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Synthesis of [L-3-Deoxymimosine[4]]-Angiotensin I as an Approach to the Preparation of Selective Protein-tyrosine Kinase (PTK) Inhibitors

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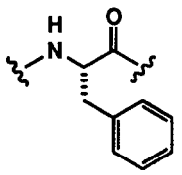
Abstract: *tert-Boc-L-3-Deoxymimosine (13) and tert-Boc-D-Deoxymimosine (16) were prepared in two steps from tert-Boc-L-asparagine and tert-Boc-D-asparagine, respectively. Both 13 and 16 were determined to be optically pure by derivatization with Marphey's reagent. Activation and coupling of 16 to L-isoleucine methyl ester resulted in a diastereomeric mixture of products containing 96% of the DL diastereomer and 4% of the LL diastereomer. [L-3-Deoxymimosine[4]]-angiotensin I was synthesized from 13 as an approach to the design and synthesis of selective protein-tyrosine kinase (PTK) inhibitors.*

Protein-tyrosine kinases (PTK's), which catalyze the transfer of the terminal phosphate of ATP to tyrosyl residues in substrate proteins, play key roles in signal transduction pathways that regulate the proliferation of both normal and neoplastic cells. This has led to recent interest in the design and synthesis of PTK inhibitors as potential anticancer agents, modulators of immune system function, and as molecular probes for elucidating the roles of PTK's in signal transduction systems. Studies of PTK's at both the genetic and protein level have led to the identification of multiple families of kinases, many of which are expressed at the same time in the same cell type. Differentiating between the various roles that these different families of kinases play in regulating cell proliferation in both normal and neoplastic cells requires the availability of inhibitors that exhibit high potency and selectivity. For example, in B lymphocytes, antigens lead to the activation of at least two antigen receptor-associated PTK's, $p56^{lyn}$ and $p72^{syk}$, but the direct consequences of the activation of neither of these kinases is known.[1,2] In many human malignancies, a specific PTK is activated or overexpressed. Examples include chromosomal translocation of *c-abl* in chronic myelogenous leukemia[3] and Ph[1]-positive acute lymphocytic leukemia,[4] amplification of *c-erb-B-2* in human breast cancer,[5] activation of $pp60^{c\text{-}src}$ in colon carcinoma,[6] and overexpression of the epidermal growth factor (EGF) receptor in squamous cell carcinoma.[7,8] This activation of different PTK's in distinct tumor types further establishes the need for selective inhibitors. It has also been suggested that one of the most common cellular lesions found in human cancers is the coexpression of growth factors such as TGFα, PDGF-A, PDGF-B, basic FGF and acidic FGF and their specific receptor PTK's on the same cell type.[9] Such expression leads to cell proliferation by an autocrine mechanism.[10,11]

Three recent comprehensive reviews have appeared on PTK inhibitors.[12-14] These reviews document the fact that there are presently no highly selective and potent suicide substrates (mechanism based inhibitors) of PTK's, and that a limited amount of work has been reported on peptide-based inhibitors. Such inhibitors would offer the advantage that they could be designed to bind selectively to the active site of a particular protein-tyrosine kinase. In certain cases, peptide fragments derived

from PTK's themselves have displayed PTK inhibitory activity. For example, a decapeptide fragment containing residues 415-424 of Rous sarcoma virus has been shown to inhibit the protein-tyrosine kinase activities of $pp60^{src}$, P90 of Y73 avian sarcoma virus, and P140 of Fujinami sarcoma virus,[15] while a peptide corresponding to residues 1142-1153 of the insulin proreceptor requence inhibits the autophosphorylation of the insulin receptor, EGF receptor, and $pp60^{v\text{-}src}$.[16] In addition, a peptide corresponding the residues 137-157 of the noncatylitic domain of $pp60^{v\text{-}src}$ inhibits the tyrosine kinase activity of $pp60^{v\text{-}src}$ and the autophosphorylation of the EGF receptor.[17] The peptide Ac-Phe-Gly-Ala-Leu-CH_2Cl, containing a chloroketone moiety, was reported to inhibit the EGF receptor tyrosine kinase derived from A-431 cell membranes.[18] Several synthetic peptides derived from substrate sequences and containing tyrosine analog residues in place of the phosphorylated tyrosine residue have also been reported to possess PTK inhibitory activity. These include peptides containing phenylalanine (**1**),[16] 4-methoxyphenylalanine (**2**),[16] tetrafluorotyrosine (**3**),[19] dehydrophenylalanine (**4**),[20] and β-(4-pyridyl-1-oxide)-L-alanine (**5**)[21] residues. The latter modification, which was introduced in order to produce a potential suicide substrate (mechanism based inhibitor), resulted instead in a weak competitive inhibitor of $p56^{lck}$.[21]



