The Use of N^{α},N^{im}-Bis(*tert*-butyloxycarbonyl)histidine and N^{α}-2-(*p*-Biphenylyl)isopropyloxycarbonyl-N^{im}-*tert*-butyloxycarbonylhistidine in the Solid-Phase Synthesis of Histidine-Containing Peptides[1]

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Abstract: Crystalline N^{α}-Bpoc(Im-Boc)histidine and the dicyclohexylamine salt of N^{α}-Boc(Im-Boc)histidine have been synthesized and employed in the solid-phase synthesis of the heptapeptide alanylhistidylarginylleucylhistidylglutaminylleucine (I) which corresponds to positions 17–23 in human growth hormone. Synthesis of I first by N^{α}-Bpoc protection and then by N^{α}-Boc protection led to preparations which were identical with each other. In the case with N^{α}-Boc protection synthesis of I was accomplished both by tosyl and by nitro protection of the arginine side chain; however, use of nitroarginine led to I in diminished yield and the formation of ornithine-containing material.

The protecting groups which have been used with histidine in the solid-phase synthesis[2] of peptides are Im-benzyl,[3] Im-dinitrophenyl,[4] and Im-tosyl.[5] Use of histidine derivatives whose imidazole moiety is unprotected[6,7] will, of course, obviate the problem of deblocking the histidine side chain. However, the coupling of N^{α}-Boc-histidine to the peptide resin is not always successful.[4,8] We now report the use of the Im-Boc group for protection of the side chain of histidine, in conjunction with the N^{α}-Boc and N^{α}-Bpoc groups, and the application of these derivatives to the solid phase synthesis of alanylhistidylarginylleucylhistidylglutaminylleucine (I) corresponding to amino acid residues 17–23 in the human growth hormone molecule.[9,10] In addition, we observed a side reaction in the synthesis of arginine peptides with N^{G}-nitroarginine derivatives.

Synthesis of I with Bpoc Amino Acids. Reaction of the triethylamine salt of Boc-leucine with chloromethylated polystyrene gave Boc-leucyl resin, which was then subjected to the usual procedure for solid-phase peptide synthesis.[2] Coupling was achieved with Bpoc amino acids[11,12] and dicyclohexylcarbodiimide;[13] the exception was glutamine which was coupled to the peptide resin with Bpoc-glutamine *p*-nitrophenyl ester. Side-chain protecting groups employed were the nitro group for the guanidino function of arginine and the *tert*-butyloxycarbonyl group for the imidazole moiety of histidine. The synthesis of N^{α}-Bpoc(Im-Boc)histidine was accomplished by consecutive reactions of histidine with 1 equiv of Bpoc azide and 1 equiv of Boc azide. The resulting derivative was soluble in methylene chloride and coupled well to the peptide resin in this solvent. Deblocking of the Bpoc groups was effected with 0.05–0.10 *N* HCl in chloroform. Collection of the filtrate from the deblocking treatment and measurement of the optical density[11] at 253 mμ indicated the amount of Bpoc moiety which had been cleaved, and afforded a measure of the coupling efficiency in the previous step. For 0.23 mmol of Boc-leucyl resin the results were 0.25, 0.24, 0.26, 0.26, 0.30, and 0.34 mmol of coupling, respectively, for the six successive cycles of synthesis. The significant increase in the last two couplings is probably due to partial deblocking of the histidine side chain during Bpoc cleavage, followed by coupling to the free imidazole group in addition to normal coupling to the terminal amino group.

Treatment of the protected heptapeptide resin with hydrogen fluoride,[14] and purification by chromatography on carboxymethylcellulose[15] (see Figure 1) gave a 54% yield of peptide I. Subjection of this material to partition chromatography on Sephadex G-25[16,17] in the solvent system 1-butanol–ethanol–0.2 *N* aqueous NH_4OH containing 0.04% acetic acid (4:1:5) gave a single symmetrical peak with R_f 0.33 (see Figure 2). This plus other analytical data as described in the Experimental Section gave no evidence of impurity which could have resulted from coupling to the free imidazole group of histidine.

Synthesis of I with Boc Amino Acids. For the synthesis of I employing N^{α}-Boc protection, N^{α}-Boc(Im-Boc)histidine and N^{α}-Boc-N^{G}-tosylarginine were used. The histidine derivative, whose synthesis with the

