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The β-Phenacyl and β-*p*-Nitrobenzyl Esters to Suppress Side Reactions during Treatment of Aspartyl Peptides with Hydrogen Fluoride[1]

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The synthetic strategy for the suppression of α to β rearrangement of aspartyl peptides during anhydrous hydrogen fluoride treatment was designed in which the carboxyl protecting groups stable to hydrogen fluoride, such as β-phenancyl or β-*p*-nitrobenzyl ester, were used and the protected peptides were treated with hydrogen fluoride-anisole mixture to remove protecting groups other than β-phenancyl or β-*p*-nitrobenzyl ester group followed by deprotection of the β-carboxyl protecting group under the mild conditions.

Thus, the model dipeptide derivative, Z(OMe)-Asp(ONb)-Ser(Bzl)-OBzl, was treated with hydrogen fluoride-anisole mixture. The product was submitted to hydrogenolysis to remove the *p*-nitrobenzyl ester group. Under such a strategy, no product due to the rearrangement was detected on paper chromatograms. Similarly, the treatment of Boc-Asp(OPac)-Ser(Bzl)-OBzl gave a satisfactory result.

H-Asp-Ser-Asp-Pro-Arg-OH, which is a fragment of immunogloblin E and is reported to be biologically active, has been synthesized from Boc-Asp(ONb)-Ser(Bzl)-Asp(ONb)-Pro-Arg(NO_2)-resin as an application of this strategy. Chemical and physical properties of the pentapeptide were in fair agreement with those of the pentapeptide which was synthesized under the mild conditions from Boc-Asp(OBzl)-Ser(Bzl)-Asp(OBzl)-Pro-Arg(NO_2)-ONb.

It has been well known that the rearrangement of α to β-aspartyl peptide occurs in either basic or acidic solution and even in hot alcohol.[3] The intermediate of this rearrangement is proved to be a succinimide derivative.[3] Recently, Baba, *et al.*[4] reported that some aspartyl peptides containing the sequences of –Asp–Gly–, –Asp–Ser– or –Asp–His– has tendency to be susceptible to the rearrangement during the anhydrous hydrogen fluoride treatment which had been widely used in both solid phase and conventional liquid phase peptide syntheses.[5] In a solid phase synthesis of bovine growth hormone-(125—133) fragment containing –Asp–Gly– sequence, the formation of a cyclic aspartoyl imide derivative was observed during the hydrogen fluoride treatment.[6] On the other hand, it was reported that treatment of Boc–Asp(OBzl)–Gly–OBzl in hydrogen fluoride and anisole mixture at correct 0° for one hour gave only H–Asp–Gly–OH.[7] On the contrary, Yang, *et al.*[8] found that Boc–Glu(OBzl)–Asp(OBzl)–

1) Abbreviations used are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **11**, 1726 (1972). Other abbreviations: OPac=phenacyl ester, DMF=dimethylformamide, DCC=dicyclohexylcarbodiimide, DCHA=dicyclohexylamine, DMSO=dimethylsulfoxide, HOBt=1-hydroxybenztriazole, TFA=trifluoroacetic acid, AP–M=aminopeptidase M, IR=infrared. The -resin represents ester bond derived from N-protected amino acid or peptide with chloromethylated polystyrene 2% divinylbenzene.

2) Location: *Komatsushima, Sendai, 983, Japan.*

3) M.A. Ondetti, A. Deer, J.T. Sheehan. J. Pluščec, and O. Kocy, *Biochemistry*, **7**, 4069 (1968) and references cited therein.

4) T. Baba, H. Sugiyama, and S. Seto, *Chem. Pharm. Bull.* (Tokyo), **21**, 207 (1973).

5) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).

6) S.S. Wang, C.C. Yang, I.D. Kulesha, M. Sonenberg, and R.B. Merrifield, *Int. J. Pept. Protein Res.*, **6**, 103 (1973).

7) Y. Ogata, K. Igano, K. Inoue, and S. Sakakibara, "Proceedings of the 12th Symposium on Peptide Chemistry," ed. by H. Yajima, Protein Research Foundation, Minoh, Osaka, 1975, p. 35.

8) C.C. Yang and R.B. Merrifield, *J. Org. Chem.*, **41**, 1032 (1976).

Gly–Thr(Bzl)–resin was derived to the corresponding aspartoyl tetrapeptide in a 99% yield under a similar condition. However, the yield of the side product was reduced to only 2.4% when Boc–Glu(OBzl)–Asp(OPac)–Gly–Thr(Bzl)–resin was first treated with sodium thiophenoxide to remove the phenacyl ester group prior to the treatment with hydrogen fluoride.[8] In spite of the application of the tactics, the yields of such side product varied 2.4 to 9.5% depending on the sequence of the peptide derivatives which contained –Asp–Gly– sequence in each peptide.[8]

Under these circumstances, it seems that suppression of the side reaction is still a problem to be solved in peptide chemistry. This paper describes a strategy for the suppression of the side reaction with a different approach from those of the other investigators[7,8] and the synthesis of immunogloblin E fragment, H–Asp–Ser–Asp–Pro–Arg–OH which has been reported to be biologically active,[9] as an application of this strategy. The synthetic strategy for the suppression of this side reaction during hydrogen fluoride treatment was designed in which the protecting groups stable to hydrogen fluoride, such as β-phenacyl or β-*p*-nitrobenzyl ester, were used and the protected peptides were treated with hydrogen fluoride-anisole mixture to remove protecting groups other than β-phenacyl or β-*p*-nitrobenzyl ester group followed by deptotection of the β-carboxyl protecting group under the mild conditions. It was found that this strategy gave satisfactory results.