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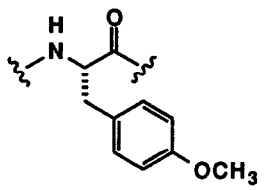

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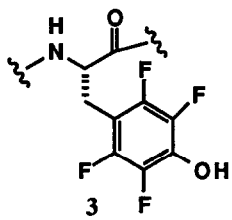

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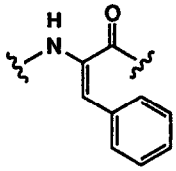

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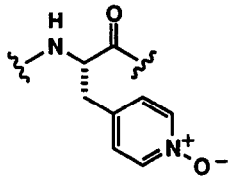

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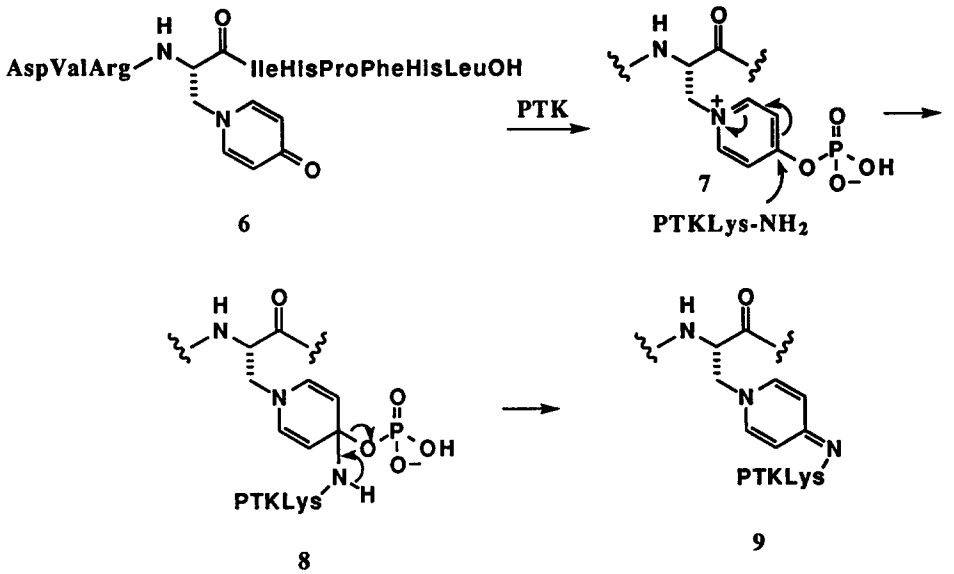
In view of these considerations, we decided to synthesize [L-3-deoxymimosine[4]]-angiotensin I (**6**) as a potential suicide substrate (mechanism-based inhibitor) for protein-tyrosine kinases. It is well known that angiotensin I is a substrate for a variety of PTK's.[22,23] The rationale for this proposal (Scheme I) is that phosphorylation of the 4-keto group of the deoxymimosine residue would activate the pyridine ring for nucleophilic attack by the lysine residue in the vicinity of the ATP binding site of the PTK, leading to intermediate **8**. The lysine is thought to be involved in the transfer of the terminal phosphate of ATP to the phenolic hydroxyl group of tyrosine in the substrate.[24] Elimination of phosphoric acid from **8** would then give **9**, in which the peptide inhibitor is covalently bound to the PTK. In order to target a particular protein-tyrosine kinase, the reactive L-3-deoxymimosine residue could be embedded in a peptide sequence resembling that of the natural substrate of the enzyme.