(1) All amino acids occurring in the peptides mentioned in this paper are of the L configuration. Abbreviations: Boc, *tert*-butyloxycarbonyl; Bpoc, 2-(biphenylyl)isopropyloxycarbonyl; Tos, *p*-toluenesulfonyl.
(2) R. B. Merrifield, *Biochemistry*, 3, 1385 (1964).
(3) (a) G. R. Marshall and R. B. Merrifield, *ibid.*, 4, 2394 (1965); (b) J. Blake and C. H. Li, *J. Amer. Chem. Soc.*, 90, 5882 (1968).
(4) F. Chillemi and R. B. Merrifield, *Biochemistry*, 8, 4344 (1969).
(5) T. Fujii and S. Sakakibara, *Bull. Chem. Soc. Jap.*, 43, 3954 (1970).
(6) B. Gutte and R. B. Merrifield, *J. Amer. Chem. Soc.*, 91, 501 (1969).
(7) A. Loffet, *Experientia*, 23, 406 (1967).
(8) We have not been able to successfully couple N^{α}-Boc-histidine to phenylalanyl resin by the use of *N,N'*-dicyclohexylcarbodiimide (D. Yamashiro, unpublished results).
(9) (a) C. H. Li, J. S. Dixon, and W.-K. Liu, *Arch. Biochem. Biophys.*, 133, 70 (1969); (b) C. H. Li and J. S. Dixon, *ibid.*, 146, 233 (1971).
(10) A preliminary account of the use of N^{α}-Boc(Im-Boc)histidine in the synthesis of an HGH-like protein has previously been reported [C. H. Li and D. Yamishiro, *J. Amer. Chem. Soc.*, 92, 7608 (1970)].
(11) P. Sieber and B. Iselin, *Helv. Chim. Acta*, 51, 622 (1968).
(12) S. Wang and R. B. Merrifield, *Int. J. Protein Res.*, 1, 235 (1969).
(13) J. C. Sheehan and G. P. Hess, *J. Amer. Chem. Soc.*, 77, 1067 (1955).
(14) (a) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc., Jap.*, 40, 2164 (1967); (b) J. Lenard and A. B. Robinson, *J. Amer. Chem. Soc.*, 89, 181 (1967).
(15) E. A. Peterson and H. A. Sober, *ibid.*, 78, 751 (1956).
(16) D. Yamashiro, *Nature (London)*, 201, 76 (1964).
(17) D. Yamashiro, D. Gillessen, and V. du Vigneaud, *J. Amer. Chem. Soc.*, 88, 1310 (1966).

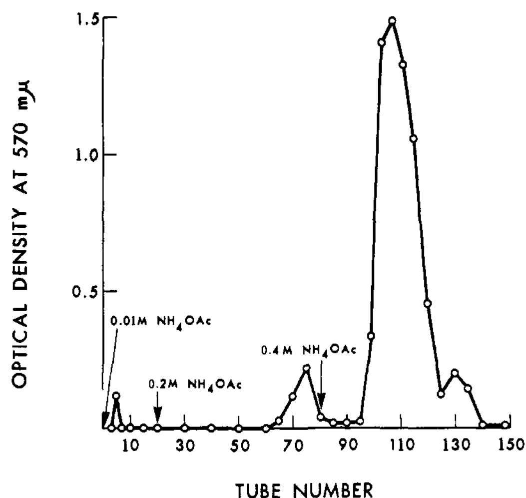

Figure 1. Carboxymethylcellulose chromatography of crude peptide I.

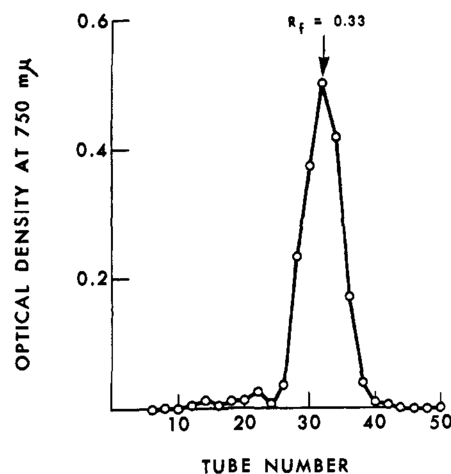

Figure 2. Partition chromatography on Sephadex G-25 of peptide I; optical density measurements by Folin–Lowry analysis.

reagent *tert*-butyloxycarbonyl fluoride has already been described,[18] was obtained in the present work as a crystalline dicyclohexylammonium salt by use of *tert*-butyloxycarbonyl azide in the pH-stat method.[19] N^{α}-Boc-N^{G}-tosylarginine[20] was obtained by an improved procedure involving tosylation of N^{α}-Boc-arginine. This arginine derivative has already been applied to solid-phase synthesis including final detosylation in liquid HF.[21] In the present work removal of the Boc group was carried out in 50% trifluoroacetic acid in dichloromethane[6] for 15 min. Coupling was achieved with dicyclohexylcarbodiimide with the exception of the glutamine residue for which its nitrophenyl ester was used. Coupling of N^{α}-Boc(Im-Boc)-histidine was effected in dichloromethane alone with an efficiency of at least 99% as judged by the test of Esko, Karlsson, and Porath.[22] Cleavage and deprotection of the finished peptide was accomplished in one step with hydrogen fluoride. Purification by carboxymethylcellulose chromatography gave results paralleling those described in the previous synthesis (Figure 1). Partition chromatography of this material, also in the same manner, gave a single symmetrical peak with R_f 0.33 (see Figure 2) and peptide I was isolated in about 63% yield (based on starting resin) in highly purified form as judged by these and subsequent analytical criteria (see Experimental Section).

It was apparent during synthesis that the Im-Boc group was removed in the deblocking steps following attachment of the histidine residues. N^{α}-Boc(Im-Boc)histidine itself is readily converted to free histidine by brief treatment with trifluoroacetic acid. During the synthesis, eluates from the deblocking steps were analyzed after leucine had been coupled to histidine. In each case the Boc amino acid that had been coupled just prior to the deblocking step appeared as the free amino acid in the eluates in amounts ranging from 0.35 to 0.50 mol of amino acid to every mole of leucine originally attached to the resin. Treatment of a portion of the finished protected peptide resin by the same deblocking procedure gave a corresponding molar content of 1.12 of alanine in the eluates. Furthermore, analysis of eluates from the neutralization step that followed the deblocking step gave an additional 0.31 mol of alanine. These data suggest that the Im-Boc group is removed during deprotection with 50% trifluoroacetic acid and that subsequently Boc amino acids alternately couple to the imidazole side chain of histidine and then are removed in the next deblocking and neutralization steps.

