Large-Scale Synthesis of a Cyclic Hexapeptide Analogue of Somatostatin

Stephen F. Brady,* Roger M. Freidinger, William J. Paleveda, Christiane D. Colton, Carl F. Homnick, Willie L. Whitter, Paul Curley, Ruth F. Nutt, and Daniel F. Veber

Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486

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A recent report from our laboratories describes the preparation and biological properties of the highly potent cyclic hexapeptide somatostatin analogue **1**. Herein we report the details of the large-scale synthesis of **1** developed to provide supplies for clinical study. The synthesis was accomplished entirely via solution chemistry, by stepwise elongation of the amino acid chain, starting from tyrosine methyl ester. Use of the mixed anhydride procedure allowed coupling without the need for protection of the tyrosine hydroxyl. Several intermediates were purified by crystallization, including the protected hexapeptide **10**. Cyclization was achieved in high yield employing an improved technique using diphenylphosphoryl azide, with sodium bicarbonate as base. Only a single chromatographic procedure was required, at the stage of the penultimate cyclic peptide **12**. Using this synthetic route we have been able to prepare >99% pure final product **1** in batches of 40–50 g. Yields based on each of the constituent residues ranged from 17% (Boc-alanine) to 52% (Boc-D-tryptophan). The process serves as an example of the kinds of considerations important for rapid and efficient large-scale synthesis of peptides.

The highly potent cyclic hexapeptide analogue of somatostatin, *cyclo*-(*N*-Me-Ala-Tyr-D-Trp-Lys-Val-Phe) (**1**) was recently disclosed.[1] This compound is undergoing clinical evaluation as an agent for improved control of glucose levels in insulin-dependent diabetic patients. Multihundred gram quantities of peptide **1** have been needed for assessment of its safety and efficacy. Herein we report the details of the synthesis developed to supply the required amounts of this material.[2]

Initial quantities of analogue **1** for biological evaluation were prepared by a procedure representative of our general approach to small cyclic somatostatin analogues,[3] wherein protected linear precursor is assembled on solid support and cyclization steps are carried out in solution, after cleavage from the resin by hydrazinolysis. While this route, as summarized in Figure 1, proved well suited for synthesizing numbers of analogues, and up to about 1 g of cyclic peptide **1** in particular, it was not feasible for larger scale work either in terms of laboratory manipulations or economics. We also realized that problems associated with the synthesis of cyclic peptides of this type, which do not contain cystine, are quite distinctive from those confronted in synthesizing somatostatin itself.[3a] Key limiting factors of our earlier approach include (1) the extensive reliance on chromatographic procedures of limited capacity, particularly gel filtration, to obtain final product of the requisite purity; (2) special difficulties associated with the removal of byproducts formed in liquid HF;[4] (3) the need for extensive utilization of protecting groups and excesses of costly reagents during couplings on solid phase; and (4) the unique problems relating to the preparation and handling of acyl azides.[5] As a result of these considerations, an alternative approach based entirely on solution chemistry was developed.

A number of structural elements in our target **1** had to be reexamined in the design of this new route. First of all, the reactivity of the side chains of tyrosine and lysine, which typically require protection during a multistage synthesis, had to be addressed. Secondly, lability of the indole in D-tryptophan to acidic and oxidative conditions requires, if not protection, limiting its exposure to such conditions. Thirdly, *N*-methylalanine, a secondary amino acid, may be subject to increased steric hindrance to coupling. Finally, of six possible linear precursors, the one expected to undergo cyclization most efficiently ought to be selected.

In our initial work we had already determined that the linear sequence depicted in Figure 1 cyclizes quite well, and, in addition, we had prepared a large number of cyclic somatostatin analogues from linear hexapeptide precursors having N-terminal D-tryptophan. This sequence offered the additional advantage of introduction of the sensitive tryptophan residue as late as possible. Thus, structure **11** (Figure 4) became the intermediary target of our new solution synthesis. The overall route is outlined in Figures 2–4.

Noteworthy features of the process include (1) the use of mixed anhydride conditions to give efficient coupling with low racemization and easily removed byproducts without the need to protect the tyrosine hydroxyl; (2) an improved mild cyclization technique using diphenylphosphoryl azide (DPPA) for carboxyl activation, with sodium bicarbonate as base;[6] and (3) removal of the lysine side-chain protection (Cbz) quantitatively by transfer hydrogenolysis[7] and direct isolation of final product without resort to column chromatography.

At a number of points in the synthesis we were able to purify intermediates by crystallization, and only a single chromatographic procedure was required, at the stage of penultimate cyclic peptide **12**. Using this synthetic route we have been able to prepare upwards of 150 g of >99% pure final product **1**. The overall yield was 23% from tyrosine methyl ester hydrochloride (11 steps) and ranged from a low of 17% based on Boc-Ala-OH (12 steps), to a high of 52% based on Boc-D-Trp-OH (7 steps), the most expensive of the six starting amino acid derivatives.

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(3) Nutt, R. F.; Veber, D. F.; Curley, P. E.; Saperstein, R.; Hirschmann, R. *Intl. J. Pept. Protein Res.* **1983**, *21*, 66 and references therein. (a) Cf. Diaz, J.; Guegan, R.; Beaumont, M.; Benoit, J.; Clement, J.; Fauchard, C.; Galtier, D.; Millan, J.; Muneaux, C.; Muneaux, Y.; Vedel, M.; Schwyzer, R. *Bioorg. Chem.* **1979**, *8*, 429. See also: Rivier, J.; Kaiser, R.; Galyean, R. *Biopolymers* **1978**, *17*, 1927.