H–Asp(ONb)–OH (I), previously synthesized by many investigators,[10] was prepared in a 44% yield based on *p*-nitrobenzyl bromide used by a modification of Ledger and Stewart's method[10a] as described in experimental section since, in our hands, it gave more satisfactory results with respects to these steps and yield than those of the other investigators. H–Asp(OPac)–OH (II)[8] was also prepared by a similar modified method in a 33% yield based on phenacyl bromide used. Z(OMe)–Asp(ONb)–OH was prepared by the reaction of H–Asp(ONb)–OH and *p*-methoxybenzyl *S*-4,6-dimethylpyrimidin-2-yl thiocarbonate[11] to give an oily product which was charactarized as its dicyclohexylammonium salt.[12] As model peptides, Boc–Asp(OBzl)–Ser(Bzl)–OBzl (V), Z(OMe)–Asp(ONb)–Ser(Bzl)–OBzl (VI) and Boc–Asp(OPac)–Ser(Bzl)–OBzl (VII), were prepared. Thus, H_2^+–Ser(Bzl)–OBzl·Cl^- (IV) was prepared with the hydrogen chloride treatment of oily Boc–Ser(Bzl)–OBzl which was prepared from Boc–Ser(Bzl)–OH[13] and benzyl bromide in the presence of triethylamine. Boc–Asp(OBzl)–OH was coupled with IV in the presence of dicyclohexylcarbodiimide and 1-hydroxybenztriazole[14] to give V. VI and VII were also prepared in a similar manner. V was treated with anhydrous hydrogen fluoride in the presence of anisole for one hour at correct 0°. After removal of hydrogen fluoride and most of anisole in vacuum, the residual peptide was dissolved in 1% acetic acid and the solution was washed with ethyl acetate and ether (1: 1 v/v). The solution was lyophilized to give crude peptide (Va), and Va was submitted to paper chromatography to identify the degree of the side reaction. VI and VII were treated in a similar manner to give the corresponding crude H–Asp(ONb)–Ser–OH (VIa) and H–Asp(OPac)–Ser–OH (VIIa) followed by submission to paper chromatography. Va was treated in 1% ammonium bicarbonate according to the directions given by Ondetti, *et al.*[3] and the resulting product (Vb) was submitted to paper chromatography. VIa was hydrogenated in the presence of 5% palladium on carbon to remove *p*-nitrobenzyl ester group and the resulting product (VIb) was submitted to paper chromatography. For the same purpose, VIIa was treated with zinc

9) R.N. Hamburger, *Science*, **189**, 389 (1975).
10) *a*) R. Ledger and F.H.C. Stewart, *Aust. J. Chem.*, **18**, 1477 (1965); *b*) M. Goodman, A.M. Felix, C.M. Deber, A.R. Brause, and G. Schwartz, *Biopolymers*, **1**, 371 (1963); *c*) R.L. Prestidge, D.R.K. Harding, J.E. Battersby, and W.S. Hancock, *J. Org. Chem.*, **40**, 3287 (1975).
11) K. Nagasawa, K. Kuroiwa, K. Narita, and Y. Isowa, *Bull. Chem. Soc. Jpn.*, **46**, 1269 (1973).
12) It is generally realized that Z(OMe)-amino acid derivatives are superior in their crystallization than those of Boc-amino acid derivatives.
13) D.A. Laufer and E.A. Blout, *J. Am. Chem. Soc.*, **89**, 1246 (1967).
14) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).

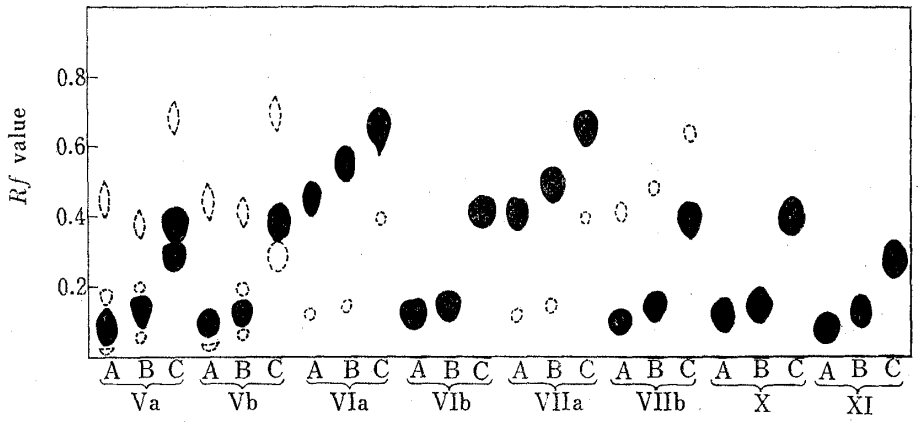

Fig. 1. Paper Chromatograms of Dipeptides treated with HF, Dipeptides Treated Further with the Other Reagents and Authentic Dipeptides

A, B and C represent the following systems: A, BuOH–AcOH–H_2O (4:1:5, upper layer); B, BuOH–AcOH–pyridine–H_2O (15:3:10:12); C, BuOH–AcOH–H_2O (2:1:1). Dotted circles represent spots stained faintly with ninhydrin.

powder in 80% acetic acid and the resulting product (VIIb) was submitted to paper chromatography. For the purpose to obtain authentic sample of H–Asp–Ser–OH (X), V was treated with trifluoroacetic acid followed by hydrogenolysis and the resulting product was purified through a column of Dowex 1×2 (acetate form) to give analytically pure dipeptide. For the same purpose, Boc–Asp(–Ser(Bzl)–OBzl)–ONb (VIII), which was prepared from Boc–Asp–ONb[15] and IV in a similar manner for the preparation of V, was treated in a similar manner for the preparation of X to give analytically pure H–Asp(–Ser–OH)–OH (XI). The paper chromatograms of these two authentic dipeptides were shown in Fig. 1 together with those of dipeptides described above. The paper chromatograms of Fig. 1 show that treatment of VI or VII with hydrogen fluoride give the expected H–Asp(ONb)–Ser–OH or H–Asp(OPac)–Ser–OH with trace of H–Asp–Ser–OH. On the other hand, treatment of V with hydrogen fluoride give the desired H–Asp–Ser–OH with considerable amount of side product as expected from the results with the use of Z–Asp(OBzl)–Ser(Bzl)–OMe reported by Baba, *et al.*[4] The crude H–Asp–Ser–OH (Va) derived from V was treated in 1% ammonium bicarbonate and the mixture was lyophilized. The contents of H–Asp–Ser–OH and H–Asp(–Ser–OH)–OH were 97% and 3% respectively as determined by amino acid analyzer.[16] Further, the chromatograms show that catalytic hydrogenolysis of crude H–Asp(ONb)–Ser–OH (VIa) or treatment of crude H–Asp(OPac)–Ser–OH (VIIa) with zinc powder in acetic acid give the desired H–Asp–Ser–OH without any detectable side products.

Another problem in the synthesis of peptide containing –Asp–Pro– sequence is that the peptide bond is very susceptible to hydrolysis in acidic solution.[17] As a preliminary experiment, Boc–Asp(OBzl)–Pro–OBzl (IX), which was prepared from Boc–Asp(OBzl)–OH and H_2^+–Pro–OBzl·Cl^-,[18] was treated with hydrogen fluoride-anisole mixture and the reaction

15) E. Schröder and E. Klieger, *Ann.*, **673**, 208 (1964).

16) H-Asp-Ser-OH and H-Asp(-Ser-OH)-OH appeared at 87 min and 35 min of retention time respectively on a long column in 4 hr standard analysis.