Since protection of the guanidino function of arginine with the nitro group is frequently employed in solid-phase synthesis in conjunction with N^{α}-Boc protection, the synthesis of peptide I was also carried out with the use of N^{α}-Boc-N^{G}-nitroarginine. With this exception, the synthesis was conducted in exactly the same manner as in the case with tosyl protection of arginine. Cleavage and deprotection of the finished peptide were again accomplished with hydrogen fluoride, and the product was subjected to carboxymethylcellulose chromatography in the same manner. When material represented by the major peak corresponding to peptide I was submitted for amino acid analysis[23] after acid hydrolysis, an amino acid close to the position of lysine was present along with a low arginine content. The ultraviolet spectrum of the product excluded the possibility of the presence of a nitroarginine residue resulting from incomplete deblocking in hydrogen fluoride. When the product was subjected to partition chromatography under exactly the same conditions previously described, the chromatogram shown in Figure 3 was obtained. Peptide I (R_f 0.33) was isolated in highly purified form in a yield of 41% (based on starting resin), significantly lower than the synthesis where tosyl protection was employed.

Material corresponding to the peak with R_f 0.25 in Figure 3 represented in part an unexpected side reaction in the synthesis employing Boc-nitroarginine. Further examination of this peptide material showed that it consisted of a mixture of peptides and that an acid hydrolysate contained ornithine as indicated by

(18) E. Schnabel, H. Herzog, P. Hoffmann, E. Klauke, and I. Ugi, *Justus Liebigs Ann. Chem.*, **716**, 175 (1968).
(19) E. Schnabel, *ibid.*, **702**, 188 (1967).
(20) J. Ramachandran and C. H. Li, *J. Org. Chem.*, **27**, 4006 (1962).
(21) R. H. Mazur and G. Plum, *Experientia*, **24**, 661 (1968).
(22) K. Esko, S. Karlsson, and J. Porath, *Acta Chem. Scand.*, **22**, 3342 (1968).
(23) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

comparisons with an authentic sample on the amino acid analyzer and by paper electrophoresis. In order to determine the full extent of this side reaction during synthesis, the crude product obtained directly after treatment of the peptide resin with hydrogen fluoride was analyzed for amino acids after acid hydrolysis. The molar ratios of ornithine and arginine were 0.16 and 0.63, respectively, with the value of leucine taken as 2, thus indicating a side reaction occurring at the expense of the arginine residue. In contrast, synthesis of peptide I employing tosyl protection of arginine gave a crude cleavage product with a value of 0.96 for arginine and no amino acid other than those expected for peptide I. In other experiments not described here protected heptapeptide resin obtained by use of Boc-nitroarginine was treated with hydrogen bromide in trifluoroacetic acid. In addition to the expected major product, alanylhistidylnitroarginylleucylhistidylglutaminylleucine, we also isolated an ornithine peptide.[24]

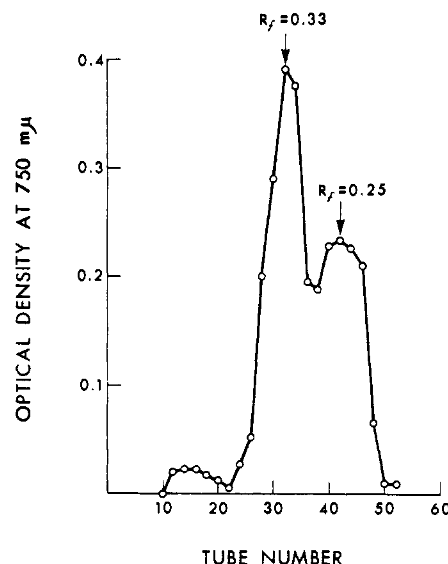

Figure 3. Partition chromatography on Sephadex G-25 of peptide I synthesized by use of Boc-nitroarginine. Optical density measurements by Folin–Lowry analysis.

Experimental Section[25]

N^{α}-Boc-arginine. A mixture of arginine free base (51 g, 0.29 mol) in 50% aqueous dioxane (*ca.* 35 ml) was treated for 3 hr at 45–50° with *tert*-butyloxycarbonyl azide (*ca.* 0.32 mol) and 4 *N* NaOH by the pH-stat procedure over the pH range 9–10. After removal of dioxane *in vacuo* and dilution of the aqueous solution with approximately an equal volume of water, the solution was washed with two 50-ml portions of ether, cooled to 0°, and acidified to pH 3 with cold 3 *N* HCl. The solution was quickly washed with ethyl acetate (100 ml) and then kept in the cold for several hours while crystallization occurred. The product was collected and washed with a minimum of ice-cold water: yield, 50 g; mp 113° dec. For analysis a sample (1.00 g) was dissolved in 20 ml of water, cooled to 0°, mixed with 10 ml of 3 *N* HCl, and stored at 0° for 22 hr. The crystalline product was collected, and washed with cold absolute ethanol (6 ml) and anhydrous ether: yield, 0.66 g; mp 117–119° dec; tlc (BPAW) R_f 0.65; $[\alpha]^{24}D$ −8.8° (*c* 2, H_2O).

