, we adopted an approach (Scheme 1) which would prevent the generation of this compound. The key step in overcoming the internal cyclization was to vitiate the nucleophilicity of the N-7 electron pair by construction of the halocarbamate **4a**,**b**. Utilizing this new approach, we synthesized both the (2*S*,9*R*)-reagent **10a** and the corresponding (2*S*,9*S*)-compound **10b**. Commercially available *N*ε-CBZ-L-lysine *tert*-butyl ester (Scheme 1) was protected with di-*tert*-butyl dicarbonate to yield **1**, which was hydrogenated to remove the CBZ protecting group. The resulting primary amine **2** was reacted with either (*S*)-(+)-epichlorohydrin to generate

Scheme 2. Verification of Stereochemical Integrity of the Hypusine Reagent[a]



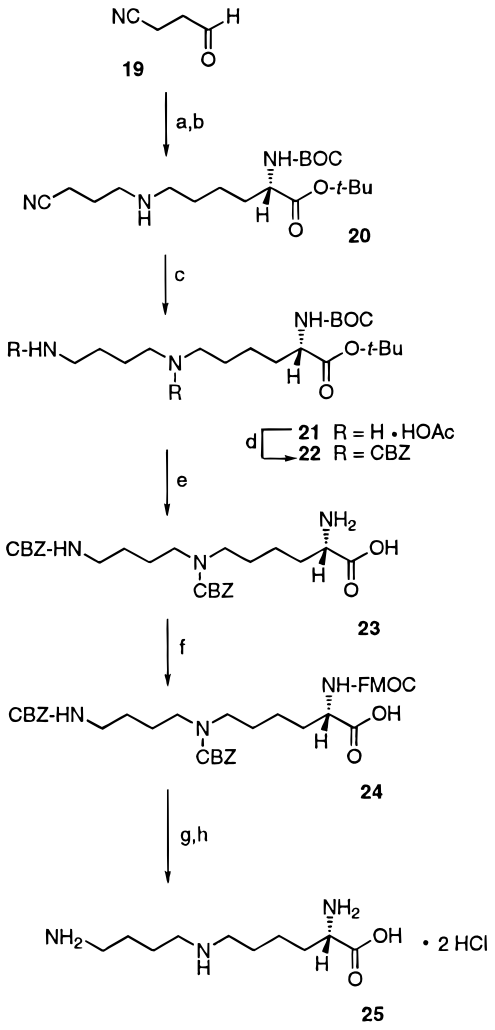
[a] Reagents: (a) L-valine *tert*-butyl ester hydrochloride, BOP, DIEA; (b) piperidine; (c) pTosOH; (d) $(BOC)_2O$, $NaHCO_3$; (e) (*R*)-(−)-Mosher's acid chloride, pyridine; (f) HBr/HOAc in TFA, phenol, pentamethylbenzene, triisopropylsilane; (g) $(BOC)_2O$, NaOH.

the (9*S*)-chlorohydrin (**3a**) or with (*R*)-(−)-epichlorohydrin to produce the (9*R*)-chlorohydrin (**3b**).[25] Benzyl chloroformate was condensed with N-7 of the chlorohydrins **3a**,**b** to yield the N^7-CBZ compounds **4a**,**b**. Again, because of the carbamate linkage at N-7, azetidinium formation is precluded. Reaction of **4a**,**b** with KCN in the presence of 18-crown-6[26] resulted in the corresponding nitriles **5a**,**b**. In each instance, the (9*R*)- and (9*S*)-hydroxy nitriles were converted to the Mosher esters **11a**,**b**, respectively,[27] in order to verify chiral integrity at C-9. The methoxy resonance in the ^1H NMR spectrum of **11a** was observed at 3.60 ppm and that of **11b** at 3.46 ppm. Both resonances were easily distinguishable in a mixture of the two compounds. The inability to observe the 3.46 ppm resonance of **11b** in the proton NMR of **11a** or the 3.60 ppm resonance of **11a** in that of **11b** confirmed that chiral integrity at C-9 had been maintained to this stage in the synthetic sequence.

Nitriles **5a**,**b** were next reduced with hydrogen and a mixed catalyst. The resulting diamine diacetates **6a**,**b** were then reacted with *N*-(benzyloxycarbonyloxy)succinimide (CBZ-ONSu) to produce the N^7,N^{12}-di-CBZ hydroxy derivatives **7a**,**b**. Treatment of these systems with trifluoroacetic acid and triethylsilane[28] released both the BOC and *tert*-butyl ester protecting groups, resulting in the di-CBZ amino acids **8a**,**b**. The C-9 hydroxyl groups were next converted to the THP ethers **9a**,**b**. Finally, both **9a**,**b** were transformed to their FMOC derivatives **10a**,**b**.[29]

An additional verification of the stereochemical integrity of the reagent and its peptide products was undertaken (Scheme 2). In the course of earlier synthetic studies we demonstrated that the extent of racemization at the α-methine of the hypusine fragment may be quantified by preparing the corresponding L-valine dipeptide.[22] Proton NMR spectra clearly distinguished between the L-lysyl-L-valine and D-lysyl-L-valine as well as between the related (2*S*,9*R*)-hypusinyl-L-valine and (2*R*,9*R*)-hypusinyl-L-valine dipeptide diastereomers. The protected hypusine reagent **10a** (2*S*,9*R*) was condensed with valine *tert*-butyl ester resulting in the α-*N*-FMOC-protected dipeptide **12** (2*S*,9*R*). The FMOC protecting group was next removed by exposure to piperidine, yielding **13**, followed by *p*-toluenesulfonic acid in acetone−water to remove the THP ether (dipeptide **14**). Protection of the free nitrogen with BOC anhydride provided Nα-BOC dipeptide **15a** (2*S*,9*R*). This dipeptide **15a** was subjected to two separate reactions: conversion to Mosher ester **16a** (2*S*,9*R*) or deprotection using HBr in acetic acid/TFA/phenol/pentamethylbenzene/triisopropylsilane to dipeptide **17a** (2*S*,9*R*). To generate the other set of diaster-

Scheme 3. Synthesis of the Deoxyhypusine Reagent and Free Deoxyhypusine[a]

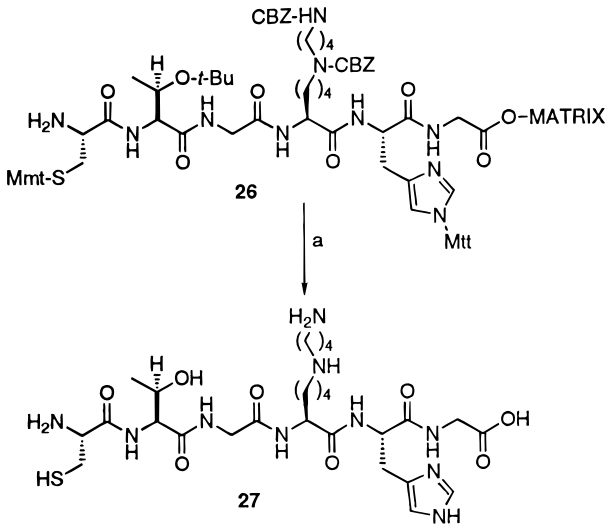


[a] Reagents: (a) **2**, benzene, molecular sieves; (b) H_2, PtO_2, THF; (c) H_2, Pd/C, PtO_2, HOAc; (d) CBZ-ONSu, $KHCO_3$; (e) TFA, triethylsilane, CH_2Cl_2; (f) FMOC-ONSu, Na_2CO_3; (g) piperidine; (h) HBr/HOAc in TFA, phenol, pentamethylbenzene, triisopropylsilane.

eomers (2*S*,9*S*), the α-amino group of the di-CBZ intermediate **8b** (2*S*,9*S*) was reacted with BOC anhydride; the acid **18** thus generated was condensed with valine *tert*-butyl ester. As with **15a**, two separate conversions were initiated: **15b** was transformed to the corresponding Mosher ester **16b** (2*S*,9*S*) and deprotected to yield dipeptide **17b** (2*S*,9*S*). Thus, three sets of diastereomers were available for comparison: **15a**,**b**, **16a**,**b**, and **17a**,**b**. Proton NMR spectra of **15a**,**b** and **17a**,**b** did not reveal the presence of any racemization at C-2 of the hypusine moiety as no (2*R*)-hypusinyl-L-valine diastereomers were detected. Furthermore, a comparison of both the ^1H and ^{19}F NMR analyses of **16a** (9*R*) and **16b** (9*S*) clearly supported the optical integrity at C-9. The Mosher ester methoxy protons and fluorine signals of **16a** (9*R*) occurred at δ 3.50 and −71.78, respectively, while the signals for **16b** (9*S*) were at δ 3.44 and −71.40.

In keeping with the ease of peptide synthesis using hypusine reagent **10a** on a Merrifield resin,[22] we elected to synthesize the analogous deoxyhypusine reagent **24** (Scheme 3). The initial step in our synthesis of the deoxyhypusine reagent involved a molecular sieve-promoted condensation of 3-cyanopropanal (**19**)[30] with the *N*α-BOC-*tert*-butyl ester of L-lysine (**2**) and reduction of the resulting intermediate imine over H_2/PtO_2[31] to the deoxyhypusine framework **20**. Further reduction of the nitrile functionality on **20** was done using H_2 over Pd/C and PtO_2 in acetic acid to **21**; the N-7 and N-12 positions were reacted with CBZ-ONSu to produce tetraprotected deoxyhypusine **22**. Both *tert*-butyl protecting groups were removed with trifluoroacetic acid and triethylsilane,[28] and the amino acid **23** was converted to the Nα-FMOC-di-CBZ-protected compound **24**. This reagent was both deprotected to yield free deoxyhypusine and utilized to generate a deoxyhypusine-containing hexapeptide.

Scheme 4. Polymer-Bound Synthesis of a Deoxyhypusine Hexapeptide[a]



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

For free deoxyhypusine (**25**), the deoxyhypusine reagent **24** was first treated with piperidine to remove the FMOC protecting group and then deprotected using 30% HBr in trifluoroacetic acid with a "cocktail" of cation scavengers (Scheme 3). Once the reaction was complete, the reactants were dissolved in water, and the nonsalts were extracted into methyl *tert*-butyl ether. After concentration, the product was chromatographed on silica, eluting with methylene chloride/methanol/ammonium hydroxide. The free amine was converted to the dihydrochloride salt **25**. Analytical data of the final compound were in agreement with the literature values.[31] The same procedure can be used to convert hypusine reagent **10a** to hypusine (data not shown).

The deoxyhypusine reagent was utilized to prepare Cys-Thr-Gly-deoxyhypusine-His-Gly. The synthesis of the hexapeptide **27** (Scheme 4) was performed on a 2-chlorotrityl resin using SPPS and FMOC chemistry with HBTU as an activating agent. The cysteine and histidine residues of the hexapeptide were protected as 4-methoxytrityl (Mmt) and 4-methyltrityl- (Mtt) group derivatives, respectively, while the threonine was protected as its *tert*-butyl ether. Deprotection of **26** was then achieved with HBr/acetic acid in TFA, 1,2-ethanedithiol to prevent disulfide bond formation, and a "cocktail" of carbocation scavengers (phenol, pentamethylbenzene, triisopropylsilane) at room temperature.

