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Synthetic Studies of Bacitracin. I. Synthesis of a Protected Intermediate Heptapeptide (5—11)

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A protected heptapeptide, the formyl-L-isoleucyl-ε-benzyloxycarbonyl-L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-L-aspartic acid α-methyl-β-benzyl ester, was synthesized by the azide method and the carbodiimide method. It is an intermediate peptide for the total synthesis of the antibiotic bacitracin A, the structure of which is still ambiguous at a few points. β-Benzyl benzyloxycarbonyl-L-aspartate was prepared by a novel method similar to that used in the synthesis of γ-benzyl glutamate. The α-methyl-β-benzyl L-aspartate prepared could be useful in the synthesis of branched aspartic acid peptide. Phenomena of dimorphism were observed in crystals of δ-tosyl-D-ornithine and δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanine ethyl ester hydrobromide, and two crystal forms of each compound were isolated in pure states.

Bacitracin is a cyclic polypeptide antibiotic produced from *Bacillus licheniformis*,[1,2] and a mixture of several peptides with similar structures.[3,4] Among the members of the bacitracin group, bacitracin A has been most often subjected to strutural study, since it is the main component and since it has the most active antibacterial effect.[5] The most probable structure proposed for bacitracin A is shown in Fig. 1,[6–10] although a few ambiguous points still remain to be resolved.[11] In Fig. 1, the dotted line represents the cyclol structure, and the directions of peptide linkages are shown by arrows, the points of which indicate the nitrogen of the peptide bond (→ : –CO–NH–).

In this molecule, some unusual structures are included which have not been found in other natural peptides. For example, it involves a thiazoline ring between the *N*-terminal isoleucine (1) and the leucine (3) residue. The thiazoline in the peptide may be formed biogenetically from the cysteine residue. Actually the formation of the thiazoline ring in the peptide was shown by two groups, using the model peptides with a cysteine residue.[10,12]

The cyclol structure assumed to be present between an amino group of *N*-terminal isoleucine (1) and a carboxyl group of phenylalanine (9) is also a very peculiar feature in the peptide structure, although ergot alkaloids certainly contain the cyclol structure in their peptide moiety.[13,14]

Concerning the linkage between an amino group of lysine (6) and a carboxyl group of aspartic acid (11), Swallow and Abraham concluded the possibility of the four partial structures shown in Fig. 2, but they could not give positive support to any one structure of the four.[11] Stoffel and Craig suggested