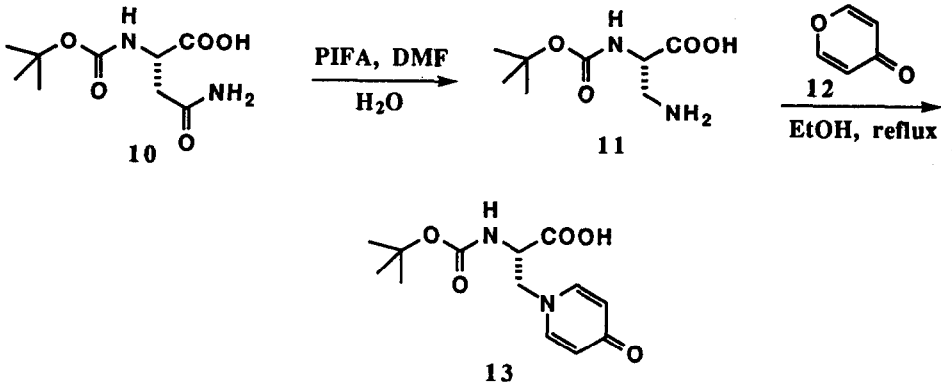
Scheme I



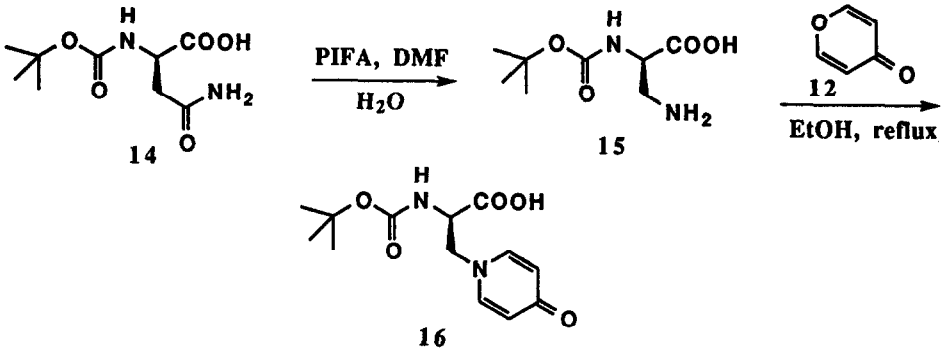
Although an improved synthesis of L-3-deoxymimosine from asparagine was reported previously by Harris,[25] it proved to be rather tedious and inefficient for the preparation of *tert*-Boc-L-3-deoxymimosine (**13**) due to its reliance on tosyl protection of the α-amino group, utilized to withstand the rather harsh conditions for the Hofmann rearrangement (Br_2, NaOH). Accordingly, a two step synthesis of *tert*-Boc-L-3-deoxymimosine (**13**) was performed (Scheme II) from commercially available *tert*-Boc-L-asparagine (**10**). This route employed *I,I*-[bis(trifluoroacetoxy)iodo]benzene (PIFA) as the reagent for the rearrangement, which required milder conditions.[26-29] Treatment of *tert*-Boc-L-asparagine (**10**) with PIFA in aqueous DMF afforded the rearrangement product **11**,[26] which was converted to *tert*-Boc-L-3-deoxymimosine (**13**) in 61% yield when heated with 4*H*-pyran-4-one (**12**) in refluxing ethanol for 10 hours. The yields obtained when **11** and **12** were stirred in aqueous sodium hydroxide solution for 7 days were much lower.[25] *tert*-Boc-D-3-Deoxymimosine (**16**) was also prepared in 87% yield from *tert*-Boc-D-asparagine (**14**) using the same chemistry (Scheme III).

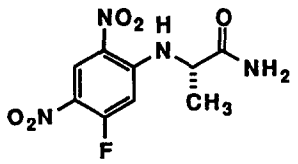
The optical purities of the intermediate *tert*-Boc-protected L- and D-2,3-diaminopropanoic acids **11** and **15**, as well as the *tert*-Boc-protected L- and D-3-deoxymimosines **13** and **16**, were determined with Marphey's reagent (**17**). The reaction of **11** or **15** with **17** in acetone under basic conditions afforded **18** or **19**. The HPLC analyses of **18** and **19** showed complete diastereomeric purity, which proves that **11** and **15** were optically pure enantiomers. Similarly, *tert*-Boc-protected D-3-deoxymimosine **16** was deprotected and derivatized with Marphey's reagent, yielding **21**, which was also diastereomerically pure by HPLC analysis, as none of the corresponding diastereomer **20** could be detected. These results indicated clearly that **13** and **16** were optically pure compounds.