Anal. Calcd for $C_{11}H_{22}N_4O_4 \cdot HCl \cdot H_2O$ (328.81): C, 40.18; H, 7.66; N, 17.04. Found: C, 40.46; H, 7.65; N, 17.48.

N^{α}-Boc-N^G-tosylarginine. A mixture of N^{α}-Boc-arginine hydrochloride hydrate (3.00 g, 9.15 mmol) and 10 ml of 80% aqueous acetone was cooled in an ice bath and the pH was adjusted to 11.5 with cold 4 *N* NaOH. While the mixture was stirred vigorously in the ice bath, solutions of *p*-toluenesulfonyl chloride (5.25 g, 27.5 mmol) in acetone (total volume of 15 ml) and 4 *N* NaOH were added in portions over a period of 15 min while the pH was maintained in the 11.0–11.3 range. After additional stirring for 5 min the pH was adjusted to 7.5 with 3 *N* HCl. Acetone was removed *in vacuo* and upon the addition of 30 ml of water a clear solution was obtained. The solution was washed with two 25-ml portions of ether and then acidified with cooling to pH 1.7 with 3 *N* HCl. The product was extracted into ethyl acetate (75 ml) and the latter solution was washed with three 10-ml portions of water and then dried over anhydrous $MgSO_4$. After removal of drying agent the product was crystallized from ethyl acetate (30 ml). Recrystallization gave: 1.53 g; mp 96–97°; tlc (CM) R_f 0.6; $[\alpha]^{24}D$ −3.6° (*c* 4, dimethylformamide) [lit.[20] mp 99–100°; $[\alpha]^{25}D$ −3.3° (*c* 4, dimethylformamide)].

A portion of the product was dried at 80° over P_2O_5 for 2 hr: mp 96–98°; tlc (CM) R_f 0.6. A sample (156 mg) was treated with liquid HF (*ca.* 10 ml) for 30 min at 0° in the presence of anisole (0.2 ml). After removal of the HF and drying *in vacuo* the residue was dissolved in 0.1 *N* HCl (10 ml) and washed with two 10-ml portions of ether. The solution was evaporated *in vacuo* to dryness. Optical rotation on the residue gave $[\alpha]^{24}D$ +26° (*c* 1.59, 5 *N* HCl) based on the amount of arginine obtainable from the sample and tlc (BPAW) R_f 0.35 corresponding to arginine with no traces of either N^{α}-Boc-N^G-tosylarginine or N^G-tosylarginine. Identical treatment of a sample (62.5 mg) of arginine free base gave $[\alpha]^{24}D$ +27° (*c* 1.56, 5 *N* HCl).

Dicyclohexylamine Salt of N^{α}-Boc(Im-Boc)histidine. Histidine free base (15.5 g, 0.1 mol) in 50% aqueous dioxane (20 ml) was treated at 45° over a 3-hr period with *tert*-butyloxycarbonyl azide (0.2 mol) while maintaining pH in the 9.0–9.5 range with 4 *N* NaOH and adding additional dioxane (40 ml). The pH was then allowed to decrease to 8.2 by addition of the azide (0.05 mol). During the next 3-hr period the azide (0.15 mol) and dioxane (20 ml) were added while maintaining pH in the 8.0–8.4 range with base. The solution was evaporated *in vacuo* to remove dioxane, mixed with water (150 ml), and washed with two 150-ml portions of ether. The solution was cooled to 0° and the pH adjusted to 3.0 with 3 *N* HCl. The product was extracted with 150- and 100-ml portions of ethyl acetate. The combined ethyl acetate extracts were washed with three 50-ml portions of saturated NaCl and dried over anhydrous $MgSO_4$. Removal of drying agent and solvent gave 10.8 g of oil. This was dissolved in 60 ml of dichloromethane, mixed with 300 ml of petroleum ether (30–60°), cooled to 0°, and mixed with 6.0 ml of dicyclohexylamine. Initial crystallization occurred on standing at −20° for 3 days. Two successive crops of crystalline material were obtained: 12.5 g (23% yield); mp 156–159°. For analysis a sample was recrystallized from dichloromethane–petroleum ether: mp 157–159°; $[\alpha]^{24}D$ +17.6° (*c* 2, $CHCl_3$).

Anal. Calcd for $C_{28}H_{48}N_4O_6$ (536.72): C, 62.66; H, 9.01; N, 10.4. Found: C, 62.71; H, 8.82; N, 10.1.

A sample (169 mg) was treated with trifluoroacetic acid (5 ml) for 6 min and evaporated *in vacuo* to dryness: tlc (BPAW) R_f 0.30 corresponding to histidine. Determination of optical rotation gave $[\alpha]^{24}D$ +13.8° (*c* 2, 6 *N* HCl) based on the amount of histidine obtainable from the sample. An equimolar mixture of histidine and dicyclohexylamine treated in the same manner gave $[\alpha]^{24}D$ +13.1° (*c* 2, 6 *N* HCl).