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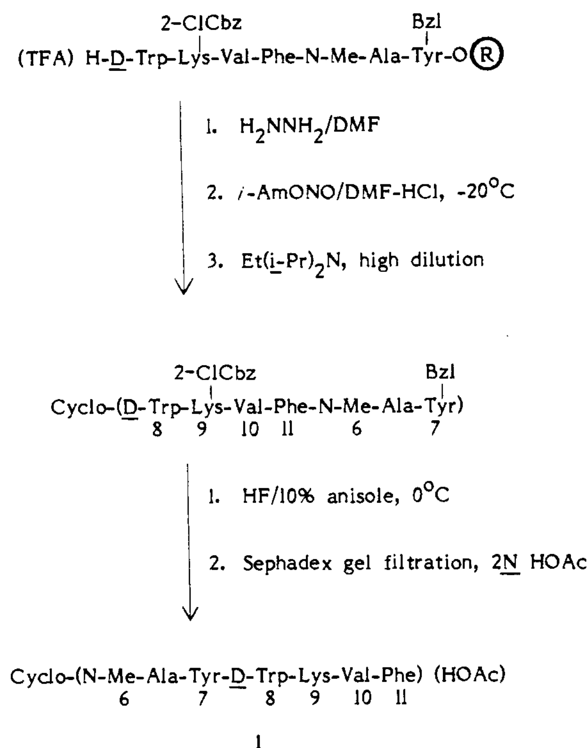

Figure 1. Synthesis of cyclic hexapeptide somatostatin analogues by combined solid phase/solution approach.[3]

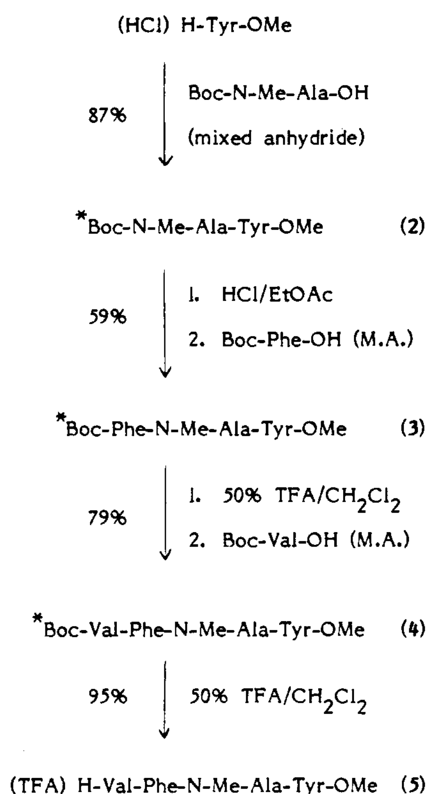

Figure 2. Synthesis of the C-terminal linear tetrapeptide fragment.

Synthesis of Linear Precursor. Since tyrosine was situated at the carboxy terminus of the target sequence, we were able to have an immediate test of the reactivity of the tyrosine hydroxyl during coupling. As anticipated, with the use of stoichiometric quantities of reagents, the mixed anhydride reaction was highly selective for α-amino over hydroxyl, and tetrapeptide **4** was readily assembled in stepwise fashion (Figure 2). In practice, both the re-

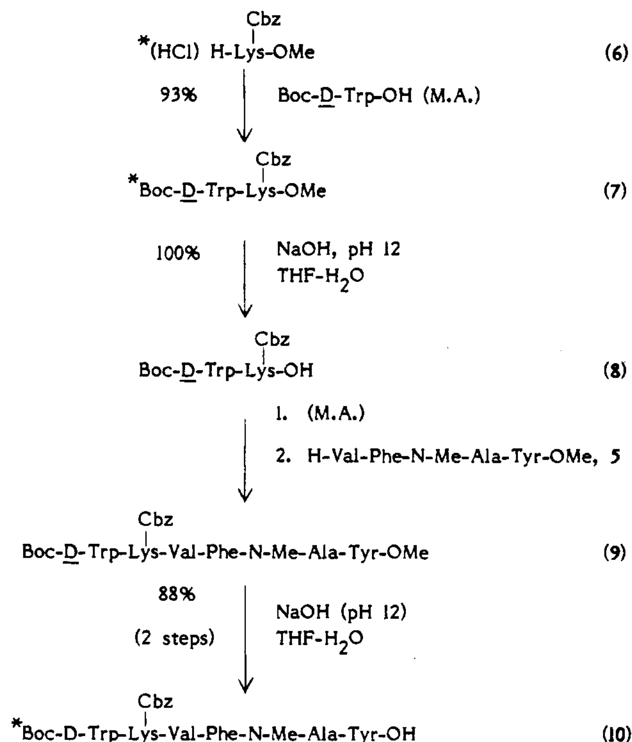

Figure 3. Synthesis of linear hexapeptide.

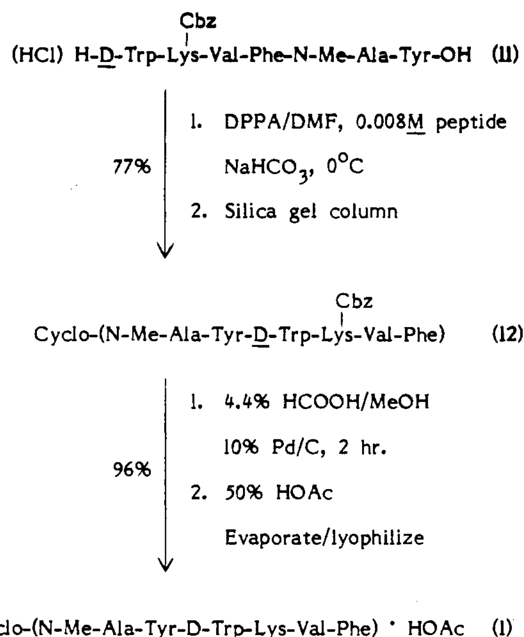

Figure 4. Cyclization and purification.

quirement for only minimal excesses of reagents during coupling and the overall simplicity of operation (convenient batch scaleup, extractive workups) proved to be important advantages. Intermediates **2**, **3**, and **4** were purified by crystallization, which greatly facilitated execution of this sequence on a large scale, and all crystalline products were obtained in purity >98%, as determined by HPLC. The somewhat lower yield in the second coupling step (Boc-Phe-OH onto *N*-methylalanyl dipeptide (cf. **3**) was not entirely unexpected, assuming some steric hindrance to acylation (see above),[8] and unreacted dipeptide was determined to be present to the extent of about 10%. Sig-

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nificantly, at no point in the sequence was O-acylation at tyrosine detected. Both the HCl and TFA methods were explored for each Boc removal, and the superior approach was chosen in each instance. Thus, in the case of tripeptide **3**, TFA was preferred because the resulting TFA salt was found to dissolve more readily in the next coupling reaction solution, whereas it was HCl treatment of dipeptide **2** that afforded a purer (crystalline) product than did the TFA procedure. Finally, we determined that when Boc removal from peptide **4** was conducted with HCl a more polar impurity (see Experimental Section) not seen when TFA was used, was evident. We did not pursue isolation of this material, but the possibility of racemization at the *N*-methylalanyl residue is not unreasonable on the basis of literature reports of racemization of *N*-methyl amino acid residues during exposure to strongly acidic conditions.[9]