Scheme II



Scheme III





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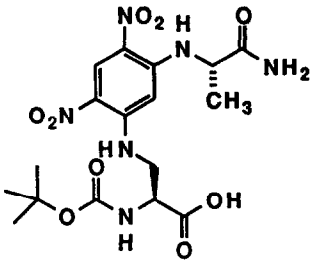

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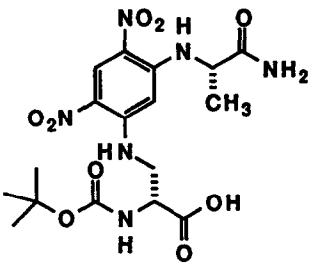

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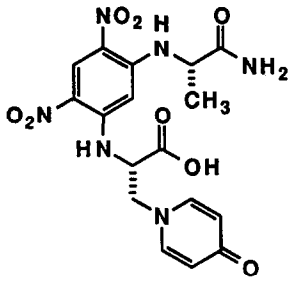

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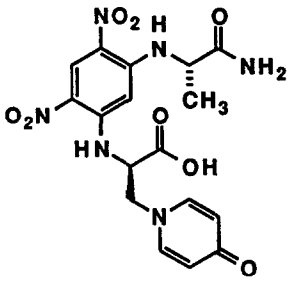

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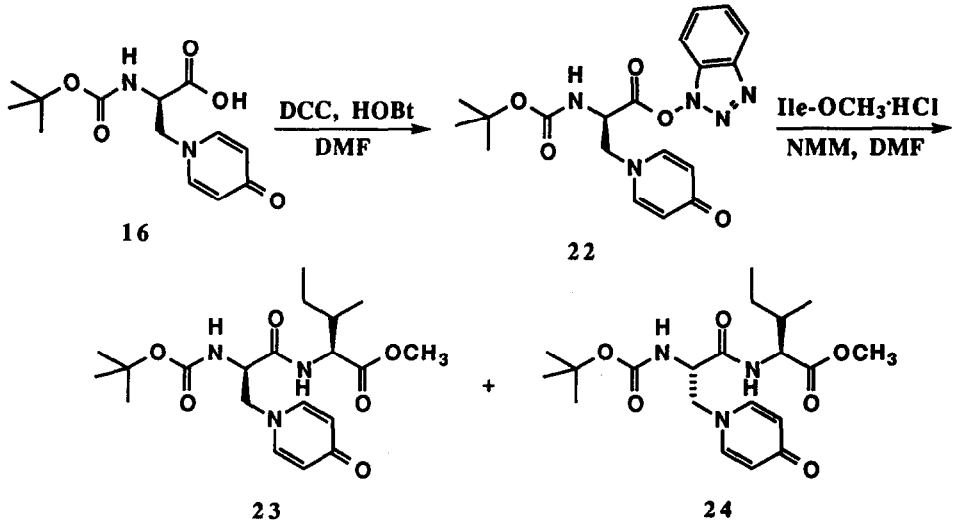
To study the effects of activation and coupling on the chiral integrity of the 3-deoxymimosine residue during peptide synthesis, *tert*-Boc-D-3-deoxymimosine (**16**) was treated with DCC/HOBt and the resulting activated ester (**22**) was coupled to L-isoleucine methyl ester hydrochloride in DMF (Scheme IV). The crude product was analyzed by HPLC, which showed a diastereomeric mixture of products whose composition was 96% DL (**23**) and 4% LL (**24**). Since the Marphey's reagent derivatization of deprotected **16** indicated complete optical purity, the formation of 4% of the LL isomer **24** must occur during formation or coupling of the active ester **22** to L-isoleucine methyl ester. It is recognized that amino acid active esters may racemize in DMF solution, and that the extent of racemization is more significant if the coupling reaction is slow due to steric hindrance in either the acylating agent or the amino component.[30] In the present case, the active ester **22** was formed separately in DMF and then reacted with L-isoleucine methyl ester, and this procedure is likely to have allowed significant racemization of the active ester to occur.

It was planned to use the 2,4-dinitrophenyl group to protect the histidine residues during the synthesis of the desired peptide **6**, so it was important to study the stability of the 3-deoxymimosine residue towards the nucleophilic deprotection conditions. The *tert*-Boc-protected amino acid **13** was incubated with an excess of thiophenol in DMF for 1 hour at room temperature and HPLC analysis indicated that it was not affected.