(24) Synthesis of the model peptide alanylarginylarginylleucylarginylarginylphenylalanine with the use of Boc-nitroarginine under exactly the same conditions described in this work gave, after HF cleavage, a product containing no trace of ornithine (D. Yamashiro, unpublished results). This suggests that the side reaction we have encountered might be sequence dependent.

(25) Melting points were determined on a Fisher-Johns block and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. Thin-layer chromatography (tlc) was run on silica gel in the following solvents: chloroform–methanol, 1:1 (CM); chloroform–acetone, 1:1 (CA); *n*-butyl alcohol–pyridine–acetic acid–water, 30:20:6:24 (BPAW); *sec*-butyl alcohol–3% ammonium hydroxide, 7:3 (SBA). The carboxymethylcellulose chromatography was performed on a column of 1.0 × 55 cm. The initial buffer was 0.01 *M* ammonium acetate, pH 4.5. After 20 tubes (10 ml/tube), a gradient with respect to pH and salt concentration was started by introducing 0.1 *M* ammonium acetate buffer of pH 6.7 through a 500-ml mixing flask containing the starting buffer. Thereafter the volume per tube was 4 ml. Later the gradient was increased as indicated in the figure. For analysis, 0.1-ml aliquots were mixed with 0.1 ml of 2 *N* sodium hydroxide. After evaporation to dryness, the residue was treated with 2 ml of 2 *N* sodium hydroxide at 100° for 75 min. After cooling and neutralization, the solutions were analyzed by the ninhydrin colorimetric method.

For use in synthesis the free acid was obtainable by shaking a sample (2.68 g) of the salt with 50 ml of ethyl acetate, 5.5 ml of 1 *N* H_2SO_4, and 7 ml of water in the cold. After removal of the aqueous layer, the organic layer was washed with four 10-ml portions of cold water and then dried over anhydrous $MgSO_4$ at room temperature. Removal of drying agent and solvent gave 1.68 g of oil which was dissolved in dichloromethane [tlc (CM) R_f 0.50] and stored in the cold. This material was readily converted back to the original salt with dicyclohexylamine.

Bpoc-glutamine *p*-Nitrophenyl Ester. To a solution of 13 g (34 mmol) of Bpoc-glutamine[12] and 4.73 g (34 mmol) of *p*-nitrophenol in 25 ml of ethyl acetate and 5 ml of dimethylformamide at −20° was slowly added 6.65 g (32 mmol) of dicyclohexylcarbodiimide in 10 ml of ethyl acetate and 1 ml of dimethylformamide. The mixture was stirred for 2 hr at −20° and stored 2 days at 4°. Filtration and evaporation of the filtrate gave a residue which was crystallized from ethanol–water. The product (10.9 g, mp 119–121°) was recrystallized from ethyl acetate–petroleum ether to give 8.9 g (52%) of Bpoc-glutamine *p*-nitrophenyl ester: mp 124–125°; tlc (CA) R_f 0.45; $[\alpha]^{24}D$ −64° (*c* 1, methanol).

Anal. Calcd for $C_{27}H_{27}N_3O_7$ (505.51): C, 64.1; H, 5.38; N, 8.31. Found: C, 63.5; H, 5.84; N, 8.26.

N^α-Bpoc-histidine. A solution of 3.1 g (20 mmol) of histidine (free base) in 9.3 ml of Triton B was evaporated to a residue which was taken up in 6 ml of dimethylformamide. The resultant mixture was evaporated and the residue was again taken up in 6 ml of dimethylformamide. Evaporation gave a residue which was treated with 12 ml of dimethylformamide, 6.7 g of Bpoc azide, and 5.5 ml of triethylamine at 40° for 90 min. The reaction mixture was distributed between 150 ml of water and 100 ml of ether; the aqueous layer was washed with an additional 100 ml of ether and cooled to 0° and the pH was adjusted to 5.0 with 1 *M* citric acid; the crystalline precipitate formed was filtered and washed with cold ethyl acetate and cold water, and dried to give 5.0 g (60%) of N^α-Bpoc-histidine hydrate: mp 146–149°; tlc (SBA) R_f 0.4; $[\alpha]^{24}D$ −13° (*c* 1, DMF).

Anal. Calcd for $C_{22}H_{25}N_3O_5$ (411.44): C, 64.2; H, 6.12; N, 10.2. Found: C, 64.0; H, 6.16; N, 10.5.

N^α-Bpoc-Im-Boc-histidine. N^α-Bpoc-histidine hydrate (5 g) was mixed to a paste with 50% dioxane, and 4 *N* sodium hydroxide was added to bring the pH to 8.5. The mixture was stirred at 45° and 2.7 g of Boc azide was added in portions; sodium hydroxide was added to maintain the pH at 8.2. After 4-hr stirring, the reaction mixture was evaporated to remove dioxane, and distributed between 100 ml of water and 50 ml of ether. The aqueous layer was washed with an additional 50 ml of ether and cooled to 0° and the pH was adjusted to 4.5 with 1 *M* citric acid. The aqueous mixture was extracted with two 75-ml portions of ethyl acetate, which were combined, dried, and evaporated to *ca.* 10 ml. Addition of petroleum ether gave a crystalline precipitate which was isolated and recrystallized from ethanol–water to give 3.0 g (50%) of N^α-Bpoc-Im-Boc-histidine: mp 133–134°; tlc (CM) R_f 0.55; $[\alpha]^{24}D$ +21° (*c* 1, methanol).