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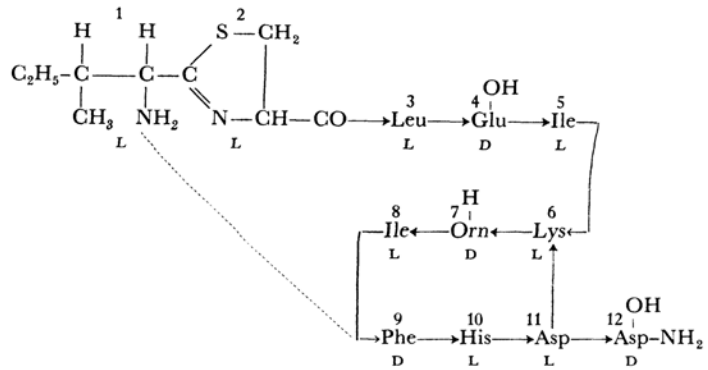
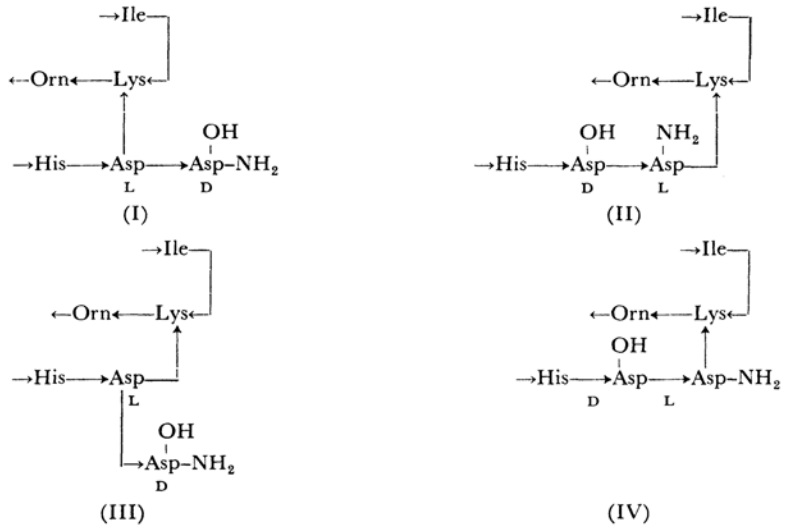
1) B. A. Johnson, H. Anker and F. L. Meleney, *Science*, **102**, 376 (1945).
2) F. L. Meleney and B. A. Johnson, *Am. J. Med.*, **7**, 794 (1949).
3) G. G. F. Newton and E. P. Abraham, *Biochem. J.*, **47**, 257 (1950).
4) G. T. Barry, J. D. Gregory and L. C. Craig, *J. Biol. Chem.*, **175**, 485 (1948).
5) L. C. Craig, J. R. Weisiger, W. Hausmann and E. J. Harfenist, *ibid.*, **199**, 259 (1952).
6) I. M. Lockhardt, G. G. F. Newton and E. P. Abraham, *Nature*, **173**, 536 (1954).
7) I. M. Lockhardt and E. P. Abraham, *Biochem. J.*, **58**, 633 (1954).
8) W. Hausmann, J. R. Weisiger and L. C. Craig, *J. Am. Chem. Soc.*, **77**, 723 (1955).
9) J. R. Weisiger, W. Hausmann and L. C. Craig, *ibid.*, **77**, 731 (1955).
10) W. Stoffel and L. C. Craig, *ibid.*, **83**, 145 (1961).
11) D. L. Swallow and E. P. Abraham, *Biochem. J.*, **72**, 326 (1959).
12) Y. Hirotsu, T. Shiba and T. Kaneko. The details will be published soon by them in This Bulletin.
13) A. Stoll, A. Hoffman and T. Petrzilka, *Helv. Ch.mi Acta*, **34**, 1544 (1951).
14) A. Stoll, "Progress in Chem. of Org. Nat. Products," Vol. 8, p. 114 (1952).



(I)

Fig. 1. Bacitracin A.



(I) (II) (III) (IV)

Fig. 2

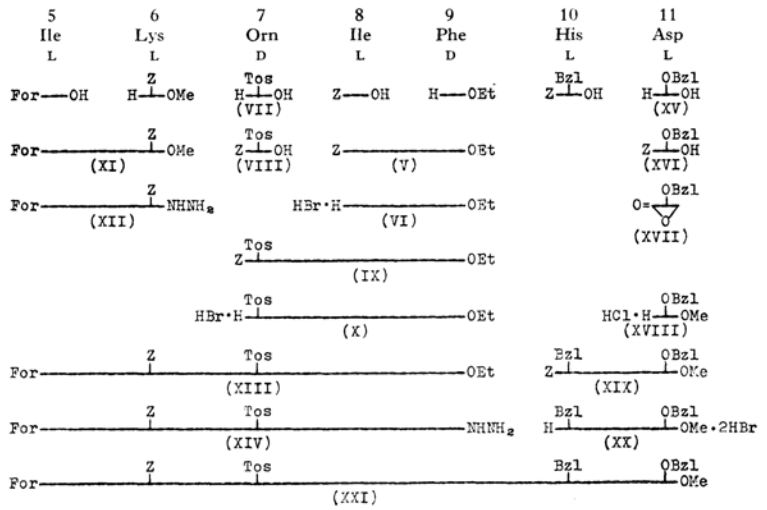


Fig. 3. Scheme for the synthesis of a protected intermediate peptide (5—11).
For=HCO–, Z=$C_6H_5CH_2OCO$–, Tos=*p*-$CH_3C_6H_4SO_2$–, Bzl=$C_6H_5CH_2$–

that the ε-amino group of lysine (6) might most likely be linked with the β-carboxyl group of aspartic acid (11); they made this suggestion on the basis of the results of their partial hydrazinolysis.[10] This total structure, shown in Fig. 1, corresponds to I in Fig. 2. However, according to a recent paper by Ressler and Kashelikar, the alternative structure II is the most possible structure of bacitracin A; this is based on the results of the reaction of commercial bacitracin A upon dehydration-reduction.[15]

The present study was undertaken to synthesize the peptides of the proposed structures and to compare them with natural bacitracin A, thus making it possible to elucidate these structural features. In this paper, the synthesis of the protected heptapeptide (5—11), namely, the formyl-L-isoleucyl-ε-benzyloxycarbonyl-L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-L-aspartic acid α-methyl-β-benzyl ester, is described; it is the intermediate peptide for the synthesis of structure I in Fig. 1. The scheme of the synthesis performed is shown in Fig. 3.