Continuation of the stepwise incorporation sequence by reaction of TFA salt **5** with Boc-ϵ-Cbz-lysine to give pentapeptide product was explored, but due to its failure to crystallize, this product would have required column chromatography for purification. Hence, the alternative (2 + 4) approach to hexapeptide **9** via dipeptide **8** was evaluated with excellent results, as shown in Figure 3. The crystallinity of dipeptide ester **7** allowed its isolation in high yield and purity, and saponification provided pure acid **8** in quantitative yield. Mixed anhydride coupling of fragments **5** and **8** proceeded, as expected under the carefully controlled activation/coupling conditions,[10] without discernible racemization at lysine. Our efforts to crystallize hexapeptide methyl ester **9** were not successful, but the acid **10** could be crystallized in a straightforward fashion to provide the fully assembled linear precursor three steps from the end in better than 98% purity.

Removal of Boc from peptide **10** to give HCl salt **11** (Figure 4) was accomplished under conditions shown to limit loss of Cbz to <5%. A factor of key importance in minimizing this side reaction was keeping the internal temperature below −10 °C throughout the operation and allowing no more than 30 min for N_2 purging of the HCl.

Cyclization. The stage was now set for cyclization of the HCl salt **11**, and this step was accomplished by adaptation of a procedure previously worked out in these laboratories entailing the use of the activating reagent diphenylphosphoryl azide (DPPA). Our experience with this reagent, which probably acts to form an acyl azide under mild conditions of carboxyl activation, suggested that it would leave the tyrosine hydroxyl untouched. A second important aim in our development effort was to be able to cyclize the linear sequence in high monomer yield while minimizing solvent volume under scale-up conditions. The conditions shown (see Figure 4) conform to those described in an earlier report,[11] except for the replacement of triethylamine as base with solid sodium bicarbonate.[6] In probe experiments we showed this substitution to effect a significant improvement in yield relative to triethylamine, about 90% monomer (**12**) being obtained, as determined by analytical gel filtration. In contrast, optimized conditions with triethylamine had been shown to give about 75% monomer.[6] As in the prior work[11] mixed acid–base ion exchange resin was employed to sequester charged byproducts. Passage through a silica gel column (the sole chromatographic procedure required during the entire synthesis) was used to separate a small amount of closely running byproduct (the D-Tyr isomer of **12**) as well as other minor components, to afford a 77% recovered yield of product **12** of >99% purity by HPLC. The rapidity and convenience of this chromatography on a large scale are noteworthy; up to 50 g of crude material could be purified on a column of 2.5 kg of adsorbent, with excellent recovery.

We feel the key advantage of bicarbonate in the cyclization lies in its *insolubility* in the reaction medium,[12] wherein a low pH of the bulk solution is maintained while at the same time acid is neutralized as it is formed during the course of the reaction. The advantages are twofold: (1) polymer formation and side reactions that may occur at the higher solution pH unavoidable with soluble base are suppressed and (2) being able to use excess insoluble base at the outset eliminates the need for frequent monitoring and periodic base addition. An important point is that the two-phase reaction requires efficient stirring to go to completion. Overall, the successful use of this modified procedure in numerous instances has convinced us that it is a valuable technique not only for cyclizations but also for peptide couplings generally, especially where mild conditions are needed.

Minimal racemization (est. <0.5%) at tyrosine was observed during the cyclization. The column chromatography served to partially purify the diastereoisomeric byproduct, which then was isolated in >90% purity from passage of enriched silica gel side cuts through Sephadex gel filtration, applied in a recycling mode.[13] This material was fully characterized by NMR and mass spectral analysis, and chirality of tyrosine was established by known methods (see Experimental Section). The finding of a low degree of racemization in this work is consistent with previous results with DPPA and soluble base.[11]

With penultimate intermediate **12** on hand, it remained to perform a single deprotection, removal of Cbz from lysine, to complete the synthesis. Initially we explored a number of methods for this purpose, including (1) metal–ammonia reduction (Na, Li, Ca); (2) trifluoroacetic acid with scavengers; (3) treatment with trimethylsilyl iodide (Me_3SiI).[14] The sodium–ammonia cleavage was unsatisfactory due to a major byproduct arising from opening the ring between Phe and *N*-Me-Ala (with reduction of Phe), a side reaction analogous to cleavage of the X-prolyl amide bond under reductive conditions.[15] Lithium likewise opened the ring, but to a lesser extent, while calcium took off Cbz too slowly to be practical. Removal in both TFA–ethanedithiol and Me_3SiI required treatment for extended periods, and numerous byproducts were seen.

It was the method of catalytic hydrogenolysis under "transfer" conditions that met the need for swift quantitative removal of Cbz and allowed recovery of product in purity essentially equivalent to that of the starting material. Thus, with 5% formic acid in methanol[16] a 1:4 (w/w) catalyst (10% Pd–charcoal)/substrate ratio sufficed for complete protecting group removal within 2 h. Other mediators (1,3-cyclohexadiene,[17] ammonium formate[18])

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(13) *Sephadex: Gel Filtration in Theory and Practice*; Pharmacia Fine Chemicals, Inc.: Uppsala, 1972; p 48.
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(15) Schön, I. *Chem. Rev.* **1984**, *84*, 287.
(16) El Amin, B.; Anantharamaiah, G. M; Royer, G. P.; Means, G. E. *J. Org. Chem.* **1979**, *44*, 3442.
(17) Felix, A. M.; Heimer, E. P.; Lambros, T. J.; Tzougraki, C.; Meienhofer, J. *J. Org. Chem.* **1978**, *43*, 4194.

were less satisfactory, with incomplete cleavage being observed, even after prolonged reaction times. Significantly, neither formylation nor reduction of the indole of D-tryptophan were seen. Isolation of final product 1 was accomplished by an uncomplicated workup procedure, in which residual formate was replaced with acetate simply by entrainment in vacuo with 50% acetic acid. The acetate salt was obtained after lyophilization, in nearly quantitative yield.