17) K.J. Fraser, K. Poulsen, and E. Haber, *Biochemistry*,, **11**, 4974 (1972).

18) R.E. Neuman and E.L. Smith, *J. Biol. Chem.*, **193**, 97 (1951).

mixture was worked up as usual. The contents of free aspartic acid and proline in the crude dipeptide were 0.25 to 0.50% based on the peptide contents as analyzed by amino acid analyzer. The minor rate of the hydrolysis is probably due to the presence of moisture contaminated in commercial anhydrous hydrogen fluoride, anisole, the protected dipeptide or reaction apparatus for hydrogen fluoride treatment. The hydrolysis could not be attributed to the treatment of the peptide in dilute acetic acid for a short time, up to ten hour in our hands, at room temperature, because any hydrolysis could not be detected when the dipeptide or H–Asp–Ser–Asp–Pro–Arg–OH was purified through a column of Dowex 1×2 (acetate form) with the use of dilute acetic acid as an elution solvent.

On the basis of these findings during the above investigations, solid phase synthesis of immunogloblin E fragment, H–Asp–Ser–Asp–Pro–Arg–OH, was designed as an application of this strategy for the suppression of the side reaction and the results were compared with that of the synthesis by usual means in which the β-carboxyl group of the amino terminal aspartic acid was protected with benzyl ester group used commonly. The β-carboxyl group of aspartic acid of the third position was protected with *p*-nitrobenzyl group, because the ester group resisted to the repeated acidolitic cleavage of *tert*-butyloxycarbonyl group in the solid phase peptide synthesis.[19] Boc–Arg(NO_2)–OH was esterified onto chloromethylated copolymer of styrene with 2% divinylbenzene in dimethylformamide and triethylamine to obtain Boc–Arg(NO_2)–resin. Boc–Ser(Bzl)–Asp(ONb)–Pro–Arg(NO_2)–resin (XII) was constructed by stepwise manner. Boc–Asp(ONb)–OH was coupled with XII to give Boc–Asp(ONb)–Ser(Bzl)–Asp(ONb)–Pro–Arg(NO_2)–resin (XIII). XIII thus obtained was treated with hydrogen fluoride-anisole mixture as usual manner and the crude peptide was submitted to hydrogenolysis to remove the *p*-nitrobenzyl ester group in the presence of 5% palladium on carbon in water. The deblocked peptide was purified through a column of Dowex 1×2 (acetate form) which was eluted with a linear gradient of water to 0.2M acetic acid to give H–Asp–Ser–Asp–Pro–Arg–OH (XVa) in a 22% yield based on Boc–Arg(NO_2)–resin. The chromatographic pattern for the purification of this peptide on a column of Dowex 1×2 (acetate form) is shown in Fig. 2. The purity was assessed by paper chromatography, paper electrophoresis and amino acid analyses of the acid hydrolysate and of the aminopeptidase

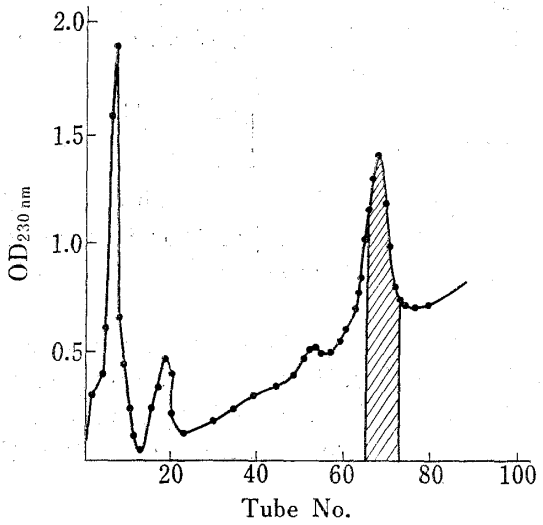

Fig. 2. Purification of XVa on a Column of Dowex 1×2 (Acetate Form)
Shaded part indicates the desired fractions.

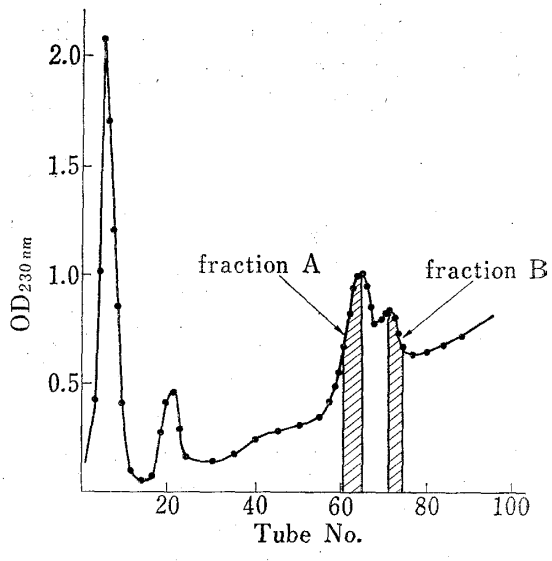

Fig. 3. Chromatographic Pattern of Crude XVb on a Column of Dowex 1×2 (Aceetat Form)

19) J. Halstrøm, O. Schou, K. Kovács, and K. Brunfeldt, *Z. Physiol. Chem.*, **351**, 1576 (1970).

M digest.[20] The chemical and physical properties of XVa are in fair agreement with those of XX prepared under the mild conditions by conventional liquid phase method as described later in this paper. Boc–Asp(OBzl)–OH was coupled with XII to give Boc–Asp(OBzl)–Ser-(Bzl)–Asp(ONb)–Pro–Arg(NO_2)–resin (XIV) which was treated just in a similar manner for the preparation of XIII. XIV thus obtained was treated with hydrogen fluoride-anisole mixture in a similar manner for the preparation of XVa to give XVb. The chromatographic pattern of crude peptide derived from XIV is shown in Fig. 3. Fraction A in Fig. 3 was lyophilized and submitted to rechromatography on a column of Dowex 1×2 (acetate form) to give the desired H–Asp–Ser–Asp–Pro–Arg–OH (XVb) which was identical with XVa and XX. Fraction B in Fig. 3 was lyophilized to give H–Asp(–Ser–Asp–Pro–Arg–OH)–OH which was assigned by amino acid analyses of the digest of the peptide with aminopeptidase M and the acid hydrolysate. Thus α-aspartyl-peptide and β-aspartyl-peptide were separated under this condition, although the separation was not satisfactory. It can be attributed to the difference of the acidity of the α-carboxyl group and β-carboxyl group in aspartic acid.