Benzyloxycarbonyl-L-isoleucine was coupled with ethyl D-phenylalaninate by means of the mixed anhydride method to form the benzyloxycarbonyl-L-isoleucyl-D-phenylalanine ethyl ester (V). The subsequent treatment of V with hydrogen bromide in acetic acid gave the dipeptide ester hydrobromide (VI) in a quantitative yield. The free dipeptide ester of VI was then coupled with benzyloxycarbonyl-δ-tosyl-D-ornithine (VIII) by *N*,*N*′-dicyclohexylcarbodiimide to form the benzyloxycarbonyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanine ethyl ester (IX). The removal of the benzyloxycarbonyl group from IX with hydrogen bromide in acetic acid afforded tripeptide ester hydrobromide (X).

On the other hand, formyl-L-isoleucine[16] was condensed with methyl ε-benzyloxycarbonyl-L-lysinate[17] in dimethylformamide by *N*,*N*′-dicyclohexylcarbodiimide to give the formyl-L-isoleucyl-ε-benzyloxycarbonyl-L-lysine methyl ester (XI). The treatment of XI with hydrazine hydrate in methanol gave the corresponding dipeptide hydrazide (XII) in a nearly quantitative yield. This compound was converted to azide, which was then condensed with the free tripeptide ester derivative of X to give the formyl-L-isoleucyl-ε-benzyloxycarbonyl-L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanine ethyl ester (XIII). The hydrazide (XIV) was obtained in a nearly quantitative yield by the treatment of XIII with hydrazine hydrate in dimethylformamide.

It was found that β-benzyl-L-aspartate (XV) could be prepared in a 43% yield by a method similar to that used in the synthesis of γ-benzyl L-glutamate.[18] The benzyloxycarbonyl group was introduced into XV by Schotten-Baumann reaction in a sodium carbonate solution to give β-benzyl benzyloxycarbonyl-L-aspartate, which showed a slightly higher melting point and optical rotation than the literature values.[19] This compound was then converted to β-benzyl *N*-carboxy-L-aspartate anhydride (XVII) by the procedure of Katchalski *et al.*[19] This was treated with anhydrous methanol containing hydrogen chloride to give α-methyl-β-benzyl L-aspartate hydrochloride (XVIII), which may be a useful intermediate for the synthesis of the branched aspartyl peptide as well as α-benzyl-β-methyl L-aspartate.[20] Benzyloxycarbonyl-*im*-benzyl-L-histidine[21] was coupled with the free ester of XVIII to the benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-aspartic acid α-methyl-β-benzyl ester (XIX) in a good yield. This was treated with hydrogen bromide in acetic acid to give the crude dipeptide ester dihydrobromide (XX), which was thoroughly washed with an aqueous sodium bicarbonate solution to remove a small amount of the corresponding carboxylic acid derivative formed by the cleavage of the benzyl ester during the procedure using hydrogen bromide. The pentapeptide hydrazide (XIV) was converted to the azide in the usual manner and then coupled with the free dipeptide ester derivative of XX to the formyl-L-isoleucyl-ε-benzyloxycarbonyl-L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-L-aspartic acid α-methyl-β-benzyl ester (XXI).

The D-ornithine used here was prepared through Walden inversion from δ-benzoyl-L-ornithine according to the direction of Izumiya,[22] with some modifications. The intermediate product, L-α-bromo-δ-benzamido-*n*-valeric acid, was isolated as colorless prisms with a melting point of 110—112°C.