Each of our isolated peptide intermediates was characterized by TLC, HPLC, optical rotation, and amino acid analysis. Nuclear magnetic resonance (proton) was especially useful for characterization of the protected cyclic product (12) and final product (1).

Conclusions. In summary, we have accomplished the preparation of a novel peptide analogue of somatostatin in yields as follows, based upon each of the six amino acid precursors: 23% (11 steps, (HCl) H-Tyr-OMe); 17% (12 steps, Boc-Ala-OH); 23% (9 steps, Boc-Phe-OH); 49% (7 steps, Boc-Val-OH); 49% (7 steps, (HCl) H-Lys(Cbz)-OMe); 51% (7 steps, Boc-D-Trp-OH). This breakdown illustrates the high efficiency of the overall process with respect to each of the constituent residues, permitting an evaluation of synthetic economy for usage of each amino acid. The synthetic protocols described above have proven amenable to scale-up to over 100-g quantities. Purification of key intermediates by crystallization and the need for only a single chromatographic procedure, are key favorable factors in this synthesis. Finally, improvements in cyclization methodology have been implemented to the point of general usefulness in peptide synthesis. The process serves as an example of the kinds of considerations that are important in large-scale synthesis of peptides and that may be of general relevance in solving problems encountered in large-scale operations in peptide chemistry.

Experimental Section

Melting points, determined on a Thomas-Hoover apparatus, are uncorrected. Spectra were obtained as follows: mass spectra by FD on a Finnegen-MAT 731 spectrometer, ^{1}H NMR spectra on a Nicolet NT360 spectrometer with δ reported in ppm relative to Me_4Si, UV spectra on a Cary 15 recording spectrophotometer, CD spectra on a Jasco J-41A spectropolarimeter. HPLC analyses were carried out on a Hewlett-Packard 1084-B liquid chromatography instrument with a Du Pont ODS column and a pH 3.2 TMA–phosphate buffer–acetonitrile or –methanol gradient elution system.

TLC analysis was performed on 250 mm 5 × 20 cm silica gel plates (Analtech) by using ultraviolet light and/or *tert*-butyl hypochlorite/starch–iodine spray for visualization. Solvent systems employed were EtOAc:HOAc:2,2,4:trimethylpentane (isooctane):H_2O, upper layer (EAIW); $CHCl_3$:CH_3OH:H_2O (CMW); $CHCl_3$:CH_3OH:concentrated NH_4OH (CMA); EtOAc:pyridine:HOAc:H_2O (EPAW); 1-butanol:HOAc:H_2O (BAW). Ratios (v/v) are as indicated in the experiment. Silica gel used for preparative scale chromatography was silica gel 60 (E. Merck, 230–400 mesh).

Amino acids and protected derivatives were all of the L configuration and obtained from commercial sources, unless otherwise indicated. ϵ-Cbz lysine methyl ester (6) was prepared in two steps from lysine hydrochloride by adaptation of published procedures.[19] *t*-Boc-*N*-methylalanine was prepared from *t*-Boc-alanine by the conventional methylation procedure using sodium hydride.[20]

Products indicated by asterisk (*) in Figures 2–4 were crystalline, as defined by birefringence under a polarizing microscope.

Boc-*N*-Me-Ala-Tyr-OMe (2). To a solution of 37.6 mL (39.6 g) of isobutyl chloroformate in 1 L of EtOAc, cooled to −5 °C under a slow stream of N_2, was added 31.9 mL (29.3 g) of *N*-methylmorpholine, which resulted in the formation of a sticky white solid.[21] After 10 min, 58.9 g (0.290 mol) of Boc-*N*-methylalanine, mp 91–92 °C, was added. The tacky solid dispersed within 5 min and after about 15 min, 57.9 g (0.196 mol) of tyrosine methyl ester HCl salt (Bachem) was added, followed by about 27 mL of *N*-methylmorpholine. The coupling, monitored by TLC (90:10:1, CMW), was complete within 15 min. After about 2 h the solution was washed with 1 N $NaHCO_3$ and then 0.5 M citric acid[22] and dried over anhydrous Na_2SO_4. Concentration in vacuo and crystallization from EtOAc gave 82.4 g (87%) of white crystals: mp 124–125 °C; $[\alpha]^{24}_D$ −34.7° (*c* 0.75, MeOH); TLC R_f 0.5 (90:10:1, CMW); HPLC 99.0%; ^{1}H NMR ($CDCl_3$) δ 1.27 (d, 7.0) (*N*MeAla$^\beta$CH$_3$), 1.45 (s) (*t*-Boc CH$_3$), 2.60 (s) (NCH$_3$), 3.00 (m) (Tyr$^\beta$CH$_2$), 3.73 (s) (OCH$_3$), 4.72 (m) (NMeAla, Tyr$^\alpha$CH).

Anal. Calcd for $C_{19}H_{28}N_2O_6$ (380.4): C, 59.99; H, 7.42; N, 7.36. Found: C, 59.64; H, 7.46; N, 7.68.

Boc-Phe-*N*-Me-Ala-Tyr-OMe (3). (a) Removal of Boc from Dipeptide 2. A solution of 82.2 g (0.216 mol) of methyl ester 2 in 1270 mL of EtOAc was cooled to below −30 °C. Gaseous HCl was then introduced at such a rate that the temperature of the reaction mixture was between 0 and −10 °C at saturation.[23] The solution was kept about 5 min at saturation and then purged with N_2 for about 30 min, maintaining the temperature at −10 °C. Addition of 150 mL of ether, followed by 750 mL of hexane, afforded a white precipitate, which after 5 min, was isolated by filtration and washed quickly three times with EtOAc/hexane (1:5 v/v). The solid was dried in vacuo to give 68.5 g (90% yield) of crystalline dipeptide HCl salt, mp 140–142 °C; TLC R_f 0.3 (90:10:1, CMA); HPLC 99.8%.