Anal. Calcd for $C_{27}H_{31}N_3O_6$ (493.54): C, 65.7; H, 6.33; N, 8.51. Found: C, 65.7; H, 6.39; N, 8.47.

Alanyl-Im-Boc-histidyl-N^G-nitroarginylleucyl-Im-Boc-histidylglutaminylleucyl Resin. A portion of the Boc-leucyl resin (1.03 g, 0.23 mmol of leucine) was treated by the following steps: (1) addition of 5 ml of methylene chloride and 5 ml of trifluoroacetic acid and shaking for 15 min; (2) washing with three 10-ml portions of methylene chloride, three 10-ml portions of ethanol, and three 10-ml portions of chloroform; (3) neutralization by the addition of 10 ml of chloroform and 0.75 ml of triethylamine and shaking for 5 min; (4) washing with three 10-ml portions of chloroform and three 10-ml portions of dimethylformamide; (5) addition of 1.6 mmol of Bpoc-glutamine nitrophenyl ester in 7 ml of dimethylformamide and shaking for 7 hr; (6) washing with three 10-ml portions of dimethylformamide; (7) acetylation by the addition of 8 ml of dimethylformamide, 0.5 ml of acetic anhydride, and 0.2 ml of *N*-methylmorpholine and shaking for 20 min; (8) washing with two 10-ml portions of dimethylformamide and four 10-ml portions of ethanol.

After the first cycle the deblocking procedure was changed as follows: (1a) washing with three 10-ml portions of chloroform; (1b) treatment with 10 ml of chloroform and 0.1 ml of 4.5 *N* HCl–dioxane for 2 min, and then adding 0.11 ml of 4.5 *N* HCl and shaking for 6 more min; (1c) washing with five 10-ml portions of chloroform; (1d) treatment with 10 ml of chloroform and 0.11 ml of 4.5 *N* HCl for 4 min; (1e) washing with three 10-ml portions of chloroform. The filtrates from the first and second acid treatments were isolated and spectroscopically analyzed (253 mμ) for biphenyl content. A value of 2% or less Bpoc cleavage in the second acid treatment (with respect to the first acid treatment) was taken to indicate virtually complete deblocking of the Bpoc group.

The following additional changes were made after the first cycle: in step 2 chloroform was substituted for methylene chloride; in step 4 methylene chloride was substituted for dimethylformamide; in step 5 coupling was achieved by shaking for 3 hr with 0.8 mmol of Bpoc amino acid and dicyclohexylcarbodiimide in 10 ml of methylene chloride; and in step 7 this step was deleted after the first cycle. In the last two deblocking reactions (after the coupling of the second histidine residue and the N-terminal alanine) it was necessary to repeat step 1d to ensure complete deblocking.

After the final deblocking, the resin was washed with ethanol and dried: yield, 1.25 g.

Boc-alanylhistidyl-N^G-nitroarginylleucylhistidylglutaminylleucyl Resin. Starting with Boc-leucyl resin (1.50 g, 0.33 mmol of leucine), the following cycle of steps was carried out for the introduction of each residue: (1) washing with four 15-ml portions of dichloromethane (retention volume of the resin for dichloromethane was 5 ml after filtration); (2) removal of the Boc group by addition of 15 ml of trifluoroacetic acid–dichloromethane (3:1) followed by a 15-min shaking period; (3) washing with three 15-ml portions of dichloromethane; (4) washing with three 15-ml portions of 50% absolute ethanol in dichloromethane; (5) washing with three 15-ml portions of dichloromethane; (6) neutralization with 0.30 ml (1.75 mmol) of diisopropylethylamine in 15 ml of dichloromethane; (7) washing with six 15-ml portions of dichloromethane with the exception that three 15-ml portions of dimethylformamide replaced the last three washings for the glutamine cycle; (8) addition of 1.36 mmol of the appropriate Boc amino acid in 11 ml of dichloromethane and shaking for 10 min, with the exceptions indicated subsequently; (9) addition of 1.33 mmol of dicyclohexylcarbodiimide in 3.2 ml of dichloromethane and shaking for the periods indicated subsequently; (10) washing with three 15-ml portions of dimethylformamide; (11) washing with three 15-ml portions of absolute ethanol.

For introduction of the glutamine residue, steps 8 and 9 were replaced by a treatment with 3.4 mmol of the *p*-nitrophenyl ester of Boc-glutamine which was added in 11 ml of dimethylformamide. The histidine residues were incorporated by use of Boc(Im-Boc)-histidine in step 8; for the nitroarginine residue, 2.04 mmol of Boc-nitroarginine was employed by addition in 9 ml of 80% dimethylformamide in dichloromethane in step 8. Coupling times for the individual residues were, in order, as follows: Gln, 17 hr; His, 3 hr; Leu, 2.5 hr; (NO_2)Arg, 5.5 hr; His, 3 hr; Ala, 2 hr. After the coupling of each of the histidine residues an aliquot of the resin (*ca.* 10 mg) was removed to test for coupling efficiency. These reactions were carried out with 1 *M* 2-hydroxy-1-naphthaldehyde in dichloromethane for 1.5–2.0 hr and with 1 *M* benzylamine in dichloromethane for 20 min. The value obtained in the test for both histidine coupling steps was 0.002 mmol/g as compared to values of 0.001 and 0.23 for starting Boc-leucyl resin before and after the deprotection, respectively. At the end of the last cycle the polypeptide resin was dried in *vacuo:* yield, 1.80 g.