In the course of the synthesis of the intermediate peptide from D-ornithine, it was found that crystals of δ-tosyl-D-ornithine (VII) and δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanine ethyl ester hydrobromide (X) showed certain phenomena of polymorphism. Two different forms of the crystals of VII were isolated on recrystallization from water by inoculation in the saturated solution. Although, in the literature,[23] only one melting point (212°C (decomp.)) is given for δ-tosyl-L-ornithine, one form obtained here melted at 231—233°C (decomp.), and the

15) C. Ressler and D. V. Kashelikar, *J. Am. Chem. Soc.*, **88**, 2025 (1966).
16) J. C. Sheehan and D.-D. H. Young, *ibid.*, **80**, 1154 (1958).
17) R. A. Boissonnas, St. Guttmann, R. L. Huguenin, P. A. Jaquenoud and E. Sandrine, *Helv. Chim. Acta*, **41**, 1867 (1958).
18) St. Guttmann and R. A. Boissonnas, *ibid.*, **41**, 1852 (1958).
19) A. Berger and E. Katchalski, *J. Am. Chem. Soc.*, **73**, 4084 (1951).
20) L. Velluz, G. Amiard, J. Bartos, B. Goffinet and R. Heymes, *Bull. Soc. Chim. France*, **1956**, 1464.
21) B. G. Overell and V. Petrow, *J. Chem. Soc.*, **1955**, 232.
22) N. Izumiya, *Nippon Kagaku Zasshi* (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **72**, 149 (1951).
23) B. F. Erlanger, H. Sachs and E. Brand, *J. Am. Chem. Soc.*, **76**, 1806 (1954).

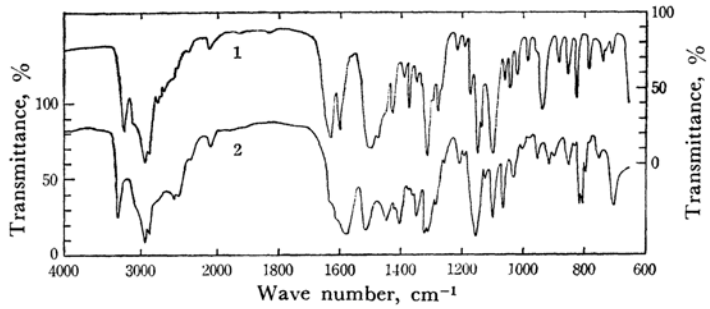


Fig. 4. Infrared spectra of δ-tosyl-D-ornithine.
1, mp 234.5—235.5°C (decomp.) 2, mp 231—233°C (decomp.)

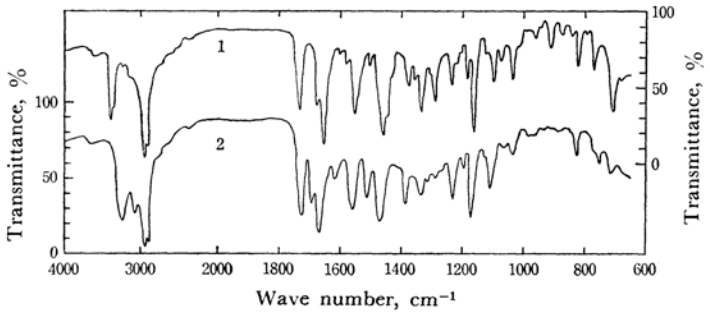


Fig. 5. Infrared spectra of δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanine ethyl ester hydrobromide.
1, mp 212.5—213.5°C 2, mp 171—172°C

other, at 234.5—235.5°C(decomp.). They are identical in elementary analysis, but quite different in infrared spectra (Fig. 4). Two forms of X, melting at 171—172°C and at 212.5—213.5°C, were also isolated on recrystallization from ethanol-ether in a similar way. The crystal with the lower melting point was transformed into the one with the higher melting point after fusion above its own melting point (171—172°C) and resolidification, or after storage for several months at room temperature. The infrared spectra of the two forms of the crystals of X are shown in Fig. 5.

Experimental

All melting points are uncorrected. The infrared absorption spectra were measured in Nujol mull on a Hitachi EPI-2 spectrophotometer.