(b) Coupling with Boc-Phe-OH. To a solution of 30.7 mL (32.3 g) of isobutyl chloroformate in 1 L of EtOAc, cooled to −5 °C, was added 26.0 mL (23.9 g) of *N*-methylmorpholine. A sticky solid formed (see above), and then 62.7 g (0.236 mol) of *t*-Boc phenylalanine was added as a solid and, after 15 min, 68.0 g (0.215 mol) of crystalline dipeptide ester (HCl) H-*N*-Me-Ala-Tyr-OMe. An additional 30 mL of *N*-methylmorpholine was added (reaction mixture at "pH 7.5", as determined with moistened E. Merck narrow-range pH sticks). After 4 h, TLC (90:10:1, CMA) still showed a small amount of starting dipeptide. Workup proceeded as with the preparation of 2 to give, upon crystallization from EtOAc, 73.9 g (65% yield) of white crystals: mp 145–146 °C; $[\alpha]^{25}_D$ −24.3° (*c* 0.72, MeOH); TLC R_f 0.6 (90:10:1, CMW), HPLC 98.8%; ^{1}H NMR ($CDCl_3$) δ 1.28 (s) (*t*-Boc CH$_3$), 1.32 (d, 7.0) (NMeAla$^\beta$CH$_3$), 2.20/2.60 (s, ca. 4:1) (NCH$_3$), 3.72 (s) (OCH$_3$), 4.42, 4.72 (m) (Phe, NMeAla, Tyr$^\alpha$CH), 5.45 (d, 6.0) (*t*-BocNH), 6.93, 7.27 (m) (Phe, Tyr aromatic).

Anal. Calcd for $C_{28}H_{37}N_3O_7$ (527.6): C, 63.74; H, 7.07; N, 7.96. Found: C, 63.76; H, 7.25; N, 8.25.

Incompletely dissolved starting dipeptide was recovered by filtration of the reaction mixture and amounted to 6.3 g (9.3%).

Boc-Val-Phe-*N*-Me-Ala-Tyr-OMe (4). (a) Removal of Boc from Tripeptide 3. A solution of 55.8 g (0.106 mol) of methyl ester 3 in 300 mL of CH_2Cl_2, cooled to 5 °C in an ice bath, was treated (stirring) with 300 mL of 100% TFA. After 35 min at 0–5 °C, the solution was poured all at once into 2.4 L of ether (precooled to −20 °C), with vigorous stirring. To the resulting precipitate was added 2.5 L of petroleum ether (30–60 °C), and the mixture was allowed to stand 30 min at 0 °C and then filtered; and the solid was washed three times with 1:1 ether/petroleum ether and dried in vacuo to give 48.6 g (85% yield) of tripeptide TFA salt, an amorphous solid; TLC R_f 0.6 (90:10:1, CMA).

(b) Coupling with Boc-Val-OH. A solution of 21.5 g (0.099 mol) of Boc-valine in 400 mL of EtOAc was cooled to −5 °C and treated with 10.9 mL of *N*-methylmorpholine, followed by 12.8 mL (13.5 g) of isobutyl chloroformate. The mixture, which became slightly cloudy upon addition of the chloroformate, was stirred

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(19) Coggins, J.: Demayo, R.; Benoiton, L. *Can. J. Chem.* **1970**, *48*, 385.
(20) Cheung, S. T.; Benoiton, L. *Can. J. Chem.* **1977**, *55*, 906.

(21) The difficulty of stirring caused by this gummy solid prompted us to change the order of reagent addition in later mixed anhydride couplings, to: (1) Boc amino acid; (2) *N*-methylmorpholine; (3) isobutyl chloroformate.
(22) The order of basic and acidic washes was reversed in later couplings to avoid residual citric acid in the product.
(23) The considerable heat of solution of HCl gas in EtOAc results in the observed 20–30 °C temperature rise during HCl addition.

for 10 min; then 48.6 g (0.090 mol) of solid tripeptide ester (TFA) H-Phe-*N*-Me-Ala-Tyr-OMe was introduced, followed immediately by another 10.9 mL of *N*-methylmorpholine (reaction mixture at "pH 7", see above). After 1.5 h, TLC (90:10:1, CMA) showed complete reaction, and 200 mL of water was added. After standing overnight, the aqueous layer was drawn off and the EtOAc solution washed in turn with three portions each of 0.5 M citric acid and 1 N $NaHCO_3$ and once with water and saturated NaCl, then dried over anhydrous $MgSO_4$, and evaporated at reduced pressure to a volume of approximately 200 mL. The product came out gradually as a white crystalline solid: mp 115–125 °C, yield 52.4 g (93%); TLC R_f 0.5 (90:10:1, CMW), 0.6 (9:2:5:10, EAIW); HPLC 93.1% (15.2 min).

Recrystallization from EtOAc gave a sample 97.3% pure by HPLC; mp 130–136 °C dec; $[\alpha]^{25}_D$ −63.8° (*c* 0.56, MeOH).

Amino acid analysis: $Val_{0.99}Tyr_{1.00}Phe_{1.02}$.

Anal. Calcd for $C_{33}H_{46}N_4O_8$ (626.8): C, 63.24; H, 7.40; N, 8.94. Found: C, 63.07; H, 7.54; N, 8.98.

^1H NMR (CD_3OD) δ 0.23, 1.23 (2 d, 7.0) (NMeAla$^{\beta}CH_3$), 1.43, 1.47 (2 s) (*t*-Boc), 1.90, 2.05 (2 m) (Val$^{\beta}$CH), 2.13, 2.71 (2 s) (NCH_3), 3.70, 3.72 (2 s) (OCH_3), 3.81, 3.93 (2 d, 8.0) (Val$^{\alpha}$CH), 4.5, 4.6, 5.0 (m) (Phe, NMeAla, Tyr$^{\alpha}$CH), 6.65 (d, 8.5) (Tyr $H_{3,5}$), 6.98 (d, 8.5) (Tyr $H_{2,6}$).

(TFA) H-Val-Phe-*N*-Me-Ala-Tyr-OMe (5). A sample of 41.6 g (0.066 mol) of methyl ester **4**, suspended in 190 mL of CH_2Cl_2 in a 1-L Ehrlenmeyer flask, was cooled to 5 °C and treated with 190 mL of TFA, followed by stirring at 0–5 °C for 35 min (solution complete in <1 min). The reaction mixture was then poured rapidly into 1500 mL of briskly stirred ether (precooled to −20 °C), whereupon the product came out as a white solid. After 5 min, 1500 mL of petroleum ether (30–60 °C) was added, and the precipitate was let settle for 30 min at 0 °C and then collected by filtration and washed three times with 1:1 ether/petroleum ether (30–60 °C). Drying in vacuo gave 40.3 g (94.8% yield) of amorphous product. TLC R_f 0.2 (12:2:2:10, EAIW), 0.5 (90:10:1, CMA), HPLC 97% (9.7 min).