H–Asp–Ser–Asp–Pro–Arg–OH (XX) was also synthesized by conventional liquid phase method under the mild conditions to compare precisely the chemical and physical properties with those of XVa synthesized by solid phase method, because it had been generally realized that the purity of the peptides synthesized by conventional liquid phase method was more believable than those of the peptides synthesized by solid phase method. Thus, Z–Arg(NO_2)–ONb[21] was debenzyloxycarbonylated with hydrogen bromide-acetic acid solution and the resulting product was coupled with Z–Pro–ONp[22] to give Z–Pro–Arg(NO_2)–ONb (XVI). The debenzyloxycarbonylated XVI was coupled with Boc–Asp(OBzl)–OH in the presence of dicyclohexylcarbodiimide and 1-hydroxybenztriazole to give Boc–Asp(OBzl)–Pro–Arg(NO_2)–ONb (XVII). XVII was de-*tert*-butyloxycarbonylated with 4N hydrogen chloride in dioxane and the resulting product was coupled with Boc–Ser(Bzl)–OH in a similar manner for the preparation of V to give Boc–Ser(Bzl)–Asp(OBzl)–Pro–Arg(NO_2)–ONb (XVIII). Boc–Asp-(OBzl)–OH was coupled with the de-*tert*-butyloxycarbonylated XVIII just in a similar manner for the preparation of V to give Bos–Asp(OBzl)–Ser(Bzl)–Asp(OBzl)–Pro–Arg(NO_2)–ONb (XIX). Since the coupling reaction mixtures in the presence of 1-hydroxybenztriazole were often colored in light brown, the products extracted in ethyl acetate were needed to be decolored with activated charcoal. After the removal of the *tert*-butyloxycarbonyl group in XIX, the resulting pentapeptide derivative was dissolved in water and methanol mixture and hydrogenated in the presence of 5% palladium on carbon for seven days.[23] The crude peptide was purified through a column of Dowex 1×2 (acetate form) in a similar manner for the purification of XVa to give XX in 25% yield. When the hydrogenolysis of the de-*tert*-butyl-oxycarbonylated XIX was discontinued after two days, H–Asp–Ser–(Bzl)–Asp–Pro–Arg–OH, which was assigned by amino acid analyses of the digests with aminopeptidase M and acid hydrolysates of both the peptide itself and the further hydrogenated peptide, was isolated in the fractions corresponding to those of the shaded part in Fig. 2. Only traces of the desired H–Asp–Ser–Asp–Pro–Arg–OH were detected on paper chromatography of the fractions. The unusual long time required to hydrogenolysis of XIX and the low yield of XX could be assumed to be attributed to the sequence dependent of the peptide derivative.

20) AP-M (Lörm & Haas Co., Darmstadt, lot No. 4330139) was purchased through Protein Research Foundation, Minoh, Osaka. The cluster of -Asp-Pro- in the molecule resisted to digestion with this enzyme preparation, although synthetic H-Thr-Pro-Gly-Ser-Arg-OH [K. Suzuki, N. Endo, R. Tani, and H. Kikuchi, *Chem. Pharm. Bull.* (Tokyo), **22**, 2462 (1974)] was digested completely with the same enzyme.

21) R.A. Boissonnas. St. Guttmann, and P.A. Jaquenoud, *Helv. Chim, Acta*, **43**, 1349 (1960).

22) M. Itoh, *Chem. Pharm. Bull.* (Tokyo), **18**, 784 (1970).

23) When Z-Arg(NO_2)-ONb was hydrogenated to verify the catalytic activity of the catalyst in a similar condition for 22 hr, main product was arginine and minor Sakaguchi reaction negative product was detected by means of paper electrophoresis under the conditions described in the experimental section.

Experimental

All melting points are uncorrected. Unless otherwise mentioned, Boc-group and Z(OMe)-group of the protected peptide were deblocked with 4 N HCl in dioxane and Z-group with 5.7 N HBr in AcOH, and the paper chromatograms were performed on Toyo Roshi No. 51 with the following solvents: *Rf* (A), BuOH–AcOH–H_2O (4: 1: 5, upper layer);[24] *Rf* (B), BuOH–AcOH–pyridine–H_2O (15: 3: 10: 12),[25] and on Toyo Roshi No. 50 with the following solvent: *Rf* (C), BuOH–AcOH–H_2O (2: 1: 1),[4] at room temperature by asending procedure. Paper electrophoreses were carried out in pyridine–AcOH–H_2O (10: 0.4: 90, pH 6.5) using Toyo Roshi No. 51; 500 V, 90 min. Electrophoretic mobilities are expressed as fractions, E_{Asp} of the distances traveled by aspartic acid in the same system. Amino acid analysis was carried out on a Hitachi Model KLA-3B amino acid analyzer according to the directions given by Moore, *et al.*[26] For amino acid analysis, a sample was hydrolyzed by constant boiling HCl in an evacuated sealed tube at 110° for 22 hr and AP-M digestion was carried out by the procedure given by Hofmann, *et al.*[27]

H-Asp(ONb)-OH (I)——To a solution of H-Asp-OH (6.65 g) in 2 N NaOH (50 ml) was added a solution of $CuCl_2 \cdot 2H_2O$ (8.5 g) in H_2O (50 ml) and the mixture was stirred for 1 hr at room temperature. The precipitate formed was collected and washed with H_2O. Wet copper aspartate was added to a solution of disodium salt of H-Asp-OH which was prepared from H-Asp-OH (5.0 g), 2 N NaOH (40 ml) and H_2O (210 ml). The solution was stirred for 30 min and the insoluble material was removed by filtration. To the filtrate diluted with DMF (350 ml) was added a solution of *p*-nitrobenzyl bromide (17.2 g) in DMF (150 ml) with vigorous stirring, and the mixture was stirred for 24 hr at 40°. The precipitate formed was collected and washed with 80% DMF, H_2O and acetone. The copper complex of H-Asp(ONb)-OH was added to a boiling solution of ethylenediaminetetraacetic acid tetrasodium salt (40 g) in H_2O (1500 ml) of which pH was adjusted to 4.5—5.0 with AcOH. The solution was kept at 4° overnight. Crystals formed were collected and washed with H_2O; needles, yield, 8.8 g (44%); mp 188—189° (lit.189—190°,[10a] 191°,[10b] 193—195°[10c]); $[\alpha]_D^{13}$ +15.7° (c=1.0, AcOH), [lit. $[\alpha]_D^{21}$ +12.1° (c=1.0, AcOH),[10a] $[\alpha]_D$ +15.13°[10b]]; *Rf* (A) 0.42, *Rf* (B) 0.58, single spot positive to ninhydrin.