The final peptide **27** was purified by reverse-phase HPLC. Both high-field (600-MHz) ^{1}H NMR at two different temperatures and amino acid analysis revealed the correct structure for the hexapeptide.

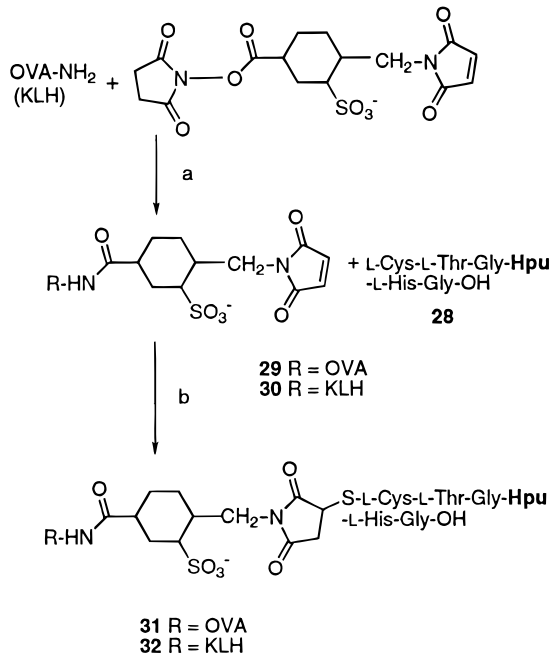
Hypusine Monoclonal Antibody Generation

To assess whether small hypusine- or deoxyhypusine-containing peptide fragments are transported into or out of the nucleus under the conditions of microinjection experiments, it was clear that antibodies to the peptides would be valuable as an analytical tool. This would allow us to confirm, using a different method, the work of Bevec et al.[20] in the determination of nuclear import/export properties of these peptides. Microinjection of these peptides into the nucleus or cytoplasm of *Xenopus* oocytes, time-dependent fixation and treatment of cells with the appropriate hypusine/deoxyhypusine peptide-directed murine IgG, and use of a FITC-labeled anti-mouse antibody should make it possible to localize the peptides. In so doing, the identification of the minimal nuclear import/export structures should be possible. Recall that the mutant studies[20] have demonstrated that eIF-5A fragments with nuclear import signals, once transported into the nucleus, bind to the HIV Rev-RRE complex and prevent nuclear export and thus viral replication. Given the conflicting views of eIF-5A localization using polyclonal antisera,[10,11] we instead developed monoclonal antibodies to the hypusine-containing fragment of eIF-5A.

To produce antibodies in animals to haptens such as synthetic peptides (e.g., a peptide containing hypusine), conjugation to a larger carrier protein (e.g., ovalbumin (OVA)) is generally required.[32] The hypusine reagent **10a** was employed for synthesis of a hypusine-containing hexapeptide; cysteine replaced the lysine residue on the amino terminus (L-Cys-L-Thr-Gly-Hpu-L-His-Gly-OH, **28**, Scheme 5).[22] The peptide was prepared in this manner in order to facilitate conjugation to a maleimide-derivatized OVA.[33] The amino terminal end of OVA was covalently linked to a bifunctional cross-linking agent via an amide linkage to produce a derivatized OVA containing maleimide coupling sites. After reaction with **28**, the final product was the **28**−OVA conjugate **31**.

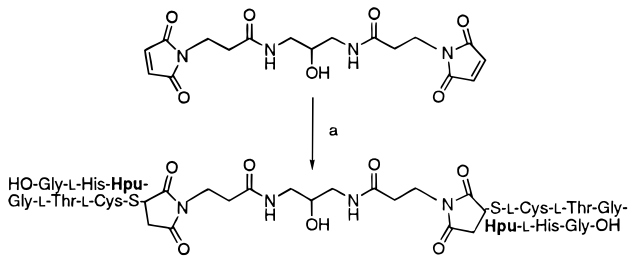
The conjugate **31** combined with Ribi adjuvant was used to immunize four Balb/c BYJ mice subcutaneously. Sera were obtained after four immunizations and tested in a series of enzyme-linked immunosorbent assays (ELISAs). Because ELISA screens of antibodies elicited by **31** could be directed to either the OVA- or the hypusine-containing peptide regions of the conjugate molecule, we needed to be sure that there was a response against **28**. Therefore, suitable ELISA antigens containing the hapten **28** were required. The hexapeptide **28** itself is too small to reliably remain attached to the 96-well plate as required by the ELISA. Thus, **28** was (1) attached to an antigenically neutral macromolecular carrier, keyhole limpet hemocyanin (KLH), and (2) cross-linked to itself to form a bis-hexapeptide, now large enough to remain attached for ELISA. The **28**−KLH conjugate **32** was made utilizing procedures identical with those for **31** (Scheme 5). Formation of the bis-maleimide complex **33** employed a sulfhydryl-specific homobifunctional cross-linking reagent (Scheme 6).

Scheme 5. Coupling the Hypusine Peptide Hapten to Macromolecular Carriers[a]



[a] The OVA conjugate **31** was used to immunize mice for antibody production; the KLH conjugate **32** was used as an antigen in an ELISA. Reagents: (a) pH 7.4 0.1 M Na phosphate buffer, 3 h; (b) after determination of maleimide content; 90% of the theoretical amount of **31**.

Scheme 6. Formation of the Bis-Hypusinyl Hexapeptide **33** for Use as an ELISA Antigen[a]



[a] Reagents: (a) **28**, 2 mmol.

The murine sera were assayed by ELISA for reactivity to the following: unconjugated KLH (neutral carrier control), **32** (to check for reactivity of the sera to the peptide coupled to a neutral carrier), and **33**. In the ELISA assay employed, a test serum absorbance value 3 times above that of the normal serum background was considered a positive response at the dilution of serum tested. For example, testing of the normal serum control at a 1:800 dilution yielded an absorbance value of 0.1 against the bis-hexapeptide conjugate; the serum from mouse #4 at a 1:800 dilution gave a greater than 10-fold increase. By the use of class-specific secondary antibodies in the ELISA, it was also possible to determine whether the class of antibody elicited by immunization has switched from the lower-affinity IgM to the higher-affinity IgG; such a class switch was detected in these immunized mice.

Once sufficient and specific reactivity to the hypusine peptide in the sera of the immunized mice was established, a fusion could be performed. The fusion was performed by standard procedures[34] followed by a 10-

Table 1. Reactivity of Monoclonal Antibodies with Hypusine-Containing Peptide Conjugates and Related Antigens as Measured by ELISA

	A_{410} versus antigen[a]					
clone	OVA	KLH	Lys−KLH	**27**−KLH	**32**	**33**
medium	0.104	0.109	0.101	0.100	0.108	0.104
imm mouse serum	2.529	0.192	2.575	2.575	2.684	2.751
1D9	0.112	0.116	0.235	0.261	0.244	0.226
1F6	0.111	0.120	0.112	0.114	0.150	0.128
4H3	0.108	0.115	2.108	1.760	2.523	2.108
4A6	0.110	0.129	0.106	0.112	0.130	0.105
1F12	0.102	0.109	0.102	1.522	2.233	1.902
5A4	0.116	0.115	0.107	2.442	2.777	2.710
4E3	0.111	0.207	0.128	0.137	0.150	0.129
2A3	0.104	0.125	0.108	0.267	2.611	2.265
5E10	0.102	0.114	0.109	1.452	2.452	1.592
4E12	0.103	0.113	0.102	0.704	2.383	1.848
3C9	0.101	0.113	0.104	1.580	2.575	1.600
5F10	0.111	0.150	0.242	0.489	2.078	1.521
4D4	0.105	0.112	0.106	1.355	2.411	1.435
1B3	0.097	0.116	0.937	0.928	0.835	0.737
4D11	0.099	0.106	0.098	0.122	0.168	0.172
1C3	0.107	0.116	2.561	2.285	2.670	2.474
4E9	0.115	0.111	0.106	2.474	2.670	2.612
1E6	0.117	0.111	0.114	2.196	2.520	2.219
4F4	0.111	0.120	0.112	1.298	1.530	0.657

[a] A_{410} determined after 1-h incubation with *p*-nitrophenyl phosphate substrate; murine immune serum (positive control), medium alone (negative control), and hybridoma culture supernatants were undiluted. The antibody-containing hybridoma culture supernatants were obtained 10 days after fusion of spleen cells (from a mouse immunized with **31**) with the SP2/0 cell line.

day incubation of the cultures. A primary ELISA screen was then done on the hybridoma culture supernatants to detect whether cells were secreting antibody to **28**, in the form of either **32** or **33**. Those wells that were positive in this assay were screened again 1 week later to determine the class of antibody (IgG or IgM). All but 4 of the 19 positive clones secreted only IgG; this indicates that the antibodies secreted were of high affinity and should therefore be useful in immunodetection systems.

To assess whether the monoclonal antibodies can differentiate between eIF-5A peptide fragments containing unmodified lysine, eIF-5A peptide fragments containing deoxyhypusine, and eIF-5A peptide fragments containing hypusine, we have also synthesized the lysine- and deoxyhypusine-containing hexapeptides with the same sequence as **28**, including the cysteine residue at the amino terminus. Recall that substitution of the cysteine residue for serine is to facilitate coupling by the procedures already described for **28**. The panel of monoclonal antibodies was screened against these conjugates in the ELISA (Table 1). Most of the clones tested positive with more than one of the peptide antigens but not with either of the carriers. Specifically, those antibodies reactive with the lysine-containing peptide (Lys−KLH) were also reactive with the deoxyhypusine- and hypusine-containing peptides; the clones that recognized the deoxyhypusine-containing peptide **27**−KLH conjugate also recognized **32** and **33**. These antibodies were likely binding to epitopes common to all three peptide antigens. Of interest were clones 4E12 and 5F10 which, while somewhat reactive with the **27**−KLH conjugate with A_{410} values of 0.7 and 0.5, respectively (versus a medium control value of 0.1), gave a strong signal against the hypusine-containing peptide antigens **32** and **33** with A_{410} values of greater than 2.0 to **32** and greater than 1.4 to **33**. However, clone 2A3 is possibly the most useful; the antibodies did not appreciably bind to the **27**−KLH conjugate, yet the A_{410} values of 2.6 to **32** and 2.3 to **33** indicate the secretion of high-affinity antibodies specific to the hypusine-containing peptide. It is important to point out that none of the antibodies cross-reacted with the eIF-5A parent polypeptides as tested by ELISA against a recombinant human eIF-5A precursor polypeptide in which Lys-50 had not been posttranslationally modified and against a protein fraction from CHO cells known to contain hypusinated eIF-5A. Since the isolated antibodies do not cross-react with these constitutively expressed cellular proteins, the antibodies should prove to be useful in experiments tracking the localization of microinjected hypusine-containing peptides.