Boc-alanylhistidyl-N^G-tosylarginylleucylhistidylglutaminylleucyl Resin. Starting with Boc-leucyl resin (1.51 g, 0.33 mmol of leucine) the same cycle of steps for each residue was carried out as described in the previous section with the following exceptions. The arginine residue was incorporated by adding 1.37 mmol of N^α-Boc-N^G-tosylarginine in step 8 in 12 ml of 10% dimethylformamide in dichloromethane and coupling in step 9 for 5.5 hr. The aforementioned coupling test gave a value of 0.001 mmol/g for this coupling. At the end of the last cycle the dried resin weighed 1.83 g.

During synthesis eluates were collected at various stages and analyzed for amino acids as follows. After the leucine residue had been coupled to the histidine, eluates from the next deblocking and washing steps (steps 2 and 3) were collected, evaporated to dryness, and analyzed on the amino acid analyzer; 0.12 mmol of leucine was found. In similar manner 0.16 mmol of N^G-tosylarginine was found in these eluates after the coupling of N^α-Boc-N^G-tosylarginine. After coupling of the second histidine residue, 0.16 mmol of histidine was found. An aliquot of the finished protected peptide resin was then carried through steps 1–7. The eluates of steps 2 and 3 contained 0.37 mmol of alanine and those of steps 4–7 contained 0.10 mmol, as calculated for the entire run.

Alanylhistidylarginylleucylhistidylglutaminylleucine (I). A. From Boc-alanylhistidy-N^G-tosylarginylleucylhistidylglutaminylleucyl Resin. A mixture of 765 mg of Boc-alanylhistidyl-N^G-tosylarginylleucylhistidylglutaminylleucyl resin, 0.70 ml of anisole,

and about 15 ml of liquid hydrogen fluoride was stirred for 30 min at 0°. The hydrogen fluoride was evaporated with a stream of nitrogen at 0° (*ca.* 10 min). The residue was dried *in vacuo* over NaOH and then stirred with 10 ml of trifluoroacetic acid for 15 min. The polymer support was filtered off and rinsed with two 5-ml portions of trifluoroacetic acid. The filtrate was evaporated *in vacuo*, and the resulting oily residue was taken up in a mixture of 10 ml of 0.2 *N* acetic acid and 10 ml of ether. The ether layer was discarded and the aqueous phase was washed again with a 5-ml portion of ether, and lyophilized to give crude peptide I. Amino acid analysis of an acid hydrolysate gave $His_{1.92}Arg_{0.96}Glu_{0.99}Ala_{2.70}Leu_{2.00}$. Chromatography on CM-cellulose gave 80 mg of peptide I.

For further purification partition chromatography on Sephadex G-25 was employed. A 1.92 × 63 cm column was equilibrated with the solvent system 1-butanol–ethanol–0.2 *N* aqueous NH_4OH containing 0.04% acetic acid (4:1:5). The column was thoroughly equilibrated with organic phase (330 ml) before carrying out chromatography. The material from carboxymethylcellulose chromatography was subjected to partition chromatography in two batches with collection of 5.55 ml fractions. Only one peak was detected in each case by the Folin–Lowry method[26] with R_f 0.33 (Figure 2). Isolation of the two batches gave 78 mg of I (63% yield based on starting resin): tlc (BPAW) R_f 0.40; $[\alpha]^{24}D$ −56° (*c* 1, 1 *N* acetic acid); $[\alpha]^{24}D$ −57° (*c* 0.33, 10% acetic acid). For analysis a sample was dried at 100° for 6.5 hr *in vacuo* over P_2O_5.

Anal. Calcd for $C_{38}H_{63}N_{15}O_9 \cdot 3H_2O$ (928.09): C, 49.2; H, 7.49; N, 22.6. Found: C, 49.3; H, 7.19; N, 23.0.

Paper electrophoresis in pyridine acetate buffer (pH 3.7, 400 V, 4 hr) and in collidine acetate buffer (pH 6.9, 400 V, 4 hr) showed single ninhydrin and Pauly positive spots with mobilities of R_f 0.94 and 0.55, respectively, relative to lysine. Amino acid analyses of an acid hydrolysate and a leucine aminopeptidase digest (pH 8, 24 hr, 37°) gave $His_{1.97}Arg_{1.02}Glu_{1.01}Ala_{0.95}Leu_{2.00}$ and $His_{2.02}Arg_{0.91}Gln_{0.74}Ala_{0.94}Leu_{2.00}$, respectively.