Amino acid analysis: $Val_{1.01}Tyr_{0.99}Phe_{0.99}$.

When the Boc removal was done using HCl in EtOAc (see above), a significant impurity was seen on HPLC (2.8 min) and TLC (R_f 0.15 (12:2:2:10, EAIW)), to the extent of about 10%. Identification of this contaminant, none of which was evident when TFA was used, was not pursued.

Boc-D-Trp-Lys(Cbz)-OMe (7). A sample of 39.5 g (0.130 mol) of Boc-D-tryptophan suspended (part-solution) in 730 mL of CH_2Cl_2 was treated with 14.4 mL (13.2 g, 0.13 mol) of *N*-methylmorpholine. Complete dissolution occurred during the base addition, and the solution was treated, after cooling to −5 °C, with 16.9 mL (17.8 g, 0.13 mol) of isobutyl chloroformate over 1–2 min. Ten minutes later, 38.4 g (0.116 mol) of solid N^{ϵ}-(Cbz)lysine methyl ester HCl salt (**6**)[19] was added, followed by 14.8 mL of *N*-methylmorpholine all at once, and ca. 3 mL in increments (to pH 7.2 on moist pH paper). TLC indicated nearly complete absence of starting ester (R_f 0.6, 90:10:1, CMA). After 2 h, 100 mL of H_2O was added and the mixture was stirred overnight. The layers were separated and the organic phase was washed with water, twice with 0.5 M citric acid, once with water, three times with 0.5 N $KHCO_3$, then water, and saturated NaCl, drying over anhydrous $MgSO_4$ (all aqueous extracts were back-washed with CH_2Cl_2). Solvent removal in vacuo afforded a gummy residue, which was crystallized from approximately 700 mL of hot (55–60 °C) $MeOH/H_2O$ (ca. 4:1 v/v). After standing overnight the solid was collected, washed with 2:1 $MeOH/H_2O$, and dried in vacuo to give 62.3 g (92.5% yield) of needles: mp 141–142 °C; TLC R_f 0.6 (9:2:5:10, EAIW), 0.73 (90:10:1, CMA); HPLC 98.5% (17.1 min); ^1H NMR (CD_3OD) δ 5.05 (s, $C_6H_5CH_2$), 4.23 (m, Lys$^{\alpha}$CH), 4.38 (t, Trp$^{\alpha}$CH), 3.00 (t, Lys CH_2), 3.18 (d,d, Trp$^{\beta}CH_2$), 3.65 (s, CH_3O); $[\alpha]^{25}_D$ −13.4° (*c* 0.73, MeOH).

Anal. Calcd for $C_{31}H_{40}N_4O_7$ (580.7): C, 64.12; H, 6.94; N, 9.65. Found: C, 64.07; H, 7.28; N, 10.03.

Boc-D-Trp-Lys(Cbz)-OH (8). To a solution of 57.1 g (0.098 mol) of dipeptide ester **7** in 800 mL of THF (peroxide-free) was added 400 mL of water; then 2.5 N NaOH was added to pH 11 (pH meter, calibrated with 1:1 THF/pH 10.00 buffer standard). The pH was maintained at 10.5–11.0, and TLC (90:10:1, CMW) indicated completion by approximately 6 h. The solution was neutralized with 3 N HCl and concentrated under reduced pressure to remove most of the THF, followed by partition with 700 mL of EtOAc and portionwise shaking with 2.5 N HCl until the aqueous layer was about pH 3. The organic layer was washed with water, 50% saturated NaCl, and saturated NaCl (each was back-extracted with EtOAc), dried over anhydrous $MgSO_4$, and evaporated under reduced pressure to give the crude acid as a stiff foam, which afforded 57.2 g (100% yield) of white amorphous solid upon trituration: TLC R_f 0.6 (80:25:5, CMA), 0.5 (9:2:5:10, EAIW), 0.7 (40:5:1:1, EPAW); HPLC 97.7% (14.8 min). A sample was obtained crystalline from CH_2Cl_2:ether: mp 90–95 °C dec; $[\alpha]^{25}_D$ −3.0° (*c* 1.09, MeOH).

Anal. Calcd for $C_{30}H_{38}N_4O_7$ (566.6): C, 63.59; H, 6.76; N, 9.65. Found: C, 63.18; H, 7.06; N, 9.28.

Boc-D-Trp-Lys(Cbz)-Val-Phe-*N*-Me-Ala-Tyr-OH (10). (a) Coupling of Dipeptide Acid 8 with Tetrapeptide Ester 5. A solution of 44.2 g (0.078 mol) of dipeptide **8** in 750 mL of EtOAc was treated with 8.3 mL (7.5 g, 0.075 mol) of *N*-methylmorpholine and then cooled to −5 °C. To this mixture was added 9.7 mL (10.2 g, 0.075 mol) of isobutyl chloroformate, followed by stirring with the reaction temperature below −5 °C for another 12 min (some white precipitate formed). Then a solution of 46 g (0.072 mol) of tetrapeptide ester TFA salt (**5**) in 150 mL of DMF plus 6.5 mL of *N*-methylmorpholine was added at such a rate that the temperature stayed below −5 °C (two 30-mL 1:1 EtOAc:DMF rinses used). Additional *N*-methylmorpholine (3.5 mL) was added portionwise to a final pH ca. 7.5. TLC after 1.5 h showed small amounts (est. <5%) of both starting materials **5** and **8** remaining (9:2:5:10, EAIW; 90:10:1, CMW), neither of which completely disappeared on continued stirring. The mixture was treated with 600 mL of water and 200 mL of EtOAc for partition; the organic phase washed with 400 mL each of 0.3 M citric acid, H_2O, 0.5 M $KHCO_3$ (2×), H_2O, and 50% saturated NaCl and dried over anhydrous Na_2SO_4. Evaporation afforded a stiff foam, which was triturated to give a white solid, 79.8 g (100% yield): R_f 0.4 (9:2:5:10, EAIW), 0.6 (90:10:1, CMW). This crude ester (**9**) was saponified directly.