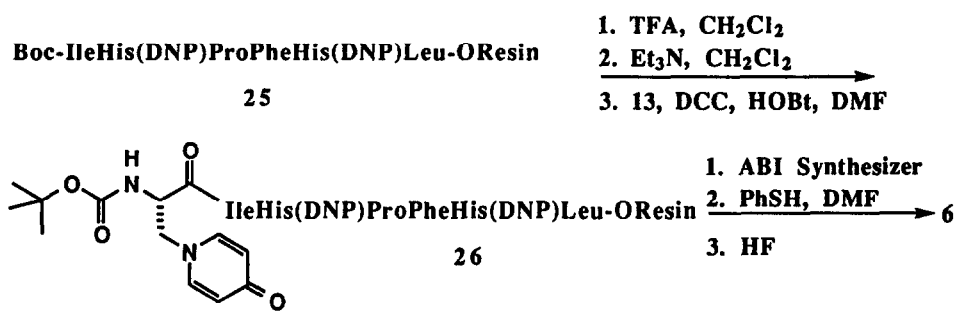
With the information concerning the optical purity of **13**, the chiral integrity of the 3-deoxymimosine residue during coupling, and its stability during histidine deprotection in hand, the synthesis of the desired peptide **6** was attempted. The resin-linked peptide **25** (Scheme V) was assembled on an ABI peptide synthesizer using the *N*-methylpyrrolidone (NMP)/HOBt coupling protocol. The *tert*-Boc group was removed from the peptide **25** and the product coupled manually with **13** in DMF using DCC/HOBt as the coupling agent to give the peptide-resin **26**. A quantitative

ninhydrin test showed that the coupling efficiency was greater than 99%. The remaining amino acid residues were then coupled automatically using the ABI peptide synthesizer, and the peptide was cleaved from the resin with HF. The HPLC analysis of the crude peptide mixture indicated the presence of one major component. The ratio of **6** to the corresponding D-deoxymimosine-containing peptide was 98.5 to 1.5, respectively. The pure product **6** was isolated by preparative reverse phase HPLC on a Vydac 218P C-18 column, using a gradient elution of 16% to 32% aqueous acetonitrile containing 0.1% trifluoroacetic acid. The amino acid analysis results and high resolution FABMS data were in agreement with the assigned structure **6**. The HPLC trace produced by the pure product is displayed in Figure 1.

Scheme IV



Scheme V



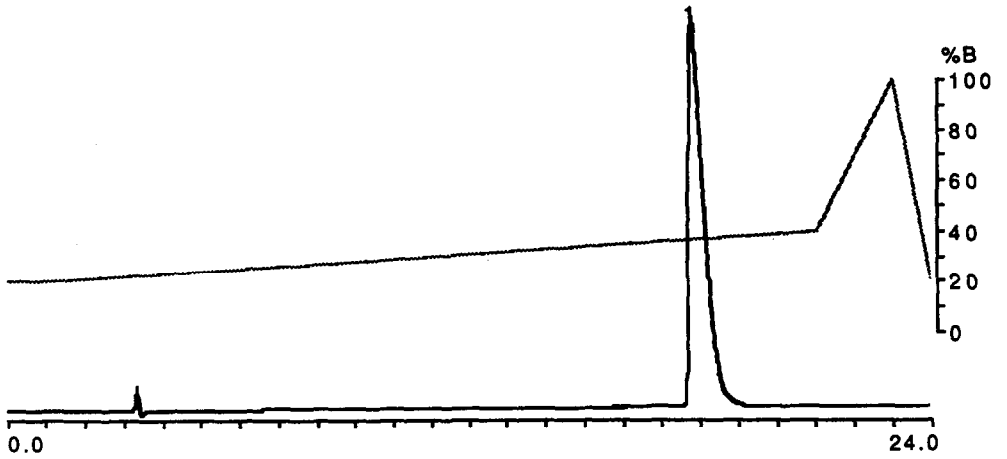


Figure 1. HPLC analysis of [L-3-deoxymimosine[4]]-angiotensin I (**6**). Column: Vydac 218 TP, C-18, 5 μ, 4.6 x 250 mm. A gradient elution of 16% CH_3CN-H_2O-0.1% TFA to 32% CH_3CN-H_2O-0.1% TFA in 40 min at a flow rate of 1.0 mL/min was used (detector 230 nm).

In conclusion, *tert*-Boc-L-3-deoxymimosine (**13**) has been prepared in two steps from *tert*-Boc-L-asparagine and it has been demonstrated that its behavior during the synthesis of peptide **1** resembles that of an ordinary amino acid. Studies of the interactions of **6** with a variety of PTK's are presently being conducted.