B. From Bpoc-alanyl-Im-Boc-histidyl-N^G-nitroarginylleucyl-Im-Boc-histidylglutaminylleucyl Resin. A portion of the above dried heptapeptide resin (485 mg) was treated with 0.5 ml of anisole and 10 ml of hydrogen fluoride for 30 min at 0°. After evaporation of the hydrogen fluoride at 0° and thorough drying, the resin was stirred for 15 min with 10 ml of trifluoroacetic acid. The mixture was filtered and the filtrate was evaporated to a residue which was distributed between 10 ml of 0.1 *N* acetic acid and 10 ml of ether. The aqueous layer was washed with 5 ml of ether, and lyophilized to a residue which was purified by carboxymethylcellulose chromatography as above to give 55 mg of peptide. Rechromatography gave 47 mg (54% yield based on starting Boc-leucyl resin) of I: $[\alpha]^{24}D$ −56° (*c* 0.3, 10% acetic acid). Paper electrophoresis in pyridine acetate buffer (pH 3.7, 400 V, 4 hr) showed one ninhydrin positive, Pauly positive spot at R_f 0.93 (with respect to lysine). Paper electrophoresis in collidine acetate buffer (pH 6.9, 400 V, 4 hr) showed one ninhydrin positive, Pauly positive spot at R_f 0.51. Amino acid analyses of an acid hydrolysate and a leucine aminopeptidase digest gave $Leu_{2.00}His_{2.02}Arg_{1.03}Glu_{1.04}Ala_{0.95}$ and $Leu_{2.00}His_{2.04}Arg_{1.02}Gln_{0.80}Ala_{0.97}$, respectively.

C. From Boc-alanylhistidyl-N^G-nitroarginylleucylhistidylglutaminylleucyl Resin. A sample (767 mg) of Boc-alanylhistidyl-N^G-nitroarginylleucylhistidylglutaminylleucyl resin was treated with liquid HF in exactly the same manner as described in part A. Isolation of the crude cleavage product in the same way gave 164 mg of solids. Amino acid analysis of an acid hydrolysate of this material gave $Orn_{0.16}His_{1.84}Arg_{0.63}Glu_{1.04}Ala_{2.88}Leu_{2.00}$. Chromatography on CM-cellulose gave two major peaks as analyzed at 240 mμ. The faster moving peak (fraction A) gave 27.6 mg after lyophilization; the slower moving peak (fraction B) gave 44.1 mg after lyophilization.

Fraction A was subjected to partition chromatography under conditions identical with those described above. A major peak with R_f 0.33 was detected along with a substantial peak with R_f 0.25 (Figure 3). Isolation of materials corresponding to these peaks gave 15.5 and 9.2 mg, respectively. In like manner fraction B was chromatographed to give a major peak with R_f 0.33 and a smaller peak with R_f 0.27. The yields in this case were 36.7 and 5.2 mg, respectively.

The materials represented by the major peak with R_f 0.33 were pooled and rechromatographed in exactly the same manner to give one peak with the same R_f. Recovery of peptide I was 43.8 mg (41% yield based on starting resin): tlc (BPAW) R_f 0.40; $[\alpha]^{24}D$ −55° (*c* 0.32, 10% acetic acid). Paper electrophoresis performed under conditions identical with those described in part A gave exactly the same results. Amino acid analyses of an acid hydrolysate and a leucine aminopeptidase digest gave $His_{1.97}Arg_{0.99}Glu_{0.93}Ala_{0.97}Leu_{2.00}$ and $His_{2.21}Arg_{0.97}Gln_{0.77}Ala_{1.02}Leu_{2.00}$, respectively.

Materials represented by the minor peak with low R_f value (Figure 3) were combined along with the same material obtained after HF cleavage from a second batch of protected peptide resin (506-mg run) and rechromatographed on the partition column. An unsymmetrical peak with R_f 0.27 was obtained and isolation gave 20.1 mg. Amino acid analysis of an acid hydrolysate showed a peak running 4.8 min before the histidine peak. Authentic samples of ornithine and lysine ran 4.8 and 4.0 min, respectively, ahead of histidine. Paper electrophoresis (collidine acetate buffer of pH 6.9, 400 V, 6.5 hr) of the acid hydrolysate gave a ninhydrin spot with color and mobility identical with an authentic sample of ornithine, while authentic samples of lysine and arginine had mobilities of R_f 0.90 and 0.80, respectively, relative to ornithine.

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(26) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193, 265 (1951).

Communications to the Editor

Effect of Ligand Electronegativity on the Inversion Barriers of Arsines[1]

Sir:

We have recently shown[2] that, by analogy with the planarity or near planarity at nitrogen in silylamines,[3] the barrier to pyramidal inversion at phosphorus is markedly lowered by the incorporation of silyl substituents. We now report the operation of a similar effect when arsenic is the inversion center. The inversion barrier in isopropylphenyltrimethylsilylarsine (**2a**) (Table I) represents a decrease of *ca.* 18 kcal/mol relative to ethylmethylphenylarsine (**1a**). Moreover, the trend in barrier heights within the arsine series **1a**–**4a** (Table I) exactly parallels the trend previously observed[4] in the analogous phosphines **1b**–**4b** (Table I). Hence, the predominant influence upon pyramidal stability in these systems appears to be the atomic electronegativity[5] of the

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(2) R. D. Baechler and K. Mislow, *J. Amer. Chem. Soc.*, 92, 4758 (1970).

(3) C. Glidewell, D. W. H. Rankin, A. G. Robiette, and G. M. Sheldrick, *J. Mol. Struct.*, 6, 231 (1970), and references cited therein.

(4) R. D. Baechler and K. Mislow, *J. Amer. Chem. Soc.*, 93, 773 (1971).

(5) A. L. Allred, *J. Inorg. Nucl. Chem.*, 17, 215 (1961).