Discussion

The synthetic methods described in this paper allow for the assembly of both deoxyhypusine- and hypusine-containing peptides. Although a synthetic approach for the hypusine reagent was previously described,[22] in the course of scaleup racemization problems were noted at C-9. The key step in the current approach, N-7-carbobenzoxylation of the halohydrin intermediate **3a**,**b** (Scheme 1), prevented any racemization at C-9. This is in keeping with the idea that azetidinium ring formation was the source of C-9 epimerization. Thus, the stereochemical integrity of both C-2 and C-9 in the hypusine reagent and the ensuing peptides is now easily controllable. The deoxyhypusine reagent, lacking the C-9 problem, also lends itself nicely to matrix methodologies. Both reagents were successfully employed in Merrifield syntheses of hexapeptides (**27**, Scheme 4, and **28**[22]). Furthermore, the schemes allowed access to the parent amino acids deoxyhypusine and hypusine.

Finally, the availability of deoxyhypusine- and hypusine-containing peptides with an N-terminal cysteine residue has made it possible to conjugate such peptide fragments to OVA for monoclonal antibody production. The resulting monoclonal antibodies will now make it possible to detect deoxyhypusine and hypusine and determine whether the deoxyhypusine- or hypusine-containing peptide fragments accessible via the methods described herein contain the necessary structural information for nuclear import, nuclear export, or both.

Experimental Section

Chemical reagents were purchased from Aldrich, Fluka, or Sigma Chemical Co. and 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 magnesium sulfate. Acetonitrile was distilled from NaH. THF was distilled from sodium and benzophenone immediately before use. KCN was finely ground and dried for 20 h at 145 °C in vacuo. Silica gel 32−63 (40-μm "flash") from Selecto, Inc. (Kennesaw, GA) was used for flash column chromatography, and Supelclean LC-18 gel from Supelco, Inc. (Bellefonte, PA) was used for reversed-phase column chromatography. ^{1}H NMR spectra were recorded at 300 MHz and ^{13}C NMR spectra at 75 MHz in CD_3OD and at 25 °C, unless otherwise specified. Chemical shifts for proton NMR 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, unless otherwise specified. ^{13}C NMR chemical

shifts in CD_3OD are calibrated on the CD_3OD resonance at 49.0 ppm. Coupling constants (*J*) are in Hz. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. FAB mass spectra were run in a 3-nitrobenzyl alcohol matrix. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA.

Conjugation of Hypusine-Containing Peptide to Carriers. We conjugated the hypusine-containing peptide **28** to the carrier protein ovalbumin (OVA) via a maleimide procedure as pictured in Scheme 5.[33] Reagent **10a** was employed for synthesis of a hypusine-containing hexapeptide with cysteine instead of the normal lysine on the amino terminus (**28**) in order to facilitate conjugation to OVA. In the first coupling step, the amino terminal end of OVA was covalently linked to the cross-linking agent via an amide linkage to produce a derivatized OVA containing maleimide moieties. The coupling reaction was run in phosphate buffer (pH 7.4) with OVA at a concentration of 10 mg/mL. The bifunctional cross-linking agent 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylic acid 3-sulfo-*N*-hydroxysuccinimide ester (sodium salt) was added at a final concentration of 4 mM (Scheme 5). After a 3-h reaction the OVA maleimide derivative **29** was immediately purified by gel filtration chromatography (Sephadex G-25) to remove starting materials which could interfere with the sulfhydryl peptide coupling reaction. The maleimide content of the purified OVA derivative was then quantitated by reacting with a known excess of a sulfhydryl-containing peptide (glutathione), and the remaining sulfhydryl content was measured using Ellman's reagent.[35] Derivative **29**, containing about 1.8 maleimides/mol of OVA, was then incubated with 90% of the theoretical amount of peptide **28** (the theoretical amount determined on the basis of sulfhydryl content) overnight to allow the cysteine-SH of **28** to be alkylated by the maleimide double bond. The OVA−hexapeptide conjugate **31** was then purified by Sephadex G-25 chromatography, lyophilized, and stored desiccated at −20 °C. Additional conjugations were performed to generate antigens for the ELISA, i.e., **28** to keyhole limpet hemocyanin (KLH) and **28** to itself. The KLH conjugate **32** was made utilizing procedures and conditions identical with those for **31** (Scheme 5). Formation of the bis-maleimide complex **33** employed the sulfhydryl-specific homobifunctional cross-linking reagent *N*,*N*′-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine(Scheme 6) at a concentration of 1 mM in phosphate buffer, pH 7.4. The theoretical amount (2 mmol) of **28** was added, and the reaction mixture was stirred overnight. The product was passed though a P-4 column.

Monoclonal Antibody Production. The conjugate **31** combined with Ribi adjuvant (Ribi Immunochemicals, Hamilton, MT) was used to immunize four Balb/c BYJ mice subcutaneously with 50, 100, 150, or 200 μg of the peptide conjugate. Sera were obtained after four immunizations spaced 2 weeks apart and tested in an ELISA (described below) for reactivity to the following: unconjugated KLH (neutral carrier control), **32** (to check for reactivity of the sera to the peptide coupled to a neutral carrier), and **33**. Three days before fusion, the mice were immunized intraperitoneally with 100 μg of **31** without adjuvant. On the day of the fusion, one mouse was sacrificed; the spleen was removed. Half of the spleen was used in the fusion. The fusion was performed by procedures described in Simrell and Klein[34] except the fusion partner was SP2/0 (a murine aminopterin-resistant cell line with a defect in purine metabolism). After a 10-day incubation, a primary ELISA screen was done on the culture supernatants to detect whether cells were secreting antibody to **28**, presented as either **32** or **33**. Those wells that were positive in this assay were screened again 1 week later to determine the class of antibody (IgG or IgM). These clones were also tested for reactivity to the deoxyhypusine-containing peptide (Cys-Thr-Gly-dHpu-His-Gly)− and lysine-containing peptide (Cys-Thr-Gly-Lys-His-Gly)−KLH conjugates as well as to carrier controls.

Enzyme-Linked Immunosorbent Assay (ELISA). The ELISA was performed essentially as described by Kao and Klein.[36] Briefly, Nunc Maxisorp plates (Nunc, Naperville, IL) were coated with 50 μL/well of the antigens at a concentration of 10 μg/mL. Pooled sera from strain-, age-, and sex-matched unimmunized mice were run as a background control when immune sera were screened; a test serum absorbance value 3 times above that of the normal serum background was considered a positive response at the dilution of serum tested. Postimmunization sera were tested at 2-fold dilutions ranging from 1:200 to 1:1600. When hybridoma culture supernatants were analyzed, undiluted medium was the background control, and test hybridoma culture supernatants were also undiluted. Rabbit anti-mouse IgG (whole molecule), goat anti-mouse IgG (γ-chain-specific), and goat anti-mouse IgM (μ-chain-specific), all alkaline phosphatase-conjugated (Sigma), were utilized at 1:1000, 1:8000, and 1:8000 dilutions, respectively. The absorbance at 410 nm (A_{410}) was read after a 1-h incubation at room temperature with *p*-nitrophenyl phosphate (Sigma).

Isolation of Recombinant eIF-5A. A plasmid, containing the eIF-5A cDNA sequences (pBKS(−)/4D), was a gift from Dr. John Hershey, University of California at Davis.[37] *Escherichia coli* HB101 competent cells were from Gibco/BRL (Gaithersburg, MD). *E. coli* competent cells were transformed; the plasmid was isolated and subjected to restriction enzyme (Promega, LaJolla, CA) mapping and sequencing using a T3 primer (DNA Sequencing Core Laboratory, University of Florida). Amplification of the cDNA fragment was performed using a polymerase chain reaction (PCR) kit (Perkin-Elmer, Norwalk, CT). The PCR primers were from Gibco: (1, 5′ end) 5′-CGT GGA TCC ATG GCA GAT GAC TTG GAC TTC-3′, (2, 3′ end) 5′-AGA GAA TTC TTA TTT TGC CAT GGC CTT GAT TGC-3′, (3, 5′ end) 5′-CGT GGA TCC GCA GAT GAC TTG GAC TTC GAG-3′. For amplification, 29 cycles of 95 °C for 2 min, 52 °C for 2 min, and 72 °C for 2 min, followed by a final extension at 72 °C for 7.5 min, were run. An expression vector, pGEX-2T, was obtained from Pharmacia (Piscataway, NJ), and the PCR-amplified fragment and expression plasmid were digested with *Eco*RI and *Bam*HI. The digested nucleic acids were purified, combined in a fragment-to-vector molar ratio of 1:4, and ligated using T4 DNA ligase (Gibco/BRL). The ligation product was transformed into competent HB101, *E. coli* JM 109, or *E. coli* BL 21 cells. Sequence confirmation was performed by PCR sequencing (using primers 1 and 2 described above) of DNA isolated from transformed cells using a Wizard megapreps DNA purification kit (Promega, Madison, WI). One clone containing the correct DNA sequence for eIF-5A except for a deleted methionine codon and the addition of codons for Gly-Ser at the amino terminus was utilized for large-scale expression in either JM109 or BL21 cells under the following conditions. Cultures were incubated at 30 °C with shaking at 300 rpm until late log phase, 0.1 mM isopropylthio-β-D-galactoside was added, and the culture was incubated 6 h. The glutathione *S*-transferase (GST)−eIF-5A protein was isolated from cell extracts in the following manner. After centrifugation at 7700*g* for 10 min at 4 °C and resuspension of the pellet in 1/20 volume of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), the cells were lysed by sonication (5 × 40 s) (Sonifier 450, Branson Ultrasonics Corp., Danbury, CT). The crude homogenate was centrifuged at 23000*g* for 10 min at 4 °C; the supernatant was centrifuged at 26 000 rpm (Beckman SW 28 tubes) for at least 6 h at 4 °C.

The GST fusion protein (43 kDa) was purified from the supernatant by affinity chromatography on glutathione Sepharose 4B. The GST (26 kDa) was cleaved from the N-terminal end of the eIF-5A fusion protein by thrombin protease. The product, recombinant human eIF-5A precursor polypeptide (17 kDa), was purified to homogeneity by FPLC on MonoQ Sepharose.

Preparation of Cell Extracts Containing eIF-5A. Approximately 1×10^8 CHO cells in midlogarithmic growth were homogenized in 50 mM sodium phosphate buffer, pH 7.4, containing 0.9% NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride (1.5 mL). This homogenate was fractionated by ammonium sulfate precipitation as described by Abbruzzese et al.[38] and dialyzed.