EXPERIMENTAL

***N^2*-(*tert*-Butoxycarbonyl)-L-2,3-diaminopropanoic Acid (11).** Compound **11** was obtained as described previously[26]: mp 197-198 °C (dec.) (lit.[26] 198-200 °C); TLC (silica gel, *n*-butanol/AcOH/pyridine/water = 4:1:1:2, v:v), R_f 0.55; $[\alpha]_D^{20}$ = - 7.22° (c 1.1, AcOH) [lit.[26] - 2.7° (c 1.0, AcOH)]; FABMS *m/e* 205 (MH^+); ^1H NMR (500 MHz, D_2O) δ 1.25 (s, 9 H, CH_3), 3.01 (dd, J = 13.0, 8.5 Hz), 3.21 (dd, J = 13.0, 5.5 Hz), 4.01 (dd, J = 8.5, 5.5 Hz); ^{13}C NMR (125 MHz, D_2O) δ 28.45, 41.84, 53.71, 82.47 ,158.24 ,175.38.

***N*-(*tert*-Butoxycarbonyl)-L-3-deoxymimosine (13).** A suspension of **11** (31 mg, 0.152 mmol) and 4*H*-pyran-4-one (**12**, 24.5 mg, 0.255 mmol) in absolute ethanol (1.5 mL) was heated at reflux for 10 h. The yellow solution was cooled and activated charcoal (50 mg) was added. The mixture was heated at reflux for 5 min, cooled, filtered, and concentrated. The residue was triturated with ether (2 x 10 mL), dissolved in 50% aqueous methanol (4 mL) and purified by reverse phase HPLC on a C-18, 12 μ, 10 x 350 mm Dynamax 300A column using a gradient elution of 10% aqueous acetonitrile-1% AcOH to 20% aqueous acetonitrile-1% AcOH in 15 min at a flow rate of 4.5 mL/min. The fraction containing the desired product was lyophilized to give a white solid (26 mg, 60.6%): mp 211-213 °C (dec); TLC (silica gel, *n*-butanol/AcOH/pyridine/water = 4:1:1:2, v:v) R_f 0.41, (silica gel, *n*-butanol/AcOH/water = 4:1:1, v:v) R_f 0.42; $[\alpha]_D$ = -24.8° (*c* 1.0, MeOH); FABMS

0.41, (silica gel, *n*-butanol/AcOH/water = 4:1:1, v:v) R_f 0.42; $[\alpha]_D$ = -24.8° (*c* 1.0, MeOH); FABMS *m/e* 283 (MH^+); ^1H NMR (500 MHz, D_2O) δ 1.136 (s, 9 H), 3.870 (dd, 1 H, J = 9.79, 14.10 Hz), 4.194 (m, 1 H), 4.350 (dd, 1 H, J = 14.10, 4.03 Hz), 6.362 (d, 2 H, J = 7.05 Hz), 7.635 (d, 2 H, J = 7.05 Hz).

***N*^{2}-(*tert*-Butoxycarbonyl)-D-2,3-diaminopropanoic Acid (15).** *N*-*tert*-Butoxycarbonyl-D-asparagine (**14**, 100 mg, 0.431 mmol) was added to suspension of *I,I*-[bis(trifluoroacetoxy)iodo]benzene (PIFA, 274 mg, 0.637 mmol) in 1:1 DMF-water (2 mL). After 15 min, pyridine (0.069 mL) was added causing the mixture to turn yellow in color and become homogeneous. The reaction mixture was stirred for 4 h at room temperature and the solvent was removed under high vacuum. The residue was dissolved in water (10 mL) and washed with ether (3 x 10 mL). The aqueous layer was lyophilized to give a gummy material which was dissolved in water and filtered through a C-18 Sep-Pak cartridge. The eluate was chromatographed on a C-18, 12 μ, 10 x 350 mm Dynamax 300A column using water as solvent at a flow rate of 3 mL/min. The desired fraction was lyophilized to give a white solid (43.1 mg, 49%): mp 200-202 °C; $[\alpha]_D$ = +5.19° (*c* 1.04, AcOH). The chromatographic data were identical with those detailed for **6** above.