(b) Saponification of Methyl Ester 9. A composite sample of 158 g (ca. 0.14 mol) of crude ester **9** was dissolved in 1300 mL of THF. Water (650 mL) was added, followed by 2.5 N NaOH to effect saponification, running the reaction under conditions parallel to those described for dipeptide ester **7**. After acidification to pH 3 (2.5 N HCl) and evacuation to a volume of ca. 1 L, a gummy residue had separated. The mixture was partitioned with 1800 mL of EtOAc and 500 mL of H_2O, adjusting the aqueous layer to pH <3. The EtOAc was washed with 800-mL portions of H_2O (2×) and 50% saturated NaCl, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure to a volume of ca. 600 mL. Another 800 mL of EtOAc was added, followed by CH_2Cl_2 with continuous swirling, to induce crystallization (up to 2 L of CH_2Cl_2). After allowing the thick mixture to stand several hours at 0 °C, the product was collected by filtration, pressed into a compact bed, and washed through with two 500-mL portions of CH_2Cl_2:EtOAc (2:1 v/v). The solid mass was dried in vacuo to give 118.9 g (80% yield) of white microcrystalline solid: mp 120–135 °C dec; TLC R_f 0.4 (10:2:4:10, EAIW), 0.4 (90:10:1:1, CMWA), 0.6 (75:25:2.5, CMA); ^1H NMR (CD_3OD) δ 3.32 (s) (NMeAla NCH_3), 0.17 (d, 5.0) (NMeAla$^{\beta}CH_3$); HPLC 99.1% (17.0 min); $[\alpha]^{25}_D$ −55.7° (*c* 0.70, MeOH).

Anal. Calcd for $C_{61}H_{80}N_8O_{14}$ (1061.2 + EtOAc): C, 63.75; H, 7.02; N, 9.75. Found: C, 63.35; H, 7.25; N, 9.62.

Amino acid analysis: $Lys_{0.98}Val_{0.99}Tyr_{1.01}Phe_{1.03}$.

Further product was obtained by concentration of the mother liquor, dissolving the residue in 250 mL of EtOAc and proceeding as above to give 18.0 g (12% yield) of second crop: mp 115–135 °C dec; HPLC 99.3% (17.0 min).

***cyclo*-(D-Trp-Lys(Cbz)-Val-Phe-*N*-Me-Ala-Tyr) (12). (a) Boc Removal from Hexapeptide 10.** A solution of 68.4 g (0.060 mol) of hexapeptide acid **10** in 1100 mL of EtOAc was cooled to −50 °C and treated with HCl gas (under rapid mechanical stirring) at such a rate that the internal temperature reached −10 °C in about 15 min (approximately saturation). After 10-min saturation, at ≤−10 °C, N_2 was introduced to vigorously purge most of the HCl, while the temperature was kept at −10 °C using an ice bath. After about 45 min 1000 mL of cold (0 °C) ether was added to cause precipitation of the product HCl salt (**11**). The solid was collected by filtration, washed three times with ether, and dried

in vacuo to yield 56.5 g (88%) of white solid: TLC R_f 0.5 (80:20:2, CMW), 0.4 (75:25:2.5, CMA), 0.5 (15:5:1:1, EPAW); HPLC ca. 94% (14.6 min).[24]

Amino acid analysis: $Lys_{1.01}Val_{0.99}Tyr_{0.99}Phe_{1.01}$.

A second portion, deposited from the filtrate, was similarly isolated to afford another 3.5 g (5.5%) of HCl salt **11**, of comparable quality to the main batch.

Samples of this intermediate were found to contain, despite exhaustive drying under vacuum, an average of about 15% of excess chloride (HCl) based on elemental analysis. The material was submitted to cyclization without further purification.

(b) Cyclization of Hexapeptide 11. A solution of 24.0 g (24.1 mmol) of HCl salt **11** in 3 L of DMF (degassed) was cooled to 0 °C under a stream of N_2, and 5.71 mL (equivalent to 7.29 g, 26.5 mmol) of diphenylphosphoryl azide (DPPA) was slowly added, followed by 10.1 g (120 mmol) of solid $NaHCO_3$. The mixture was stirred briskly for 3 days at 0–5 °C, at which time TLC (EPAW, 15:5:1:1) showed <2% of starting peptide **11** left. Then 750 mL of H_2O was added and the mixture was combined with four other similarly processed 24-g runs for treatment in a 5-gal polypropylene carboy, with 5 lb of Bio-Rad AG501-X8D mixed bed (indicating) ion exchange resin, stirring 3 h. The resin was separated by filtration and washed with 4:1 DMF/H_2O, and the combined filtrate was concentrated in vacuo to give a viscous oil. Addition of 500 mL of water and trituration afforded white solid, which on isolation by filtration gave 111.0 g (97.8% yield) of crude cyclic peptide **12**.

(c) Purification of 12. The crude product was chromatographed in two equal portions, each charged through a column of 2.5 kg of silica gel packed in 7 L of $CHCl_3$ (bed dimensions 13.5 × 55 cm) and washed with 500 mL of 98:2 $CHCl_3$:CH_3OH; eluting with 7 L each of 98:2, 97:3, and 96:4. Product was collected in 1-L fractions from 96:4, monitoring by TLC (CMW, 90:10:1) and HPLC. The major product, R_f 0.72, was followed by fractions containing increasing amounts of an impurity, R_f 0.62. Fractions containing a main product of ≥99% purity (HPLC) were pooled and concentrated in vacuo; the residue was dissolved in methanol and evaporated to an oil three times. Addition of water then brought down white solid, which was triturated and isolated by filtration, washed thoroughly with water, and dried in vacuo. The combined total yield, including material obtained on reprocessing of less pure fractions, was 86.9 g (76.6%): TLC R_f 0.7 (90:10:1, CMW), 0.75 (60:5:1:1, EPAW); HPLC 99.3% (15.7 min); ^1H NMR (CD_3OD) δ 7.02 (d, 9.0) (Tyr $H_{2,6}$), 6.72 (d, 9.0) (Tyr $H_{3,5}$), 6.94 (s) (Trp H_2), 5.07 (s) (Cbz CH_2), 4.60 (t, 9.0) (Tyr$^\alpha$CH), 4.78 (q, ~7.0) (NMeAla$^\alpha$CH), 4.85, 4.38 (d/d) (Phe, Trp$^\alpha$CH), 4.45 (d, 6.0) (Val$^\alpha$CH), 3.82 (d/d, ~11.0, 3.0) (Lys$^\alpha$CH), 2.33 (s) (NCH_3), 2.08 (m) (Val$^\beta$CH), 0.95 (d, ~7.0), 0.88 (d, ~7.0) (Val CH_3), 0.6 (env.) Lys$^\gamma CH_2$, 0.30 (d 6.8) (NMeAla$^\beta CH_3$); $[\alpha]^{25}_D$ −70.0° (c 0.82, MeOH).