***N*α-BOC-*N*ε-CBZ-L-lysine *tert*-Butyl Ester (1).** Sodium hydrogen carbonate (2.81 g, 33.47 mmol) in water (75 mL) was added to *N*ε-CBZ-L-lysine *tert*-butyl ester hydrochloride (12.00 g, 32.18 mmol) in chloroform (100 mL). The mixture was stirred at room temperature 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 was refluxed for 1.5 h and allowed to cool to room temperature. The layers were separated, the aqueous layer was extracted with chloroform (3 × 100 mL), and the combined organic layers were dried. Concentration in vacuo followed by flash chromatography (25% ethyl acetate/hexane) gave **1** (13.82 g, 98%) as a colorless oil: ^{1}H NMR δ 1.30−1.83 (m, 6 H), 1.43 (s, 9 H), 1.45 (s, 9 H), 3.11 (t, 2 H, J = 6.7), 3.94 (dd, 1 H, J = 8.3, 5.1), 5.06 (s, 2 H), 7.24−7.37 (m, 5 H); ^{13}C NMR δ 24.0, 28.3, 28.7, 30.4, 32.4, 41.4, 55.7, 67.3, 80.4, 82.5, 128.7, 128.9, 129.4, 138.4, 158.1, 158.8, 173.8; HRMS *m*/*z* calcd for $C_{23}H_{37}N_2O_6$ 437.2652, found 437.2643. Anal. ($C_{23}H_{36}N_2O_6$) C, H, N. $[\alpha]^{27}_D$ +5.0° (*c* 2.00, $CHCl_3$).

***N*α-BOC-L-lysine *tert*-Butyl Ester Hydrochloride (2).** To a solution of **1** (34.51 g, 79.05 mmol) in ethanol (300 mL) 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 was dried in vacuo to give **2** as the hydrochloride salt (26.59 g, 99%): ^{1}H NMR δ 1.30−1.84 (m, 6 H), 1.43 (s, 9 H), 1.45 (s, 9 H), 2.93 (t, 2 H, J = 7.7), 3.95 (dd, 1 H, J = 8.8, 5.0); ^{13}C NMR δ 23.9, 28.3, 28.7, 32.1, 40.6, 55.5, 79.5, 80.5, 82.7, 158.2, 173.5; HRMS *m*/*z* calcd for $C_{15}H_{31}N_2O_4$ 303.2284, found 303.2272. $[\alpha]^{23}_D$ −21.2° (*c* 1.00, CH_3OH).

(2*S*,9*S*)-2-[(*tert*-Butoxycarbonyl)amino]-10-chloro-9-hydroxy-7-azadecanoic Acid *tert*-Butyl Ester (3a). A solution of **2** (4.83 g, 14.3 mmol) in chloroform (100 mL) was extracted with saturated $NaHCO_3$ solution (2 × 100 mL) and water (100 mL). The organic layer was dried, concentrated, and further dried in vacuo. The resulting oil was dissolved in cyclohexane (26 mL), and (*S*)-(+)-epichlorohydrin (1.58 g, 17.1 mmol) was added under an Ar atmosphere. The precipitated product was filtered after 27 h, washed with cold cyclohexane, and dried in vacuo to give **3a** (2.20 g, 39%) as a fine, white powder: mp 87−88 °C; ^{1}H NMR δ 1.30−1.83 (m, 6 H), 1.44 (s, 9 H), 1.46 (s, 9 H), 2.57−2.66 (m, 3 H), 2.77 (dd, 1 H, J = 12.2, 3.9), 3.51 (dd, 1 H, J = 11.2, 5.7), 3.56 (dd, 1 H, J = 11.2, 5.2), 3.84−3.91 (m, 1 H), 3.94 (dd, 1 H, J = 8.6, 5.3); ^{13}C NMR δ 24.6, 28.3, 28.7, 30.0, 32.6, 48.2, 50.3, 53.5, 55.8, 71.1, 80.4, 82.5, 158.1, 173.8; HRMS *m*/*z* calcd for $C_{18}H_{36}ClN_2O_5$ 395.2313, found 395.2303. Anal. ($C_{18}H_{35}ClN_2O_5$) C, H, Cl, N. $[\alpha]^{22}_D$ −26.1° (*c* 1.00, CH_3OH).

(2*S*,9*R*)-2-[(*tert*-Butoxycarbonyl)amino]-10-chloro-9-hydroxy-7-azadecanoic Acid *tert*-Butyl Ester (3b). Compound **3b** was prepared from reaction of **2** (5.93 g, 17.50 mmol) with (*R*)-(−)-epichlorohydrin (1.62 g, 17.50 mmol) in 44% yield using the same procedure as described for **3a**. **3b**: mp 101 °C; ^{1}H NMR δ 1.30−1.80 (m, 6 H), 1.44 (s, 9 H), 1.46 (s, 9 H), 2.61 (m, 3 H), 2.77 (dd, 1 H, J = 12.3, 4.0), 3.49 (dd, 1 H, J = 11.3, 5.8), 3.57 (dd, 1 H, J = 11.3, 5.3), 3.88 (m, 1 H), 3.94 (dd, 1 H, J = 8.6, 5.0); HRMS *m*/*z* calcd for $C_{18}H_{36}ClN_2O_5$ 395.2313, found 395.2303. Anal. ($C_{18}H_{35}ClN_2O_5$) C, H, N. $[\alpha]^{22}_D$ −15.5° (*c* 0.96, CH_3OH).

(2*S*,9*S*)-2-[(*tert*-Butoxycarbonyl)amino]-7-(carbobenzyloxy)-10-chloro-9-hydroxy-7-azadecanoic Acid *tert*-Butyl Ester (4a). A solution of benzyl chloroformate (1.21 g, 7.09 mmol) in chloroform (10 mL) was added over a period of 15 min to an ice-cold solution of **3a** (2.16 g, 5.46 mmol) in chloroform (40 mL) under an Ar atmosphere. After dropwise additon of triethylamine (1.11 g, 11.0 mmol) in chloroform (10 mL), the reaction mixture was stirred for 4.5 h at room temperature. The reaction mixture was extracted with 1 N HCl (60 mL) and water (60 mL), dried, and concentrated. The residue was purified by flash chromatography (33% ethyl acetate/hexane) to give **4a** (2.69 g, 93%) as a colorless oil: ^{1}H NMR δ 1.30−1.83 (m, 6 H), 1.44 (s, 9 H), 1.45 (s, 9 H), 3.20−3.64 (m, 6 H), 3.93 (dd, 1 H, J = 8.2, 5.5), 4.00 (m, 1 H), 5.12 (s, 2 H), 7.26−7.40 (m, 5 H); ^{13}C NMR δ 24.1, 28.3, 28.8, 32.5, 49.4, 51.4, 52.2, 55.7, 68.4, 71.0, 80.4, 82.5, 129.0, 129.1, 129.6, 138.0, 157.5−158.4 (br), 158.1, 173.7; HRMS *m*/*z* calcd for $C_{26}H_{42}ClN_2O_7$ 529.2681, found 529.2694. Anal. ($C_{26}H_{41}ClN_2O_7$) C, H, N. $[\alpha]^{23}_D$ −21.8° (*c* 1.00, CH_3OH).

(2*S*,9*R*)-2-[(*tert*-Butoxycarbonyl)amino]-7-(carbobenzyloxy)-10-chloro-9-hydroxy-7-azadecanoic Acid *tert*-Butyl Ester (4b). According to the method described for the preparation of **4a**, **3b** (2.93 g, 7.42 mmol) was reacted with benzyl chloroformate (1.60 g, 9.38 mmol) and triethylamine (1.50 g, 14.81 mmol) to obtain **4b** (3.35 g, 85%) as a colorless oil: ^{1}H NMR δ 1.24−1.82 (m, 6 H), 1.44 (s, 9 H), 1.45 (s, 9 H), 3.15−3.64 (m, 6 H), 3.92 (m, 1 H), 4.00 (m, 1 H), 5.12 (m, 2 H), 7.25−7.40 (m, 5 H); ^{13}C NMR δ 24.0, 28.3, 28.8, 32.5, 42.4, 51.3, 52.3, 55.7, 68.4, 71.0, 71.1, 80.4, 82.5, 129.0, 129.1, 129.6, 138.0, 158.1, 158.4, 173.7; HRMS *m*/*z* calcd for $C_{26}H_{42}ClN_2O_7$ 529.2681, found 529.2691. Anal. ($C_{26}H_{41}ClN_2O_7$) C, H, N. $[\alpha]^{23}_D$ −1.7°(*c* 0.98, CH_3OH).

(2*S*,9*R*)-2-[(*tert*-Butoxycarbonyl)amino]-7-(carbobenzyloxy)-10-cyano-9-hydroxy-7-azadecanoic Acid *tert*-Butyl Ester (5a). A mixture of **4a** (2.65 g, 5.02 mmol), dry KCN (3.45 g, 53.0 mmol), and 18-crown-6 (279 mg, 1.05 mmol) in dry acetonitrile (100 mL) was stirred at 60 °C for 16 h under an Ar atmosphere. The reaction mixture was cooled, filtered through Celite, and concentrated. The residue was purified by flash chromatography (33% ethyl acetate/hexane, then 50% ethyl acetate/hexane) to give **5a** (1.84 g, 70%) as a colorless oil: ^{1}H NMR δ 1.25−1.80 (m, 6 H), 1.44 (s, 9 H), 1.45 (s, 9 H), 2.42−2.76 (m, 2 H), 3.20−3.58 (m, 4 H), 3.92 (m, 1 H), 4.08 (m, 1 H), 5.13 (s, 2 H), 7.26−7.48 (m, 5 H); ^{13}C NMR δ 24.0, 24.2, 28.2, 28.7, 32.4, 52.9, 53.8, 55.7, 67.1, 68.4, 80.4, 82.5, 118.9, 129.0, 129.2, 129.6, 138.0, 157.5−158.4 (br), 158.1, 173.7; HRMS *m*/*z* calcd for $C_{27}H_{42}N_3O_7$ 520.3023, found 520.3013. $[\alpha]^{22}_D$ −19.8° (*c* 1.00, CH_3OH).

(2*S*,9*S*)-2-[(*tert*-Butoxycarbonyl)amino]-7-(carbobenzyloxy)-10-cyano-9-hydroxy-7-azadecanoic Acid *tert*-Butyl Ester (5b). According to the method described for the preparation of **5a**, **4b** (3.23 g, 6.10 mmol) was reacted with KCN (3.96 g, 60.82 mmol) and 18-crown-6 (242 mg, 0.92 mmol) to obtain **5b** (1.62 g, 51%) as a colorless oil: ^{1}H NMR δ 1.20−1.84 (m, 6 H), 1.44 (s, 9 H), 1.45 (s, 9 H), 2.55 (m, 2 H), 3.20−3.50 (m, 4 H), 3.92 (dd, 1 H, J = 8.2, 5.1), 4.07 (m, 1 H), 5.13 (s, 2 H), 7.26−7.41 (m, 5 H); ^{13}C NMR δ 24.1, 24.2, 28.3, 28.8, 32.4, 55.7, 67.1, 68.4, 80.4, 82.5, 118.9, 129.0, 129.2, 129.6, 138.0, 158.1, 173.7; HRMS *m*/*z* calcd for $C_{27}H_{42}N_3O_7$ 520.3023, found 520.3048. Anal. ($C_{27}H_{41}N_3O_7$) C, H, N. $[\alpha]^{22}_D$ −5.3° (*c* 1.15, CH_3OH).