***N*-(*tert*-Butoxycarbonyl)-D-3-deoxymimosine (16).** A suspension of **15** (30 mg, 0.147 mmol) and 4*H*-pyran-4-one (**12**, 24 mg, 0.252 mmol) in absolute ethanol (1.5 mL) was heated at reflux for 10 h. The yellow solution was cooled and activated charcoal (50 mg) was added. The mixture was heated at reflux for 5 min, cooled, filtered, and concentrated. The residue was triturated with ether (2 x 10 mL) and recrystallized from 1:1 ethanol-ethyl acetate to give a white solid (10.7 mg). The mother liquor was concentrated and purified by reverse phase HPLC on a C-18, 12 μ, 10 x 350 mm Dynamax 300A column using a gradient elution of 8% aqueous acetonitrile-0.1% TFA to 40% aqueous acetonitrile-0.1% TFA in 15 min at a flow rate of 4.5 mL/min. The fraction containing the desired product was lyophilized to give a white solid (25.5 mg, total yield 87.3%): TLC (silica gel, *n*-butanol/AcOH/water 4:1:1) R_f 0.42 ; $[\alpha]_D$ = +29.6° (*c* 1.0, MeOH). This product had the same R_f value as the L-enantiomer that was prepared as described above and both gave a greenish blue stain with ninhydrin while the starting material **15** gave a red stain.

Derivatization of 11 and 15 with Marphey's Reagent. A sample of **11** or **15** (0.5 mg) was dissolved in water (50 μL) and to the solution was added a 1% solution of Marphey's reagent (**17**) in acetone (100 μL) and 1 M sodium bicarbonate (40 μL). The mixture was heated at 40 °C for 1 h, cooled and acidified with 2 M hydrochloric acid (20 μL). The volatiles were removed on a rotary evaporator followed by lyophilization. The solid residue was dissolved in 1:1 DMSO-H_2O and analyzed by HPLC on a C-18, 5 μ,4.6 x 250 mm Vydac 218 TP column using a gradient elution of 10% acetonitrile-triethylamine phosphate buffer solution (pH 3.0) to 50% acetonitrile-triethylamine phosphate buffer solution (pH 3.0) in 60 min at a flow rate of 2.0 mL/min with the effluent being monitored at 340 nm. Under these conditions, the adduct **18** produced from **11** had a retention time of 32.14 min and the adduct **19** produced from **15** eluted at 31.08 min.

Derivatization of 13 and 16 with Marphey's Reagent. A sample of **13** or **16** (0.7 mg) was treated with TFA (75 μL) and methylene chloride (225 μL) at room temperature for 30 min. The volatiles were removed on a rotary evaporator, and the residue was triturated with anhydrous ether (2 x 1 mL) and dried under vacuum. The residue was dissolved in water (50 μL) and to the solution was added a 1% solution of Marphey's reagent in acetone (100 μL) and 1 M sodium bicarbonate (40 μL). The mixture was heated at 40 °C for 1 h, cooled and acidified with 2 M hydrochloric acid (20 μL). The volatiles were removed on a rotary evaporator followed by lyophilization. The solid residue was dissolved in 1:1 DMSO-H_2O and analyzed using the same conditions as above. Under these conditions the adduct **20** synthesized from **16** produced only one peak at a retention time of 12.6 min and the adduct **21** prepared from **13** produced only one peak at a retention time of 11.2 min.

Activation of 16 and Coupling to L-Isoleucine Methyl Ester Hydrochloride. Under an atmosphere of argon, a solution of DCC in DMF (0.485 mL of a stock solution that contained 194 mg of DCC in 6.84 mL of DMF, 0.066 mmol) was added to a mixture of acid **16** (16.9 mg, 0.06 mmol) and HOBt·H_2O (10.1 mg, 0.066 mmol). The mixture was stirred at 0 °C for 10 min and at room temperature for 30 min. The mixture was rapidly filtered into a vial containing L-isoleucine methyl ester hydrochloride (10.9 mg, 0.06 mmol). The activation vial containing **22** was rinsed with DMF (0.1 mL) and the rinse was added to the reaction vial. The mixture was cooled to 0 °C, *N*-methylmorpholine (NMM, 6.6 μL, 0.06 mmol) was added, and the mixture was stirred for 21 h at room temperature. The mixture was diluted with ethyl acetate (2 mL) and filtered. The filter cake was washed with ethyl acetate (1 mL) and the filtrate was concentrated and dried under high vacuum. The residue was triturated with ice cold water (3 mL), filtered and analyzed by reverse phase HPLC on a C-18, 5 μ, 4.6 x 250 mm Vydac 218 TP column using a gradient elution of 26% acetonitrile-H_2O to 52% acetonitrile-H_2O in 15 min at a flow rate of 1.0 mL/min (detector 230 nm). The HPLC results indicated that the product mixture consisted of 96% **23** (retention time 13.85 min) and 4% **24** (retention time 14.86 min). TLC (silica gel, EtOAc / MeOH = 2:1) peptide **23** and **24**, R_f 0.49; ^{1}H NMR (500 MHz, $CDCl_3$) peptide **23** δ 0.84 (m, 6 H), 1.18 (m, 1 H), 1.30 (s, 9 H), 1.40 (m, 1 H), 1.80 (m, 1 H), 3.62 (s, 3 H), 3.78 (dd, 1 H, J = 10.6, 13.8 Hz), 4.07 (dd, 1 H, J = 4.0, 13.9 Hz), 4.24 (t, 1 H, J = 7.4 Hz), 4.44 (t of d, 1 H, J = 4.0, 9.98 Hz), 6.05 (d, 2 H, J = 7.51 Hz), 7.13 (d, 1 H, J = 9.15 Hz), 7.59 (d, 2 H, J = 7.51 Hz), 8.31 (d, 1 H, NH, J = 8.0 Hz), peptide **24** δ 0.82 (t, J = 7.2 Hz, 6 H), 1.15 (m, 1 H), 1.29 (s, 9 H), 1.34 (m, 1 H), 1.77 (m, 1 H), 3.651 (s, 3 H), 3.763 (dd, 1 H, J = 10.7, 13.6 Hz), 4.053 (dd, 1 H, J = 4.3, 13.7 Hz), 4.28 (dd, 1 H, J = 6.3, 8.4 Hz), 4.51 (t of d, 1 H, CH, J = 4.2, 10.2 Hz), 6.04 (d, 2 H, J = 7.6 Hz), 7.08 (d, 1 H, NH, J = 9.4 Hz), 7.597 (d, 2 H, J = 7.7 Hz), 8.48 (d, 1 H, J = 8.6 Hz); FABMS, peptide **23** or peptide **24**, *m/e* 410 (MH^+).