H-Asp(OPac)-OH (II)[28]——II was prepared from sodium salt of copper aspartate and phenacyl bromide (3.48 g) as described for the preparation of I. The product was recrystallized from H_2O; needles, yield, 1.5 g (33%); mp 155—157° (decomp.) (lit. 177—179°[8]); $[\alpha]_D^{14}$ −23.8° (c=1.0, AcOH); *Rf* (A) 0.41, *Rf* (B) 0.57 single spot positive to ninhydrin; *Anal.* Calcd. for $C_{12}H_{13}O_5N$: C, 57.37; H, 5.22; N, 5.58. Found: C, 57.43; H, 5.27; N, 5.51.

Z(OMe)-Asp(ONb)-OH•DCHA (III)——Z(OMe)-Asp(ONb)-OH was prepared from I (1.5 g) and *p*-methoxybenzyl S-4,6-dimethylpyrimidin-2-yl thiocarbonate (1.8 g) in DMSO (20 ml) containing Et_3N (0.82 ml) by the procedure given by Nagasawa, *et al.*[11] To a solution of this oily product in EtOH (4 ml) and ether (4 ml), was added DCHA (1.5 ml) and the mixture was stirred for 1 hr and the precipitate formed was recrystallized from EtOH and ether; yield, 1.15 g (33%); mp 112—114°; $[\alpha]_D^{17}$ −2.2° (c=0.7, DMF); *Anal.* Calcd. for $C_{32}H_{43}O_9N_3$: C, 62.62; H, 7.06; N, 6.85. Found: C, 62.30; H, 7.09; N, 6.78.

H_2^+-Ser(Bzl)-OBzl•Cl^- (IV)——A suspension of Boc-Ser(Bzl)-OH[13] (2.95 g), Et_3N (1.40 ml) and benzyl bromide (1.71 g) in EtOAc (20 ml) was heated under reflux for 5 hr and then allowed to stand overnight at room temperature. The mixture was washed with 1 N $NaHCO_3$ and H_2O. The organic phase was dried over $MgSO_4$ and evaporated to dryness in vacuum to give oily product. The product dissolved in 4 N HCl in dioxane (15 ml) was stirred for 30 min at room temperature. Dry ether was added to the reaction mixture to give crystals which were collected and washed with dry ether; plates, yield, 2.56 g (80% based on Boc-Ser-(Bzl)-OH). For analysis a sample was reprecipitated from MeOH and dry ether, mp 126—130°; $[\alpha]_D^{17}$ −29.8° (c=1.0, EtOH); *Rf* (A) 0.79, *Rf* (B) 0.90, single spot positive to ninhydrin; *Anal.* Calcd. for $C_{17}H_{20}O_3NCl$: C, 63.45; H, 6.26; N, 4.35. Found: C, 63.22; H, 6.24; N, 4.36.

Boc-Asp(OBzl)-Ser(Bzl)-OBzl (V)——To a solution of Boc-Asp(OBzl)-OH (323 mg), IV (322 mg) and HOBt (135 mg) in DMF (5 ml) containing N-methylmorpholine (0.11 ml) was added DCC (226 mg) at 0° and stirred at 4° overnight. Dicyclohexylurea was removed by filtration. EtOAc was added to the filtrate and the EtOAc solution was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The EtOAc solution was dried over $MgSO_4$ and evaporated to dryness. The residue was recrystallized from EtOAc and petroleum ether; needles, yield, 365 mg (62%); mp 40—42°; $[\alpha]_D^{15}$ −16.9° (c=0.8, DMF); *Rf* (A) 0.90, *Rf* (B) 0.97, single spot positive to ninhydrin; *Anal.* Calcd. for $C_{33}H_{38}O_8N_2$: C, 67.10; H, 6.49; N, 4.74. Found: C, 67.06; H, 6.56; N, 4.46.

24) S.M. Partridge, *Biochem. J.*, **42**, 238 (1948).
25) S.G. Waley and G. Watson, *Biochem. J.*, **55**, 328 (1953).
26) S. Moore, D.H. Spackman, and W.H. Stein, *Anal. Chem.*, **30**, 1185 (1958).
27) K. Hofmann, F.M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).
28) A part of the product was decomposed to give a light brown material during 6 months storage in a brown bottle. Aspartic acid was detected as one of the decomposed component as analyzed on paper chromatography.

Z(OMe)-Asp(ONb)-Ser(Bzl)-OBzl (VI)——VI was prepared from Z(OMe)-Asp(ONb)-OH (216 mg, prepared from III) and IV (161 mg) as described for the preparation of V. The oily product was recrystallized from EtOAc; needles, yield, 175 mg (50%); mp 131—132°; $[\alpha]_D^{15}$ −5.4° (c=0.7, DMF); *Rf* (A) 0.82, *Rf* (B) 0.94, single spot positive to ninhydrin; *Anal.* Calcd. for $C_{37}H_{37}O_{11}N_3$: C, 63.51; H, 5.33; N, 6.01. Found: C, 63.47; H, 5.33; N, 5.84.

Boc-Asp(OPac)-Ser(Bzl)-OBzl (VII)——VII was prepared from Boc-Asp(OPac)-OH[8] (176 mg) and IV (161 mg) as described for the preparation of V. The oily product was recrystallized from EtOAc and petroleum ether; yield, 196 mg (63%); mp 78—80°; $[\alpha]_D^{15}$ −36.0° (c=0.8, DMF); *Rf* (A) 0.86, *Rf* (B) 0.97, single spot positive to ninhydrin; *Anal.* Calcd. for $C_{34}H_{38}O_9N_2$: C, 66.00; H, 6.19; N, 4.53. Found: C, 65.67; H, 6.26; N, 4.37.

Boc-Asp(—Ser(Bzl)-OBzl)-ONb (VIII)——VIII was prepared from Boc-Asp-ONb[15] (246 mg) and IV (214 mg) as described for the preparation of V. The product was recrystallized from EtOAc; needles, yield, 320 mg (76%); mp 138°; $[\alpha]_D^{17}$ −19.6° (c=0.8, DMF); *Rf* (A) 0.79, *Rf* (B) 0.95, *Rf* (C) 0.93, single spot positive to ninhydrin; *Anal.* Calcd. for $C_{33}H_{37}O_{10}N_3$: C, 62.35; H, 5.87; N, 6.61. Found: C, 62.54; H, 5.90; N, 6.45.