(2*S*,9*R*)-2-[(*tert*-Butoxycarbonyl)amino]-11-amino-9-hydroxy-7-azaundecanoic Acid *tert*-Butyl Ester, Diacetate Salt (6a). To a solution of **5a** (1.74 g, 3.34 mmol) in glacial acetic acid (42 mL) were added 10% Pd−C (0.17 g) and PtO_2 (0.34 g), and H_2 gas was introduced. The catalyst was removed after 22 h by filtration through Celite. The filtrate was concentrated in vacuo. Azeotropic removal of acetic acid with toluene provided **6a** as a colorless oil (1.70 g, 100%): ^{1}H NMR (D_2O) δ 1.44 (s, 9 H), 1.47 (s, 9 H), 1.50−1.90 (m, 8 H), 1.97 (s, 6 H), 3.00−3.25 (m, 6 H), 3.97 (dd, 1 H, J = 8.8, 5.2), 4.06 (tt, 1 H, J = 9.6, 3.0); ^{13}C NMR (D_2O, internal standard CH_3OH = 49.5 ppm[39]) δ 24.3, 24.5, 26.9, 29.3, 29.7, 32.1, 33.5, 38.5, 54.1, 56.5, 66.8, 83.5, 85.8, 159.8, 176.0, 182.0; HRMS *m*/*z* calcd for $C_{19}H_{40}N_3O_5$ 390.2968, found 390.2965. $[\alpha]^{21}_D$ −16.1° (*c* 1.00, CH_3OH).

(2*S*,9*S*)-2-[(*tert*-Butoxycarbonyl)amino]-11-amino-9-hydroxy-7-azaundecanoic Acid *tert*-Butyl Ester, Diacetate Salt (6b). According to the method described for the preparation of **6a**, to a solution of **5b** (172 mg, 0.33 mmol) in glacial acetic acid (5 mL) were added 10% Pd−C (17 mg) and PtO_2 (35 mg) under an H_2 atmosphere to obtain **6b** in quantitative yield as a colorless oil: ^{1}H NMR (D_2O) δ 1.30−2.00 (m, 8 H), 1.45 (s, 9 H), 1.48 (s, 9 H), 1.92 (s, 6 H), 2.99−3.28 (m, 6 H), 3.99 (dd, 1 H, J = 9.0, 5.1), 4.06 (tt, 1 H, J = 9.5, 3.3); HRMS *m*/*z* calcd for $C_{19}H_{40}N_3O_5$ 390.2968, found 390.2967. $[\alpha]^{22}_D$ −7.5° (*c* 1.03, CH_3OH).

(2*S*,9*R*)-11-[(Benzyloxycarbonyl)amino]-2-[(*tert*-butoxycarbonyl)amino]-9-hydroxy-7-(carbobenzyloxy)-7-azaundecanoic Acid *tert*-Butyl Ester (7a). A solution of **6a** (1.20 g, 2.35 mmol) in water (35 mL) and diethyl ether (35 mL) was vigorously stirred and cooled to 0 °C under an Ar atmosphere. $KHCO_3$ (3.08 g, 30.8 mmol) was added, and then *N*-(benzyloxycarbonyloxy)succinimide (CBZ-ONSu; 1.70 g, 6.82 mmol) was added in five portions over 20 min. The reaction mixture was warmed to room temperature and stirred for 4 h. The layers were separated; the aqueous layer was extracted with diethyl ether (2 × 35 mL). The combined ether layers were dried and concentrated. The residue was purified by flash chromatography (50% ethyl acetate/hexane) to give **7a** (772 mg, 50%) as a colorless oil: ^{1}H NMR δ 1.22−1.82 (m, 8 H), 1.43 (s, 9 H), 1.44 (s, 9 H), 3.08−3.45 (m, 6 H), 3.81 (m, 1 H), 3.92 (m, 1 H), 5.06 (s, 2 H), 5.10 (s, 2 H), 7.24−7.38 (m, 10 H); ^{13}C NMR δ 24.1, 28.3, 28.8, 32.5, 35.9, 38.6, 54.0, 54.7, 55.7, 67.4, 68.3, 69.0, 80.4, 82.5, 128.8, 128.9, 129.1, 129.4, 129.6, 138.1, 138.4, 158.1, 158.4, 158.9, 173.7; HRMS *m*/*z* calcd for $C_{35}H_{52}N_3O_9$ 658.3704, found 658.3767. Anal. ($C_{35}H_{51}N_3O_9$) C, H, N.

(2*S*,9*S*)-11-[(Benzyloxycarbonyl)amino]-2-[(*tert*-butoxycarbonyl)amino]-9-hydroxy-7-(carbobenzyloxy)-7-azaundecanoic Acid *tert*-Butyl Ester (7b). According to the method described for the preparation of **7a**, **6b** (165 mg, 0.32 mmol) was reacted with CBZ-ONSu (178 mg, 0.71 mmol) to give **7b** (90 mg, 43%) as a colorless oil: ^{1}H NMR δ 1.18−1.82 (m, 8 H), 1.43 (s, 9 H), 1.44 (s, 9 H), 3.06−3.48 (m, 6 H), 3.83 (m, 1 H), 3.92 (m, 1 H), 5.05 (s, 2 H), 5.10 (s, 2 H), 7.22−7.40 (m, 10 H); ^{13}C NMR δ 24.1, 28.3, 28.8, 32.5, 35.9, 38.6, 49.3, 54.0, 54.7, 55.8, 67.4, 68.3, 69.0, 80.4, 82.5, 128.9, 128.9, 129.1, 129.4, 129.6, 138.1, 138.4, 158.0, 158.4, 158.9, 173.7; HRMS *m*/*z* calcd for $C_{35}H_{52}N_3O_9$ 658.3704, found 658.3702. Anal. ($C_{35}H_{51}N_3O_9$) C, H, N. $[\alpha]^{23}_D$ −5.2° (*c* 1.00, CH_3OH).

(2*S*,9*R*)-2-Amino-11-[(benzyloxycarbonyl)amino]-7-(carbobenzyloxy)-9-hydroxy-7-azaundecanoic Acid (8a). The ester **7a** (766 mg, 1.16 mmol) was added to a mixture of trifluoroacetic acid (1.73 g, 15.1 mmol), CH_2Cl_2 (1.2 mL), and triethylsilane (4.37 g, 37.5 mmol) and stirred at room temperature for 21 h under an Ar atmosphere. The reaction mixure was concentrated to dryness and stirred again in the mixture as described above for an additional 18 h. The reaction mixture was concentrated; the resultant oil was dissolved in water (4.0 mL) and adjusted to pH 7 with saturated $NaHCO_3$ solution. The solution was concentrated, and the residue was purified by chromatography on a C_{18} column (30% acetone/water, followed by 55% acetone/water) to give **8a** (421 mg, 72%) as a colorless oil: ^{1}H NMR (45 °C) δ 1.31−1.99 (m, 8 H), 3.10−3.46 (m, 6 H), 3.52 (t, 1 H, *J* = 5.9), 3.83 (m, 1 H), 5.06 (s, 2 H), 5.10 (s, 2 H), 7.22−7.36 (m, 10 H); ^{13}C NMR δ 23.5, 28.4, 29.2, 32.0, 35.9, 38.6, 54.2, 54.9, 56.0, 67.4, 68.3, 69.0, 128.7, 128.8, 128.9, 129.1, 129.4, 129.5, 138.1, 138.4, 158.0, 158.3, 158.9, 174.5; HRMS *m*/*z* calcd for $C_{26}H_{36}N_3O_7$ 502.2553, found 502.2517. $[\alpha]^{24}_D$ +5.0° (*c* 1.00, CH_3OH).

(2*S*,9*S*)-2-Amino-11-[(benzyloxycarbonyl)amino]-7-(carbobenzyloxy)-9-hydroxy-7-azaundecanoic Acid (8b). According to the method described for the preparation of **8a**, **7b** (78 mg, 0.12 mmol) was reacted with trifluoroacetic acid (704 mg, 6.20 mmol) and triethylsilane (140 mg, 11.92 mmol) in CH_2Cl_2 (0.98 mL) to give **8b** (37 mg, 62%) as a colorless oil: ^{1}H NMR δ 1.20−2.10 (m, 8 H), 3.05−3.70 (m, 7 H), 3.82 (m, 1 H), 5.05 (s, 2 H), 5.10 (s, 2 H), 7.20−7.60 (m, 10 H); HRMS *m*/*z* calcd for $C_{26}H_{36}N_3O_7$ 502.2553, found 502.2546. $[\alpha]^{23}_D$ +3.6° (*c* 1.00, CH_3OH).

(2*S*,9*R*)-2-Amino-11-[(benzyloxycarbonyl)amino]-7-(carbobenzyloxy)-9-(tetrahydropyran-2-yloxy)-7-azaundecanoic Acid (9a). Trifluoroacetic acid (164 mg, 1.44 mmol) was added to a solution of **8a** (360 mg, 0.72 mmol) in $CHCl_3$ (7 mL). The solution was concentrated in vacuo. The resultant oil was dissolved in CH_2Cl_2 (20 mL), and 3,4-dihydro-2*H*-pyran (69 mg, 75 μL, 0.83 mmol) was added at room temperature. The reaction progress was monitored by TLC, and six additional portions of 3,4-dihydro-2*H*-pyran (69 mg each) were added over the next 31 h. The reaction mixture was stirred for an additional 16 h and concentrated in vacuo. The oil was dissolved in water (4 mL) and methanol (8 mL) and then adjusted to pH 7 with saturated $NaHCO_3$ solution. The solution was concentrated, and the crude oil was purified by chromatography on a C_{18} column (30% acetone/water, followed by 55% acetone/water) to give **9a** (219 mg, 52%) as a colorless oil: ^{1}H NMR δ 1.20−1.98 (m, 14 H), 3.06−3.56 (m, 8 H), 3.68−4.02 (m, 2 H), 4.33−4.62 (m, 1 H), 5.06 (s, 2 H), 5.11 (s, 2 H), 7.24−7.36 (m, 10 H); ^{13}C NMR δ 20.7, 21.1, 21.7, 23.6, 26.3, 26.5, 32.0, 32.5, 34.2, 38.1, 38.5, 52.0, 56.2, 64.2, 65.2, 67.3, 68.3, 74.9, 100.1, 101.6, 128.79, 128.96, 129.02, 129.10, 129.20, 129.47, 129.56, 129.62, 138.3, 138.5, 158.0, 158.7, 174.8; HRMS *m*/*z* calcd for $C_{31}H_{43}N_3NaO_8$ 608.2948, found 608.2954.