Peptide-resin 26. Resin **25** (0.4 mmol/g, 0.8 g, 0.32 mmol) was subjected to the following wash and deprotection protocol in a sintered glass funnel: 1) CH_2Cl_2 (3 x 7 mL, 2 min); 2) 1:1 TFA:CH_2Cl_2 (7 mL, 5 min); 3) 1:1 TFA:CH_2Cl_2 (7 mL, 25 min); 4) CH_2Cl_2 (4 x 7 mL, 2 min); 5) 12.5% Et_3N-CH_2Cl_2 (2 x 7 mL, 5 min); 6) CH_2Cl_2 (4 x 7 mL, 2 min). After air drying a yellow resin (0.7786 g) was obtained.

Solid DCC (227 mg, 1.1 mmol) was added to an ice cold suspension of the peptide-resin **25** (0.7786 g), acid **13** (282 mg, 1 mmol) and HOBt·H_2O (306 mg, 2 mmol) in DMF (7 mL) and the mixture was stirred for 2 h at 0 °C and 36 h at room temperature. The resin was filtered, washed successively with DMF (7 mL), methanol (4 x 7 mL), ethanol (2 x 7 mL), DMF (2 x 7 mL), and methylene chloride (3 x7 mL) and air dried to give 0.8543 g of a yellow resin **26**. A quantitative ninhydrin test showed that the coupling efficiency was > 99%.

Purification of Peptide 6. The crude peptide that was delivered to us weighed 234 mg. A 52.6 mg portion was dissolved in doubly distilled water (4 mL), filtered, and loaded onto a C-18, 15-20 μ, 22.5 x 250 mm Vydac 218TP column that had been equilibrated with 16% CH_3CN-H_2O-0.1% TFA. The column was eluted with a gradient of 16% CH_3CN-H_2O-0.1% TFA to 32% CH_3CN-H_2O-0.1% TFA in 40 min at a flow rate of 10 mL/min. Several fractions were collected and analyzed by analytical HPLC on a C-18, 5 μ, 4.6 x 250 mm Vydac 218 TP column using a gradient elution of 16% CH_3CN-H_2O-0.1% TFA to 32% CH_3CN-H_2O-0.1% TFA in 40 min at a flow rate of 1.0 mL/min. The desired fractions were pooled and lyophilized to give 24.4 mg of peptide which was 100% pure and 14.1 mg that was 93% pure. High resolution FABMS *m/e* ($C_{61}H_{88}O_{14}N_{18}$) calculated 1297.68 (MH^+); found 1297.66 (MH^+). Amino acid analysis (expected/found) Asp (1.0/0.9), Pro (1.0/1.1), Val (1.0/1.0), Ile (1.0/1.0), Leu (1.0/1.1), Phe (1.0/1.0), His (2.0/2.0), Arg (1.0/1.1).

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