Boc-Asp(OBzl)-Pro-OBzl (IX)——IX was prepared from Boc-Asp(OBzl)-OH (323 mg) and H_2^+-Pro-OBzl·Cl^-[18] (242 mg) as described for the preparation of V. The resulting oil was washed with petroleum ether by decantation and dried in vacuum to give an oily product; yield, 364 mg (71%); $[\alpha]_D^{13}$ −50.0° (c=1.0, DMF); *Rf* (A) 0.71, *Rf* (B) 0.87, single spot positive to ninhydrin; *Anal.* Calcd. for $C_{28}H_{34}O_7N_2$: C, 65.86; H, 6.71; N, 5.49. Found: C, 65.58; H, 6.77; N, 5.43.

H-Asp-Ser-OH (X)——VII (66.2 mg) was treated with TFA (1 ml) for 10 min at room temperature. The solvent was evaporated to dryness in vacuum. The residue dissolved in MeOH–H_2O (1: 1 v/v, 15 ml) was hydrogenated over Pd–C (100 mg) under atmospheric pressure for 5 hr. The catalyst was removed by the aid of Cellite. The solution was evaporated to dryness; yield, 23 mg (86%). For analysis a sample dissolved in H_2O (3 ml) was applied to a column (1.7×10 cm) of Dowex 1×2 (acetate form), which was eluted with H_2O (100 ml), 1% AcOH (100 ml) and 6% AcOH (100 ml). Fractions of 5.4 ml each were collected. Fractions positive to Cl-*o*-tolidine reagent were pooled and lyophilized; mp 119—129°; $[\alpha]_D^{16}$ +23.9° (c=0.6, H_2O); *Rf* (A) 0.12, *Rf* (B) 0.14, *Rf* (C) 0.39, single spot positive to ninhydrin; amino acid ratios in the acid hydrolysate: Asp 1.02, Ser 0.98 (average recovery 85%); amino acid ratios in AP-M digest: Asp 0.94, Ser 1.06 (average recovery 90%); *Anal.* Calcd. for $C_7H_{12}O_6N_2 \cdot H_2O$: C, 35.29; H, 5.92; N, 11.76. Found: C, 34.89; H, 5.76; N, 11.59.

H-Asp(—Ser-OH)-OH (XI)——XI was prepared from VIII (113 mg) as described for the preparation of X. In this case, the crude peptide dissolved in H_2O (3 ml) was applied to a column (1.7×16 cm) of Dowex 1×2 (acetate form), which was eluted with H_2O (150 ml), 1% AcOH (150 ml) and 6% AcOH (150 ml). Fractions of 5.5 ml each were collected. Fractions positive to Cl-*o*-tolidine reagent were pooled and lyophilized; yield, 40.2 mg (95%); mp 115—125°; $[\alpha]_D^{17}$ −4.1° (c=0.9, H_2O); *Rf* (A) 0.08, *Rf* (B) 0.12, *Rf* (C) 0.27, single spot positive to ninhydrin; amino acid ratios in the acid hydrolysate: Asp 1.06, Ser 0.96 (average recovery 91%); *Anal.* Calcd. for $C_7H_{12}O_6N_2 \cdot H_2O$: C, 35.29; H, 5.92; N, 11.76. Found: C, 35.26; H, 5.73; N, 11.80.

HF Treatment of Protected Dipeptides (V, VI, VII)

Preparation of Va——V (127 mg) was stirred for 1 hr at 0° in HF (5 ml) containing anisole (5 eq.). The HF was completely distilled off in vacuum. The residue was dissolved in 1% AcOH and extracted with EtOAc–ether (1: 1 v/v). The aqueous layer was lyophilized. The product was applied on paper chromatography (see Fig. 1).

Preparation of Vb——Va (5 mg) dissolved in aqueous 1% ammonium bicarbonate was allowed to stand at 40° for 24 hr and lyophilized. The product was applied on paper chromatography (see Fig. 1).

Preparation of VIa——VI (100 mg) was treated in a similar manner as described above. The product was applied on paper chromatography (see Fig. 1). IR ν_{max}^{Nujol} cm^{-1}: 1735, 1600, 1520, 1350, 1195, 850.

Preparation of VIb——VIa (10 mg) was hydrogenated as described for the preparation of X. The product was applied on paper chromatography (see Fig. 1).

Preparation of VIIa——VII (110 mg) was treated in a similar manner as described above. The product was applied on paper chromatography (see Fig. 1). IR ν_{max}^{Nujol} cm^{-1}: 1735, 1600, 1195, 755, 690.

Preparation of VIIb——VIIa (10 mg) in 80% AcOH was treated with zinc powder in three portions at 5 min intervals and stirred for 1 hr at room temperature. The product was applied on paper chromatography (see Fig. 1).

HF Treatment of IX——IX (100 mg) was treated with HF as described in the above experiment; yield, 43.2 mg (96%); *Rf* (A) 0.19, *Rf* (B) 0.19, single spot positive to ninhydrin; amino acid ratios in the acid hydrolysate: Asp 1.05, Pro 0.95 (average recovery 85%); in the amino acid analysis of the crude dipeptide (peptide content=9.6 μmole), Asp and Pro were detected 0.25—0.50%. For analysis a sample (36 mg) was purified in a similar manner as described for the preparation of X; mp 110—115°; $[\alpha]_D^{12}$ −94.2° (c=1.0, H_2O); amino acid ratios in the acid hydrolysate: Asp 0.91, Pro 1.09 (average recovery 91%); *Anal.* Calcd. for $C_9H_{14}O_5N_2$: C, 46.95; H, 6.13; N, 12.17. Found: C, 46.56; H, 6.42; N, 12.26.

Synthesis of H-Asp-Ser-Asp-Pro-Arg-OH by Solid Phase Method

Boc-Ser(Bzl)-Asp(ONb)-Pro-Arg(NO_2)resin (XII)——Boc-Arg(NO_2)-resin was prepared from Boc-Arg(NO_2)-OH and dry chloromethylated polystyrene 2% divinylbenzene in DMF according to the procedure described by Sakakibara[29] and Marglin.[29] The amino acid content so obtained was 0.263 mmole Arg/g.