(2*S*,9*S*)-2-Amino-11-[(benzyloxycarbonyl)amino]-7-(carbobenzyloxy)-9-(tetrahydropyran-2-yloxy)-7-azaundecanoic Acid (9b). According to the method described for the preparation of **9a**, **8b** (37 mg, 73.8 μmol) was reacted with trifluoroacetic acid (17 mg, 0.15 mmol) and 3,4-dihydro-2*H*-pyran (46 mg, 50 μL, 0.55 mmol) in CH_2Cl_2 (2 mL) to give **9b** (25 mg, 58%) as a colorless oil: ^{1}H NMR δ 1.26−1.96 (m, 14 H), 3.02−4.04 (m, 10 H), 4.32−4.63 (m, 1 H), 5.06 (s, 2 H), 5.11 (s br, 2 H), 7.22−7.40 (m, 10 H); HRMS *m*/*z* calcd for $C_{31}H_{44}N_3O_8$ 586.3128, found 586.3137.

(2*S*,9*R*)-11-[(Benzyloxycarbonyl)amino]-7-(carbobenzyloxy)-2-[(9-fluorenylmethoxycarbonyl)amino]-9-(tetrahydropyran-2-yloxy)-7-azaundecanoic Acid (10a). A solution of 9-fluorenylmethyl *N*-succinimidyl carbonate (203 mg, 0.60 mmol) in DMF (3.0 mL) was added to a solution of **9a** (219 mg, 0.37 mmol) in 9% Na_2CO_3 (803 mg, 0.68 mmol) at 0 °C and stirred overnight at room temperature. The pH was adjusted to 7.0 with 0.1 N HCl. The mixture was concentrated to an oil and purified by flash chromatography ($CHCl_3$, then 95% $CHCl_3$/MeOH) to give **10a** (150 mg, 50%) as a colorless oil: ^{1}H NMR δ 1.15−1.94 (m, 14 H), 3.10−3.52 (m, 7 H), 3.66−3.98 (m, 2 H), 4.05−4.21 (m, 2 H), 4.24−4.61 (m, 3 H), 5.04 (s, 2 H), 5.08 (s, 2 H), 7.21−7.40 (m, 14 H), 7.65 (dd, 2 H, *J* = 6.9, 4.2), 7.77 (d, 2 H, *J* = 7.2); ^{13}C NMR δ 21.1, 21.7, 24.1, 26.4, 26.5, 28.2, 28.9, 32.1, 32.5, 32.6, 33.2, 34.3, 38.1, 38.5, 51.9, 52.4, 55.5, 64.1, 65.2, 67.4, 67.9, 68.3, 74.9, 79.5, 100.0, 101.5, 120.9, 126.3, 128.2, 128.80, 128.97, 129.10, 129.20, 129.48, 129.57, 129.62, 138.2, 138.5, 142.6, 145.2, 145.4, 158.1, 158.6, 176.8; HRMS *m*/*z* calcd for $C_{46}H_{54}N_3O_{10}$ 808.3809, found 808.3833.

(2*S*,9*S*)-11-[(Benzyloxycarbonyl)amino]-7-(carbobenzyloxy)-2-[(9-fluorenylmethoxycarbonyl)amino]-9-(tetrahydropyran-2-yloxy)-7-azaundecanoic Acid (10b). According to the method described for the preparation of **10a**, **9b** (26 mg, 44 μmol) was reacted with 9-fluorenylmethyl *N*-succinimidyl carbonate (22 mg, 66 μmol) and Na_2CO_3 (9 mg, 88 μmol) to give **10b** (20 mg, 55%) as a colorless oil: ^{1}H NMR δ 1.14−1.96 (m, 14 H), 3.04−3.55 (m, 6 H), 3.64−3.98 (m, 3 H), 4.14 (m, 1 H), 4.20 (t, 1 H, *J* = 6.9), 4.34 (d, 2 H, *J* = 6.9), 4.38−4.61 (m, 1 H), 5.05 (s, 2 H), 5.09 (s, 2 H), 7.20−7.42 (m, 14 H), 7.65 (m, 2 H), 7.78 (d, 2 H, *J* = 7.4); HRMS *m*/*z* calcd for $C_{46}H_{54}N_3O_{10}$ 808.3809, found 808.3839. Anal. ($C_{46}H_{53}N_3O_{10}$) C, H, N.

(2*S*,9*R*)-2-[(*tert*-Butoxycarbonyl)amino]-7-(carbobenzyloxy)-10-cyano-9-[(*S*)-α-methoxy-α-(trifluoromethyl)-phenylacetoxy]-7-azadecanoic Acid *tert*-Butyl Ester (11a). The reaction was carried out in an oven-dried 5- × 175-mm NMR tube, fitted with a rubber septum, under an Ar atmosphere. The reagents were injected via syringe in the following order: anhydrous pyridine (300 μL), (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (Mosher's acid chloride; 13 μL, 70 μmol), anhydrous carbon tetrachloride (200 μL), and then a solution of **5a** (27 mg, 52 μmol) in anhydrous carbon tetrachloride (500 μL). The reaction mixture was shaken and allowed to stand at room temperature for 18 h. The reaction mixture was then taken up in chloroform (20 mL), extracted with saturated $NaHCO_3$ solution, and then extracted with saturated NaCl solution. The organic layer was dried and concentrated in vacuo. The residue was purified by flash

chromatography (33% ethyl acetate/hexane) to give **11a** (31 mg, 81%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 45 °C) δ 1.08−1.80 (m, 6 H), 1.447 (s, 9 H), 1.453 (s, 9 H), 2.52−3.12 (m, 4 H), 3.38 (dd, 1 H, J = 14.7, 6.7), 3.52 (dd, 1 H, J = 14.7, 4.6), 3.60 (s br, 3 H), 4.08 (m, 1 H), 4.92 (m, 1 H), 5.10 (s, 2 H), 5.38 (m, 1 H), 7.28−7.53 (m, 10 H); ^{19}F NMR (282 MHz, $CDCl_3$, $CFCl_3$ as internal standard, 45 °C) δ −71.86; HRMS *m*/*z* calcd for $C_{37}H_{49}F_3N_3O_9$ 736.3421, found 736.3367. $[\alpha]^{22}_D$ −29.2° (*c* 1.00, $CHCl_3$).

(2*S*,9*S*)-2-[(*tert*-Butoxycarbonyl)amino]-7-(carbobenzyloxy)-10-cyano-9-[(*S*)-α-methoxy-α-(trifluoromethyl)-phenylacetoxy]-7-azadecanoic Acid *tert*-Butyl Ester (11b). According to the method described for the preparation of **11a**, **5b** (24 mg, 46 μmol) was reacted with (*R*)-(−)-Mosher's acid chloride (14 mg, 55 μmol) to give **11b** (23 mg, 69%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 45 °C) δ 1.14−1.80 (m, 6 H), 1.44 (s, 9 H), 1.45 (s, 9 H), 2.54−2.92 (m, 2 H), 3.09 (m, 1 H), 3.27 (m, 1 H), 3.46 (s br, 3 H), 3.48 (dd, 1 H, J = 14.7, 6.9), 3.61 (dd, 1 H, J = 14.7, 5.1), 4.10 (m, 1 H), 4.94 (m, 1 H), 5.13 (s, 2 H), 5.38 (m, 1 H), 7.28−7.52 (m, 10 H); ^{19}F NMR (282 MHz, $CDCl_3$, $CFCl_3$ as internal standard, 45 °C) δ −71.85; HRMS *m*/*z* calcd for $C_{37}H_{49}F_3N_3O_9$ 736.3421, found 736.3443. $[\alpha]^{22}_D$ +16.1° (*c* 1.08, $CHCl_3$).

FMOC-(2*S*,9*R*)-Hpu[N^7,N^{12}-di-CBZ, 9-(tetrahydropyran-2-yloxy)]-Val-O-*t*-Bu (12). Under argon, **10a** (66.8 mg, 82.7 μmol) and L-valine *tert*-butyl ester hydrochloride (21.6 mg, 103 μmol) were dissolved in DMF (9.3 mL) and stirred at 0 °C. BOP reagent (42.4 mg, 95.9 mmol) was added, and the reaction mixture was stirred for 30 min before adding diisopropylethylamine (21.4 mg, 165 μmol) and allowing to warm to room temperature. After 21 h, the DMF was diluted with brine (40 mL) and extracted with ethyl acetate (3 × 30 mL). The ethyl acetate layer was then washed with 20 mL of each of the following: 1:1 10% citric acid/brine, brine, 1:1 saturated $NaHCO_3$/brine, and brine. The ethyl acetate was dried and evaporated, and the residue was purified by flash chromatography (2% MeOH/$CHCl_3$) to give **12** (66.0 mg, 83%) as a colorless oil: ^{1}H NMR δ 0.94 (d, 6 H, J = 6.8), 1.20−1.88 (m, 14 H), 1.44 (s, 9 H), 2.11 (m, 1 H), 3.13−3.49 (m, 8 H), 3.66−3.98 (m, 2 H), 4.05−4.24 (m, 2 H), 4.28−4.62 (m, 3 H), 5.05 (s, 2 H), 5.09 (s, 2 H), 7.22−7.41 (m, 14 H), 7.64 (m, 2 H), 7.77 (d, 2 H, J = 7.5).

(2*S*,9*R*)-Hpu[N^7,N^{12}-di-CBZ, 9-(tetrahydropyran-2-yloxy)]-Val-O-*t*-Bu (13). The fully protected dipeptide **12** (141.6 mg, 147.0 μmol) was dissolved in a solution of piperidine (2.0 mL) and DMF (20 mL) and stirred under argon for 4.5 h. Concentration and purification by flash chromatography (2% MeOH/$CHCl_3$) gave **13** (97 mg, 89%) as a colorless oil: ^{1}H NMR δ 0.96 (d, 6 H, J = 6.8), 1.22−1.84 (m, 14 H), 1.44 (s, 9 H), 2.13 (m, 1 H), 3.04−3.49 (m, 8 H), 3.68−4.00 (m, 2 H), 4.20 (d, 1 H, J = 5.7), 4.35−4.63 (m, 1 H), 5.06 (s, 2 H), 5.11 (s, 2 H), 7.24−7.38 (m, 10 H); ^{13}C NMR δ 18.5, 19.5, 21.7, 23.8, 26.4, 28.3, 32.0, 36.2, 36.9, 38.5, 55.7, 59.6, 64.0, 67.3, 68.2, 74.7, 82.8, 99.8, 101.5, 128.77, 128.94, 129.18, 129.46, 129.54, 129.60, 138.2, 138.5, 158.0, 158.6, 172.2, 177.6; HRMS *m*/*z* calcd for $C_{40}H_{61}N_4O_9$ 741.4438, found 741.4458.