Anal. Calcd for $C_{52}H_{66}N_8O_{11}$ (979.1 + $2H_2O$): C, 63.79; H, 6.79; N, 11.44. Found: C, 63.67; H, 6.88; N, 11.30.

Amino acid analysis: $Lys_{1.02}Val_{1.01}Tyr_{0.98}Phe_{0.99}$.

(d) Isolation of D-Tyr Isomer of 12. Combined fractions from the large scale silica gel chromatography shown to be enriched in the more polar impurity (HPLC 75% impurity vs. 25% **12**) were charged in 1:1 DMF/50% HOAc to a column of Sephadex G25F set to recycle the eluant (50% HOAc).[13] Collection of fractions was begun after the fifth pass, with the impurity emerging in the earlier fractions, followed by cyclic product **12**. The most enriched fractions were concentrated in vacuo, and water was added for trituration; filtration and drying in vacuo afforded 61 mg of white solid: TLC R_f 0.5 (60:5:1:1, EPAW); HPLC 92% (15.3 min), 5% of **12** (15.6 min); mass spectrum m/e 1076 (M^+). CD (100% trifluoroethanol) showed a similar profile to spectra of a number of other D position-7 analogues,[25] particularly in having a strong positive band at 220 nm and a strong negative band at 198 nm, both of which were absent in the spectrum of compound **12**. The ^1H NMR spectrum (CD_3OD) was confirmatory of all expected structural features, with minor shifts in certain signals relative to the L-Tyr isomer **12**: δ 3.67 (d/d) (Lys$^\alpha$CH), 2.56 (s) (NCH_3), 1.91 (m) (Val$^\beta$CH), 0.7 (env.) (Lys$^\gamma CH_2$), 0.31 (d, ~7.0) (NMeAla$^\beta CH_3$).

Amino acid analysis: $Lys_{0.98}Val_{1.00}Tyr^{26}_{0.98}Phe_{1.04}$.

***cyclo*-(*N*-Me-Ala-Tyr-D-Trp-Lys-Val-Phe)·HOAc (1).** A sample of 36.2 g (35.7 mmol) of Cbz-protected hexapeptide **12** and 8.1 g of 10% Pd–charcoal (Engelhard Ind.) were blanketed with N_2 and then treated all at once with 1600 mL of 4.4% HCOOH in methanol.[16] After 2.5 h of stirring, TLC (EAIW, 10:2:4:10) showed complete disappearance of starting material, and the reaction mixture was filtered through a Celite pad which had been prewashed first with the reaction solvent, then with methanol. The clear filtrate was evaporated to a volume of ca. 100 mL under water aspirator and then flushed and evaporated three times with 50% acetic acid; followed by dissolution of the residue in *n*-butyl alcohol, decolorization with Darco and filtering through a Celite pad (1-butanol-washed) to give a pale yellow solution. Evaporation of this filtrate afforded a residue which was lyophilized to give 31.4 g of powdery solid.

Combination with similarly prepared lyophilizate from a second run of 33.5 g (33.0 mmol) of Cbz-protected **12** was accomplished by slurrying each batch into 1300 mL of EtOAc and then after 0.5 h, isolating the homogeneous suspended solid by filtration, washing with EtOAc followed by ether, and drying in vacuo to give 61.1 g (96% yield) of nearly white amorphous powder: TLC R_f 0.6 (EPAW, 12:5:1:3), 0.4 (CMA, 70:30:3), 0.4 (BAW, 10:1:1); UV λ_{max} 2 N HOAc 278 nm (ε 6100); HPLC 99.4% (no ε-Cbz precursor **12**) (11.1 min); $[\alpha]^{25}_D$ −65.5° (c 1.07, 0.5 N HOAc); ^1H NMR (COSY) (CD_3OD) δ 7.53 (d, 8.0), 7.38 (d, 7.8) (Trp H_4, H_7), 7.00 (s) (Trp H_2), 7.04 (d, 9.0) (Tyr $H_{2,6}$), 6.72 (d, 9.0) (Tyr $H_{3,5}$), 8.86 (d, 6.0) (Tyr NH, slow-exchanging), 4.60 (d/d) (Tyr$^\alpha$CH), 4.90 (d/d) (Phe$^\alpha$CH), 4.78 (q, ~7.0) (NMeAla$^\alpha$CH), 4.50 (d, 6.0) (Val$^\alpha$CH), 4.37 (d/d, 11.0, 5.1) (Trp$^\alpha$CH), 3.84 (d/d, 11.2, 3.5) (Lys$^\alpha$CH), 3.09 (d/d), 2.86 (d/d) (Trp$^\beta CH_2$), 3.05 (m) (Phe$^\beta CH_2$), 2.87 (m) (Tyr$^\beta CH_2$), 2.60 (m) (Lys$^\epsilon CH_2$), 2.38 (s) (NCH_3), 2.12 (m) (Val$^\beta$CH), 1.95 (s) (CH_3COOH), ~1.7, 1.3 (env.) (Lys$^\delta CH_2$), 0.96 (d, 6.7), 0.91 (d, 6.8) (Val CH_3), 0.55 (env.) (Lys$^\gamma CH_2$), 0.28 (d 6.9) (NMeAla$^\beta CH_3$).

Anal. Calcd for $C_{46}H_{66}N_8O_{12}$ (869.0 + $3H_2O$): C, 59.85; H, 7.21; N, 12.13. Found: C, 60.29; H, 7.10; N, 11.87.

Amino acid analysis: $Lys_{0.99}Val_{0.99}Tyr_{0.99}Phe_{1.01}$ (peptide content = 1.03 μmol/mg sample).

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(24) Approximately 3% of an impurity (9.0 min) was noted as the main single contaminant, presumed to be a small amount of des-Cbz (free Lys) generated during the HCl treatment; in accord with this finding, a more polar spot (R_f <0.1) on TLC (est. ≤5%) was seen.

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(26) Peak corresponding to D-tyrosine found on HPLC of hydrolysate using chiral eluant.