Boc-Arg(NO_2)-resin (7.40 g, arginine content=1.95 mmole) was placed in the reaction vessel and each Boc-amino acid was incorporated as follows: (1) three washings with CH_2Cl_2; (2) a 30 min treatment with 50% TFA in CH_2Cl_2; (3) three washings with CH_2Cl_2; (4) two washings with isopropyl alcohol; (5) three washmings with CH_2Cl_2; (6) prewash for 5 min with 10% Et_3N in CH_2Cl_2 followed by a 5 min treatment with the same solution; (7) three washings with CH_2Cl_2; (8) two washings with isopropyl alcohol; (9) three washings with CH_2Cl_2; (10) addition of 5.85 mmole of Boc-amino acid in 50 ml of CH_2Cl_2 followed by a 5 min stirring; (11) a 10 ml solution of CH_2Cl_2 containing 5.85 mmole of DCC was then added and the entire mixture stirred for 300 min; (12) three washings with CH_2Cl_2; (13) three washings with isopropyl alcohol; (14) three washings with CH_2Cl_2; (15) a 60 min treatment with 3 ml of Ac_2O and 1.8 ml of Et_3N in 60 ml of CH_2Cl_2; (16) three washings with CH_2Cl_2; (17) four washings with isopropyl alcohol. Volumes of solvent and solution of reagent(s) used were 60 ml each operation. Boc-Asp(ONb)-OH was dissolved in DMF–CH_2Cl_2 (1 : 2 v/v). After all the reaction cycle were over, the resin was dried over P_2O_5 in vacuum; yield, 8.20 g.

Boc-Asp(ONb)-Ser(Bzl)-Asp(ONb)-Pro-Arg(NO_2)-resin (XIII)——XIII was prepared from XII (2.14 g) and Boc-Asp(ONb)-OH (546 mg) in a similar manner as described for the preparation of XII. Volumes of solvent and solution of reagent (s) used were 20 ml in each operation. Yield, 2.26 g.

Boc-Asp(OBzl)-Ser(Bzl)-Asp(ONb)-Pro-Arg(NO_2)-resin (XIV)——XIV was prepared from XII (2.06 g) and Boc-Asp(OBzl)-OH (462 mg) in a similar manner as described for the preparation of XIII; yield, 2.16 g.

H-Asp-Ser-Asp-Pro-Arg-OH——(A) XVa From XIII: XIII (1.97 g) was stirred for 1 hr at 0° in HF (20 ml) containing anisole (5.0 ml). The HF was completely distilled off in vacuum. The residue was extracted with 2% AcOH (20 ml, 10 ml×2) and the extract was washed well with EtOAc. Combined extract was concentrated to small volume in vacuum and passed through a column (1.7×20 cm) of Sephadex G-10[30] which was equilibrated with 1 N AcOH and eluted with 1 N AcOH. Sakaguchi test positive eluates were pooled and lyophilized; weight 322 mg. An aliquot (69.1 mg) in H_2O (15 ml) was hydrogenated over Pd–C for 7 hr. The hydrogenated product in H_2O (3 ml) was added to a column (1.7×18 cm) of Dowex 1×2 (acetate form) which was eluted with a linear gradient of H_2O (300 ml) in the mixing chamber to 0.2 M AcOH (300 ml) in the mixing chamber to 0.2 M AcOH (300 ml) in the resorvior. Fractions of 5.4 ml each were collected with an automatic fraction collector and absorbancy at 230 nm was determined on each fraction. The eluates in tubes No. 66—73 were pooled and lyophilized; yield, 12 mg (22% based on Boc-Arg(NO_2)-resin); mp 181—195° (decomp.); $[\alpha]_D^{10}$ −88.3° (c=0.6, H_2O); *Rf* (A) 0.03, *Rf* (B) 0.09, single spot positive to ninhydrin and Sakaguchi reagents; E_{Asp} 0.45; amino acid ratios in the acid hydrolysate: Arg 0.84, Asp 2.20, Ser 0.89. Pro 1.07 (average recovery 98%); amino acid ratios in AP-M digest:[20] Arg 0.95, Asp 1.05, Ser 1.00 (average recovery 92%).

(B) XVb From XIV: XIV (1.89 g) was treated with HF in a similar manner as described for the preparation of XVa; weight 270 mg. An aliquot (74.4 mg) in H_2O was hydrogenated under the same condition as described above. The hydrogenated product was chromatographed on a column (1.7×18 cm) of Dowex 1×2 (acetate form) in a similar manner as described for the preparation of XVa. The eluates in tubes No. 61—65 (fraction A) were pooled and lyophilized; weight, 7.0 mg; *Rf* (A) 0.06 (minor) and 0.04 (main), *Rf* (B) 0.12 (minor) and 0.07 (main), two spots positive to ninhydrin and Sakaguchi reagents. This material was rechromatographed on a column (1.7×16 cm) of Dowex 1×2 (acetate form) as described above. The eluates in tubes No. 60—63 were pooled and lyophilized; yield, 3.2 mg (5% based on Boc-Arg(NO_2)-resin); mp 182—196° (decomp.); *Rf* (A) 0.04, *Rf* (B) 0.08, single spot positive to ninhydrin and Sakaguchi reagents; E_{Asp}0.45; amino acid ratios in the acid hydrolysate: Arg 0.87, Asp 1.94, Ser 0.97, Pro 1.21 (average recovery 81%); amino acid ratios in AP-M digest:[20] Arg 0.88, Asp 1.07, Ser 1.04 (average recovery 93%).

H-Asp(-Ser-Asp-Pro-Arg-OH)-OH——In the above experiment (XVb), the eluates in tubes No. 71—74 (fraction B) were pooled and lyophilized; yield, 2.0 mg (3% based on Boc-Arg(NO_2)-resin); mp 180—200° (decomp.); *Rf* (A) 0.02, *Rf* (B) 0.10, single spot positive to ninhydrin (blue-grey color)[31] and Sakaguchi reagents; E_{Asp}0.52; amino acid ratios in the acid hydrolysate: Arg 0.80, Asp 2.15, Ser 1.00, Pro 1.04 (average recovery 90%): amino acid ratios in AP-M digest: Arg 1.00, Asp 0.12, Ser 0.08.

Synthesis of H-Asp-Ser-Asp-Pro-Arg-OH by Conventional Liquid Phase Method

Z-Pro-Arg(NO_2)-ONb (XVI)——Z-Arg(NO_2)-ONb (2.1 g) was dissolved in AcOH (7 ml), anisole (1 ml) and 5.7 N HBr in AcOH (7 ml). After 40 min at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate was washed with dry ether and dried over KOH pellets in vacuum.

29) A Kishi, Y. Kishida, and S. Sakakibara, "Proceedings of the 7th Symposium on Peptide Chemistry," ed. by S. Akabori, Protein Research Foundation, Osaka, 1969, p. 36; A. Marglin, *Tetrahedron Letters*, **1971**, 3145.

30) C.H. Li, D. Yamashiro, and S. Lemaire, *Biochemistry*, **14**, 953 (1975).

31) R.W. Hanson and H.N. Rydon, *J. Chem. Soc.*, **1964**, 836.