(2*S*,9*R*)-Hpu(N^7,N^{12}-di-CBZ)-Val-O-*t*-Bu (14). The free amine **13** (92 mg, 124 μmol) was dissolved in acetone (10 mL) and H_2O (1.0 mL). Under argon, *p*-toluenesulfonic acid monohydrate (64 mg, 0.34 μmol) was added, and the solution was heated and stirred at 45 °C for 2 h. The reaction mixture was diluted with H_2O (10 mL), and the pH was adjusted to 7−8 with saturated $NaHCO_3$. The acetone was evaporated under vacuum, and the remaining aqueous layer was extracted with chloroform (2 × 10 mL). Drying, evaporation, and high-vacuum drying of the organic layer gave **14** (66.7 mg, 82%) as a colorless oil: ^{1}H NMR δ 0.95 (d, 6 H, J = 6.8), 1.22−1.83 (m, 8 H), 1.45 (s, 9 H), 2.12 (m, 1 H), 3.10−3.46 (m, 7 H), 3.82 (m, 1 H), 4.20 (d, 1 H, J = 5.7), 5.05 (s, 2 H), 5.10 (s, 2 H), 7.24−7.38 (m, 10 H); ^{13}C NMR δ 18.4, 19.5, 23.8, 28.3, 29.4, 31.9, 36.0, 36.9, 38.6, 54.6, 55.7, 59.6, 67.3, 68.2, 69.0, 82.8, 128.78, 128.87, 128.91, 129.05, 129.44, 129.55, 138.1, 138.4, 158.1, 158.9, 172.2, 177.7; HRMS *m*/*z* calcd for $C_{31}H_{53}N_4O_8$ 657.3863, found 657.3928.

BOC-(2*S*,9*R*)-Hpu(N^7,N^{12}-di-CBZ)-Val-O-*t*-Bu (15a). Under argon, **14** (50.9 mg, 77.5 mmol) was dissolved in dioxane (2 mL) and H_2O (1 mL) and cooled to 0 °C. Di-*tert*-butyl dicarbonate (30 mg, 0.13 mmol) was added; the solution was allowed to stir for 5 min, then warmed to room temperature, and stirred for 5 h. Concentration followed by flash chromatography (2.5% MeOH/$CHCl_3$) gave **15a** (58 mg, 99%) as a colorless oil: ^{1}H NMR δ 0.94 (d, 6 H, J = 6.8), 1.22−1.83 (m, 8 H), 1.43 (s, 9 H), 1.45 (s, 9 H), 2.12 (m, 1 H), 3.10−3.42 (m, 6 H), 3.82 (m, 1 H), 4.06 (m, 1 H), 4.20 (d, 1 H, J = 5.7), 5.06 (s, 2 H), 5.10 (s, 2 H), 7.23−7.37 (m, 10 H); ^{13}C NMR δ 18.4, 19.5, 24.0, 27.5, 28.3, 28.7, 32.0, 35.8, 38.6, 54.4, 55.8, 59.6, 67.4, 68.3, 68.8, 80.6, 82.8, 128.80, 128.87, 128.94, 129.06, 129.44, 129.56, 138.1, 138.4, 157.9, 158.3, 158.9, 172.0, 175.2; HRMS *m*/*z* calcd for $C_{40}H_{61}N_4O_{10}$ 757.4387, found 757.4396.

BOC-(2*S*,9*R*)-Hpu[N^7,N^{12}-di-CBZ, 9-(*S*)-α-methoxy-α-(trifluoromethyl)phenylacetoxy]-Val-O-*t*-Bu (16a). Under argon, anhydrous pyridine (900 μL), anhydrous CCl_4 (600 μL), and (*R*)-(−)-Mosher's acid chloride (20 μL, 27 mg, 107 μmol) were mixed with vigorous stirring. A solution of **15a** (55 mg, 73 μmol) in CCl_4 (1.5 mL) was added. After 17 h of stirring at room temperature, the solution was diluted with chloroform (25 mL) and extracted with 25 mL of each of the following: brine, 1:1 1 N HCl/brine, brine, 1:1 saturated $NaHCO_3$/brine, and brine. The organic layer was dried and concentrated. Purification by flash chromatography (2:1 hexane/ethyl acetate) gave **16a** (55 mg, 78%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 45.0 °C) δ 0.91 (d, 3 H, J = 6.8), 0.92 (d, 3 H, J = 6.8), 1.10−1.94 (m, 8 H), 1.44 (s, 9 H), 1.46 (s, 9 H), 2.14 (m, 1 H), 2.72−3.54 (m, 7 H), 3.50 (s, 3 H), 4.01 (m, 1 H), 4.38 (dd, 1 H, J = 8.7, 4.7), 4.83−5.37 (m, 2 H), 5.09 (s, 2 H), 5.10 (s, 2 H), 6.54 (m, 1 H), 7.24−7.52 (m, 15 H); ^{13}C NMR ($CDCl_3$, 20 °C) δ 17.5, 18.8, 22.5, 26.7, 28.0, 28.3, 31.3, 32.3, 37.1, 54.3, 55.5, 57.4, 66.7, 67.3, 73.3, 79.9, 81.9, 84.6, 127.05, 127.88, 128.06, 128.23, 128.47, 128.53, 129.6, 131.8, 136.2, 136.5, 155.6, 155.9, 156.3, 166.6, 170.7, 171.8; ^{19}F NMR (282 MHz, $CDCl_3$, $CFCl_3$ = 0 ppm) δ −71.78; HRMS *m*/*z* calcd for $C_{50}H_{68}F_3N_4O_{12}$ 973.4786, found 973.4867. $[\alpha]^{21}_D$ −16.0° (*c* 0.50, $CHCl_3$).

(2*S*,9*R*)-Hypusinyl-L-valine (17a). Under argon, phenol (270 mg), pentamethylbenzene (250 mg), and **15a** (10 mg, 13 μmol) were dissolved in TFA (5 mL) at 0 °C. With vigorous stirring, triisopropylsilane (200 μL) and 30% HBr/HOAc (200 μL) were added, and the solution was allowed to stir for 5 min before being warmed to room temperature and stirred for an additional 55 min. After concentration, the reaction mixture was diluted with 10% HOAc/H_2O (10 mL) and extracted three times with methyl *tert*-butyl ether (25 mL). Evaporation of the aqueous layer gave **17a** (8 mg) as an oil: ^{1}H NMR (D_2O, NaTSP external reference) δ 0.98 (d, 3 H, J = 6.8), 0.99 (d, 3 H, J = 6.8), 1.49 (m, 2 H), 1.69−2.01 (m, 6 H), 2.22 (m, 1 H), 3.01−3.26 (m, 6 H), 4.07 (tt, 1 H, J = 9.7, 3.1), 4.13 (t, 1 H, J = 6.6), 4.32 (d, 1 H, J = 5.7); HRMS *m*/*z* calcd for $C_{15}H_{33}N_4O_4$ 333.2502, found 333.2506.

(2*S*,9*S*)-11-[(Benzyloxycarbonyl)amino]-2-[(*tert*-butoxycarbonyl)amino]-9-hydroxy-7-(carbobenzyloxy)-7-azaundecanoic Acid (18). A solution of **8b** (37 mg, 74.0 μmol) in 1,4-dioxane (2 mL) and water (1 mL) was cooled to 0 °C, and 1 N NaOH (74 μmol) and di-*tert*-butyl dicarbonate (18 mg, 82.5 μmol) were added. The reaction mixture was stirred at room temperature for 2.5 h. The organic solvent was removed under reduced pressure; the residue was taken up in ethyl acetate (4 mL) and water (4 mL) and cooled to 0 °C. This mixture was acidified to pH 2−3 with 1 N $KHSO_4$ solution under stirring. The layers were separated, the aqueous layer was extracted with ethyl acetate (3 × 5 mL), and the combined organic layers were dried. Concentration in vacuo followed by flash chromatography (10% methanol/chloroform) gave **18** (33 mg, 74%) as a colorless oil: ^{1}H NMR δ 1.25−1.95 (m, 8 H), 1.44 (s, 9 H), 3.06−3.50 (m, 6 H), 3.83 (br m, 1 H), 4.04 (br m, 1 H), 5.06 (s, 2 H), 5.10 (s, 2 H), 7.22−7.44 (m, 10 H); HRMS *m*/*z* calcd for $C_{31}H_{44}N_3O_9$ 602.3078, found 602.3053. $[\alpha]^{21}_D$ +3.6° (*c* 1.00, CH_3OH).

BOC-(2*S*,9*S*)-Hpu(N^7,N^{12}-di-CBZ)-Val-O-*t*-Bu (15b). A solution of **18** (31 mg, 51.5 μmol) and L-valine *tert*-butyl ester hydrochloride (12 mg, 57 μmol) in dry DMF (3 mL) was cooled to 0 °C under an Ar atmosphere. BOP (25 mg, 57 μmol) was added, and after 45 min DIEA (18 μL, 103 μmol) was added. The solution was warmed to room temperature and stirred overnight. The reaction mixture was diluted with saturated NaCl solution and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were successively washed with ice-cold 10% citric acid/brine (1:1, 20 mL), brine (20 mL), saturated $NaHCO_3$ solution/brine solution (1:1, 20 mL), and brine (20 mL). The organic layer was dried and concentrated in vacuo. The residue was purified by flash chromatography (98% chloroform/methanol, then 95% chloroform/methanol) to yield **15b** (23 mg, 59%) as a colorless oil: ^{1}H NMR δ 0.95 (d, 6 H, J = 6.8), 1.25−1.85 (m, 8 H), 1.43 (s, 9 H), 1.45 (s, 9 H), 2.12 (m, 1 H), 3.08−3.48 (m, 6 H), 3.82 (m, 1 H), 4.05 (m, 1 H), 4.20 (d, 1 H, J = 5.7), 5.06 (s, 2 H), 5.11 (s, 2 H), 7.22−7.42 (m, 10 H); HRMS m/z calcd for $C_{40}H_{61}N_4O_{10}$ 757.4388, found 757.4387. $[\alpha]^{21}_D$ −13.9° (c 1.17, CH_3OH).

BOC-(2*S*,9*S*)-Hpu(N^7,N^{12}-di-CBZ, 9-[(*S*)-α-methoxy-α-(trifluoromethyl)phenylacetoxy])-Val-O-*t*-Bu (16b). The reaction was carried out according to the procedure described for the synthesis of **16a**, starting with **15b** (18.6 mg, 24.6 μmol) and (*R*)-(−)-Mosher's acid chloride (10 μL, 54 μmol). After workup, the residue was purified by flash chromatography (33% ethyl acetate/hexane) to give **16b** (15 mg, 63%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 45 °C) δ 0.90 (d, 3 H, J = 6.8), 0.93 (d, 3 H, J = 6.8), 1.20−1.90 (m, 8 H), 1.44 (s, 9 H), 1.45 (s, 9 H), 2.14 (m, 1 H), 3.02 (m, 2 H), 3.14−3.58 (m, 4 H), 3.44 (s, 3 H), 4.02 (m, 1 H), 4.38 (dd, 1 H, J = 8.8, 4.6), 4.80−5.20 (m, 2 H), 5.09 (s, 2 H), 5.09 (s, 2 H), 6.52 (m, 1 H), 7.14−7.54 (m, 15 H); ^{19}F NMR (282 MHz, $CDCl_3$, $CFCl_3$ as internal standard, 45 °C) δ −71.40; HRMS m/z calcd for $C_{50}H_{68}F_3N_4O_{12}$ 973.4786, found 973.4739. $[\alpha]^{23}_D$ −21.6° (c 0.50, $CHCl_3$).

(2*S*,9*S*)-Hypusinyl-L-valine (17b). An aliquot of **15b** (7.3 mg, 9.6 μmol), phenol (250 mg), and pentamethylbenzene (250 mg) were dissolved in degassed TFA (5.0 mL) at 0 °C. Triisopropylsilane (0.1 mL) and a saturated solution of HBr in acetic acid (0.2 mL) were added under an argon atmosphere. The solution was stirred at room temperature 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 and dried in vacuo to give **17b** (6.0 mg) as a colorless oil: ^{1}H NMR δ 0.96 (d, 3 H, J = 6.8), 0.97 (d, 3 H, J = 6.8), 1.47 (m, 2 H), 1.68−2.01 (m, 6 H), 2.20 (m, 1 H), 2.99−3.25 (m, 6 H), 4.06 (tt, 1 H, J = 9.7, 3.1), 4.11 (t, 1 H, J = 6.4), 4.26 (d, 1 H, J = 5.7); HRMS m/z calcd for $C_{15}H_{33}N_4O_4$ 333.2502, found 333.2499.

3-Cyanopropanal (19).[30] A mixture of water (50 mL) and 3-cyanopropionaldehyde diethyl acetal (10.0 g, 63.6 mmol) was refluxed for 4 h under a N_2 atmosphere and then distilled at ambient pressure to remove water and ethanol. Glassware utilized for the reflux and initial distillation was acid-washed immediately prior to use by submerging in 3 N HCl for 15 min and then rinsing twice with water. The remaining oil was transferred to a dry, short-path distillation apparatus and distilled under reduced pressure to give **19** (4.15 g, 78%): bp 80−82 °C (1.3 mmHg) [lit.[40] bp 85.1−85.5 °C (3 mmHg)]; ^{1}H NMR ($CDCl_3$) 2.63 (t, 2 H, J = 7.05), 2.91 (t, 2 H, J = 7.05), 9.80 (s, 1 H).

(2*S*)-11-Amino-2-[(*tert*-butoxycarbonyl)amino]-7-azaundecanoic Acid *tert*-Butyl Ester, Diacetate Salt (21). A solution of **2** (1.00 g, 2.95 mmol) in chloroform (30 mL) was extracted with saturated $NaHCO_3$ (2 × 30 mL) and then water (30 mL). The organic layer was dried, evaporated, and dried overnight in vacuo to give the free amine of **2** (749 mg, 84%). This oil was dissolved in benzene (100 mL) containing **19** (230.5 mg, 2.77 mmol) and activated 3-Å molecular sieves (20.30 g). The reaction mixture was allowed to stir under argon for 4.5 h, yielding the imine of **20**. The solution was filtered and concentrated in vacuo. Under argon, dry THF (100 mL) and PtO_2 (150 mg) were added; this was stirred under H_2 gas for 17 h. The black suspension was filtered through Celite to give **20**. The filtrate was concentrated, and the residue was immediately dissolved in glacial acetic acid (25 mL). Under argon, PtO_2 (152 mg) and 10% Pd−C catalyst (82 mg) were added; the suspension was stirred under H_2 gas for 23 h and then filtered through Celite and washed with acetic acid. The filtrate was concentrated in vacuo. Azeotropic removal of acetic acid with toluene provided **21** as a colorless oil (650 mg, 53% calculated from the free amine): ^{1}H NMR (D_2O) δ 1.20−2.00 (m, 10 H), 1.40 (s, 9 H), 1.47 (s, 9 H), 1.97 (s, 6 H), 2.97−3.13 (m, 6 H), 3.97 (dd, 1 H, J = 9.0, 5.5).

(2*S*)-11-[(Benzyloxycarbonyl)amino]-2-[(*tert*-butoxycarbonyl)amino]-7-(carbobenzyloxy)-7-azaundecanoic Acid *tert*-Butyl Ester (22). Under argon, **21** (556 mg, 1.13 mmol) was dissolved in H_2O (15 mL) and diethyl ether (30 mL). The biphasic mixture was stirred and cooled to 0 °C, at which point $KHCO_3$ (1.19 g, 11.9 mmol) was added. Over 20 min, CBZ-ONSu (660.0 mg, 2.648 mmol) was added in five portions. The mixture was allowed to warm to room temperature and stirred for 21 h. The organic layer was separated, the aqueous layer was washed with ether, and the combined organic layers were dried. Concentration and purification by flash chromatography (2:1 hexane/ethyl acetate) gave **22** (271 mg, 37%) as a colorless oil: ^{1}H NMR δ 1.20−1.80 (m, 10 H), 1.43 (s, 9 H), 1.44 (s, 9 H), 3.09 (m, 2 H), 3.18−3.32 (m, 4 H), 3.91 (m, 1 H), 5.06 (s, 2 H), 5.09 (s, 2 H), 7.26−7.37 (m, 10 H); ^{13}C NMR ($CDCl_3$) δ 22.4, 25.4, 25.8, 27.2, 28.0, 28.3, 32.5, 40.7, 46.5, 47.0, 53.8, 66.6, 66.9, 79.6, 81.7, 127.9, 128.0, 128.5, 136.9, 155.4, 156.1, 156.4, 171.9; HRMS m/z calcd for $C_{35}H_{52}N_3O_8$ 642.3754, found 642.3746. $[\alpha]^{23}_D$ = −9.3° (c 1.01, CH_3OH).

2(*S*)-Amino-11-[(benzyloxycarbonyl)amino]-7-(carbobenzyloxy)-7-azaundecanoic Acid (23). Under argon, **22** (271 mg, 0.422 mmol) was added to a mixture of trifluoroacetic acid (770 mg, 6.7 mmol), CH_2Cl_2 (3.0 mL), and triethylsilane (330 mg, 2.8 mmol); this was stirred at room temperature for 23 h. The reaction mixture was concentrated; the resultant oil was dissolved in water (5.0 mL) and adjusted to pH 7 with saturated $NaHCO_3$ solution. This solution was then concentrated; the residue was purified by chromatography on a C_{18} column (30% acetone/water, followed by 55% acetone/water) to give **23** (137 mg, 67%) as a colorless oil: ^{1}H NMR (19.7 °C) δ 1.30−1.66 (m, 8 H), 1.78 (m, 2 H), 3.09 (m, 2 H), 3.20−3.33 (m, 4 H), 3.45 (m, 1 H), 5.05 (s, 2 H), 5.09 (s, 2 H), 7.26−7.37 (m, 10 H); HRMS m/z calcd for $C_{26}H_{36}N_3O_6$ 486.2604, found 486.2614.

11-[(Benzyloxycarbonyl)amino]-7-(carbobenzyloxy)-2(*S*)-[(9-fluorenylmethoxycarbonyl)amino]-7-azaundecanoic Acid (24). A solution of 9-fluorenylmethyl *N*-succinimidyl carbonate (143 mg, 0.42 mmol) in DMF (1.60 mL) was added to a solution of **23** (135 mg, 0.278 mmol) in 9% Na_2CO_3 (655.9 mg, 0.557 mmol) at 0 °C and stirred at room temperature for 21 h under argon. The pH was adjusted to 7.0 with 0.1 N HCl. The mixture was concentrated to an oil and purified by flash chromatography ($CHCl_3$, then 95% $CHCl_3$/MeOH) to give **24** (115 mg, 58%) as a colorless oil: ^{1}H NMR (19.2 °C) δ 1.22−1.92 (m, 10 H), 3.08 (m, 2 H), 3.16−3.30 (m, 4 H), 4.12 (m, 1 H), 4.21 (t, 1 H, J = 6.9), 4.30−4.45 (m, 2 H), 5.04 (s, 2 H), 5.08 (s, 2 H), 7.22−7.42 (m, 14 H), 7.66 (m, 2 H), 7.78 (d, 2 H, J = 7.5); HRMS m/z calcd for $C_{41}H_{46}N_3O_8$ 708.3285, found 708.3284. $[\alpha]^{22}_D$ = +9.3° (c 1.00, $CHCl_3$).

(*S*)-Deoxyhypusine Dihydrochloride (25). Reagent **24** (53 mg, 75 μmol) was deprotected according to the method described for the preparation of **17a**. The crude product was purified by flash chromatography (1:2:1 CH_2Cl_2/MeOH/NH_3-(aq), Kieselgel 60), concentrated, dissolved in 1 mL of H_2O, and adjusted to pH = 4.5 with 0.1 N HCl. Concentration and recrystallization from 1:9:15 H_2O/MeOH/Et_2O gave **25** as the dihydrochloride salt (6.6 mg, 30%): ^{1}H NMR (D_2O) δ 1.53 (m, 2 H), 1.76 (m, 6 H), 1.97 (m, 2 H), 3.01−3.15 (m, 6 H), 3.98 (t, 1 H, J = 6.3); HRMS m/z calcd for $C_{10}H_{24}N_3O_2$ 218.1868, found 218.1900. $[\alpha]^{21}_D$ = +17.0° (c 0.44, 6 N HCl).

Cys-Thr-Gly-deoxyhypusine-His-Gly (27). The polymer-bound peptide **26** (87.4 mg), phenol (250 mg), and pentamethylbenzene (250 mg) were dissolved in degassed TFA (5.0

mL) at 0 °C. Triisopropylsilane (100 μL), 1,2-ethanedithiol (100 μL), and saturated HBr in acetic acid solution (200 μL) were added under an argon atmosphere. The solution was stirred at room temperature 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 on a preparative HPLC (solvent systems A, aqueous 0.1% TFA; and B, 0.1% TFA in CH_3CN; linear gradient of 0−20% B in 85 min; flow rate 12 mL/min; detection at 214 nm; retention time = 13.7 min) using a C_{18} reverse-phase column (Vydac Protein & Peptide C_{18}) to give **27** as a colorless oil (8.0 mg, 28%, calculated as tetrakistrifluoroacetate salt): ^{1}H NMR (600 MHz, 6.0 °C) δ 1.26 (d, 3 H, J = 6.2), 1.38 (m, 2 H), 1.63−1.81 (m, 8 H), 2.98−3.11 (m, 7 H), 3.15 (dd, 1 H, J = 14.9, 5.6), 3.19 (dd, 1 H, J = 15.4, 8.1), 3.31 (dd, 1 H, J = 15.4, 6.2), 3.94−4.06 (m, 4 H), 4.23 (m, 1 H), 4.30 (m, 1 H), 4.35 (m, 1 H), 4.42 (d, 1 H, J = 4.8), 4.77 (m, 1 H), 7.32 (s, 1 H), 8.64 (s, 1 H). Amino acid analysis: Thr 0.65, Gly 2.34, His 1.02.

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