To a solution of H_2^+-Arg(NO_2)-ONb·Br^- in DMF (10 ml) was added Z-Pro-ONp (1.6 g) followed by addition of Et_3N to keep the solution slightly alkaline. After 20 hr at room temperature, the reaction mixture was diluted with 1 N NH_4OH (10 ml), stirred for 1 hr, and mixed with EtOAc. The EtOAc solution was washed successively with 1 N NH_4OH, H_2O, 1 N HCl and H_2O, and dried over $MgSO_4$. The solution was concentrated to a small volume and petroleum ether was added to the residue. The precipitate was reprecipitated from EtOAc and petroleum ether; yield, 2.5 g (99%); mp 74—77°; $[\alpha]_D^{12}$ −30.6° (c=1.0, DMF); *Rf* (A) 0.44, *Rf* (B) 0.82, single spot positive to ninhydrin; *Anal.* Calcd. for $C_{26}H_{31}O_9N_7$: C, 53.33; H, 5.34; N, 16.75. Found: C, 53.06; H, 5.71; N, 16.57.

Boc-Asp(OBzl)-Pro-Arg(NO_2)-ONb (XVII)——XVI (2.0 g) was treated with 5.7 N HBr in AcOH as described for the preparation of XVI. XVII was prepared from H_2^+-Pro-Arg(NO_2)-ONb·Br^- and Boc-Asp(OBzl)-OH (1.1 g) as described for the preparation of V. The oily product was reprecipitated from EtOAc and petroleum ether; yield, 1.6 g (62%); mp 85—89°; $[\alpha]_D^{12}$ −27.4° (c=0.9, DMF); *Rf* (A) 0.62, *Rf* (B) 0.88, single spot positive to ninhydrin; *Anal.* Calcd. for $C_{34}H_{44}O_{12}N_8$: C, 53.96; H, 5.85; N, 14.81. Found: C, 53.94; H, 6.17; N, 14.63.

Boc-Ser(Bzl)-Asp(OBzl)-Pro-Arg(NO_2)-ONb (XVIII)——XVII (1.5 g) was treated with 4 N HCl in dioxane (5 ml) as described for the preparation of IV. XVIII was prepared from H_2^+-Asp(OBzl)-Pro-Arg(NO_2)-ONb·Cl^- and Boc-Ser(Bzl)-OH (0.59 g) as described for the preparation of V. The oily product was reprecipitated from EtOAc and petroleum ether; yield, 1.1 g (59%); mp 90—95°; $[\alpha]_D^{12}$ −30.8° (c=0.8, DMF); *Rf* (A) 0.84, *Rf* (B) 0.90, single spot positive to ninhydrin; *Anal.* Calcd. for $C_{44}H_{55}O_{14}N_9$: C, 56.58; H, 5.94; N, 13.50. Found: C, 56.28; H, 6.20; N, 13.67.

Boc-Asp(OBzl)-Ser(Bzl)-Asp(OBzl)-Pro-Arg(NO_2)-ONb (XIX)——XVIII (446 mg) was dissolved in 4 N HCl in dioxane (2 ml). After 20 min at room temperature, the reaction mixture was evaporated to dryness in vacuum quickly. XIX was prepared from H_2^+-Ser(Bzl)-Asp(OBzl)-Pro-Arg(NO_2)-ONb·Cl^- and Boc-Asp(OBzl)-OH (162 mg) as described for the preparation of V. The oily product was reprecipitated from EtOAc and petroleum ether; 0.5 g (91%); mp 65—69°; $[\alpha]_D^{13}$ −34.1° (c=0.9, DMF); *Rf* (A) 0.80, *Rf* (B) 0.95, single spot positive to ninhydrin; *Anal.* Calcd. for $C_{55}H_{66}O_{17}N_{10}$: C, 57.99; H, 5.84; N, 12.30. Found: C, 57.51; H, 6.24; N, 12.27.

H-Asp-Ser-Asp-Pro-Arg-OH (XX)——XIX (93 mg) was dissolved in TFA (1 ml) and the reaction mixture was stirred for 10 min at room temperature and then treated as described for the preparation of IV. The resulting material in MeOH–H_2O (5: 4 v/v, 15 ml) was hydrogenated over Pd–C (100 mg) for 7 days. The hydrogenated product was chromatographed on a column (1.7×17 cm) of Dowex 1×2 (acetate form) as described for the preparation of XVa. Fractions of 6.0 ml each were collected. The eluates in tubes No. 48—54 were pooled and lyophilized; weight 14.6 mg; *Rf* (A) 0.20 (minor) and 0.04 (main), *Rf* (B) 0.30 (minor) and 0.08 (main), two spots positive to ninhydrin and Sakaguchi reagents. This material was purified by partition chromatography on Sephadex G-25,[32] which was equilibrated with BuOH–AcOH–H_2O (4: 1: 5, lower layer) and eluted with BuOH–AcOH–H_2O (4: 1: 5, upper layer) (660 ml) and then with BuOH–AcOH–H_2O (4: 1: 5, lower layer). Fractions of 6.0 ml each were collected. Fractions (the eluates in tubes No. 128—135) positive to Cl-*o*-tolidine reagent were pooled, evaporated to dryness and lyophilized; yield, 12 mg (25%); mp 185—200° (decomp.); $[\alpha]_D^{10}$ −87.7° (c=0.5, H_2O); *Rf* (A) 0.04, *Rf* (B) 0.09, single spot positive to ninhydrin and Sakaguchi reagents; E_{Asp} 0.45; amino acid ratios in the acid hydrolysate: Arg 0.99, Asp 1.91, Ser 0.93, Pro 1.17 (average recovery 86%); amino acid ratios in AP-M digest:[20] Arg 1.10, Asp 0.90, Ser 1.00 (average recovery 91%).

H-Asp-Ser(Bzl)-Asp-Pro-Arg-OH——In another experiment, XIX (150 mg) was treated with TFA as described above and hydrogenated for 48 hr and then the hydrogenated product was treated in a similar manner as described above, the material of *Rf* (A) 0.20 was isolated; yield, 11.0 mg (13%); mp 173—179° (decomp.); $[\alpha]_D^{12}$ −50.1° (c=0.9, H_2O); *Rf* (A) 0.20, *Rf* (B) 0.30, single spot positive to ninhydrin and Sakaguchi reagents; amino acid ratios in the acid hydrolysate: Arg 0.98, Asp 2.12, Ser 0.85, Pro 1.05 (average recovery 88%); amino acid ratios in AP–M digest: Arg 0.96, Asp 1.04 (average recovery 91%). This material (10 mg) dissolved in H_2O was further hydrogenated over Pd–C for 4 days and applied on paper chromatography; *Rf* (A) 0.20 (minor) and 0.04 (main), *Rf* (B) 0.30 (minor) and 0.09 (main), two spots positive to ninhydrin and Sakaguchi reagents.

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