Benzyloxycarbonyl-L-isoleucyl-D-phenylalanine Ethyl Ester (V). To a cold solution of 68.0 g (0.26 mol) of benzyloxycarbonyl-L-isoleucine and 25.3 g (0.25 mol) of triethylamine in 500 m*l* of chloroform, 27.5 g (0.25 mol) of ethyl chloroformate were added at −5°C over a 30 min period with vigorous stirring. After the mixture had been stirred for 30 min at −5°C, a cold solution of 57.4 g (0.25 mol) of ethyl D-phenylalaninate hydrochloride and 25.3 g (0.25 mol) of triethylamine in 500 m*l* of chloroform was added, and the reaction mixture was stirred for 2.5 hr at room temperature. The mixture was then washed successively with N hydrochloric acid, water, a 5% sodium bicarbonate solution, and water, and then dried over anhydrous sodium sulfate. After filtration, the filtrate was concentrated to dryness *in vacuo*. The crystalline residue was recrystallized from ethyl acetate to give 86.5 g (78.5%) of V as needles, mp 151—152°C. Recrystallization from the same solvent gave needles with a mp of 152.5—153°C, $[\alpha]_D^{17}+15.3°$ (*c* 3.2, dimethylformamide).

Found: C, 68.36; H, 7.24; N, 6.36%. Calcd for $C_{25}H_{32}O_5N_2$: C, 68.16; H, 7.32; N. 6.36%.

L-Isoleucyl-D-phenylalanine Ethyl Ester Hydrobromide (VI). A suspension of 73.0 g of V in 500 m*l* of 26% (w/w) hydrogen bromide in acetic acid was permitted to stand at room temperature, with occasional shaking, for 20 min, by which time the evolution of carbon dioxide had ceased. To the reaction mixture, 1.5 *l* of anhydrous ether was then added to complete the precipitation of the product. After cooling for 15 min, the crystals were collected by filtration, washed with anhydrous ether, and dried; yield 64.0 g (99.7%). Mp 219.5—220.5°C (decomp.). Recrystallization from anhydrous ethanol gave 56.3 g (87.7%) of needles, mp 222.0—222.5°C (decomp.), $[\alpha]_D^{17}$ +67.9° (*c* 2.7, dimethylformamide).

Found: C, 52.72; H, 7.04; N, 7.19; Br, 20.39%. Calcd for $C_{17}H_{27}O_3N_2Br$: C, 52.72; H, 7.02; N, 7.23; Br, 20.64%.

D-Ornithine Hydrochloride. This compound was prepared from the enantiomer according to the directions of Izumiya.[22] The intermediate, L-α-bromo-δ-benzamido-*n*-valeric acid, was now obtained as colorless

prisms,*2 while it has been described as a semisolid in the literature.[22] Mp 233—234°C (decomp.), $[\alpha]_D^{20}$ −11.1° (*c* 5.2, water); lit.[22] mp 234—235°C, $[\alpha]_D^{10}$ −11.8° (*c* 2.37, water). Conf., lit.,[24] antipode: $[\alpha]_D^{23}$ +11.0° (*c* 5.5, water).

Found: C, 35.67; H, 7.73; N, 16.56; Cl, 21.11%. Calcd for $C_5H_{13}O_2N_2Cl$: C, 35.61; H, 7.77; N, 16.62; Cl, 21.03%.

δ-Tosyl-D-ornithine (VII). This compound was prepared according to the procedure used in the synthesis of δ-tosyl-L-ornithine by Erlanger *et al.*[23] Seventy-two grams of the copper complex of δ-tosyl-D-ornithine prepared from 33.7 g of D-ornithine hydrochloride were dissolved in 400 m*l* of 2 N hydrochloric acid, and then hydrogen sulfide was passed into the solution for 2 hr. The copper sulfide was removed by filtration with aid of Hyflosupercel, and pyridine was added to the filtrate until the pH was reached 5.6. The crystals formed were collected by filtration, washed with water, and dried *in vacuo* over concentrated sulfuric acid and soda lime; yield 48.5 g (84.7%), mp 232—233°C (decomp.). The infrared spectrum of this compound is identical with that of the crystal with the lower melting point (see the following experiment). Recrystallization from water gave 40.0 g (69.8%) of fine plates, mp 234—235.5°C (decomp.). $[\alpha]_D^{17}$ −20.0° (*c* 2.2, 6 N hydrochloric acid): Conf. lit.,[23] antipode: mp 212°C (decomp.), $[\alpha]_D^{23}$ +20.8° (*c* 2, 6 N hydrochloric acid).

Found: C, 50.22; H, 6.36; N, 10.01; S, 11.00%. Calcd for $C_{12}H_{18}O_4N_2S$: C, 50.32; H, 6.33; N, 9.78; S, 11.19%.

The infrared spectrum of this crystal is shown in Fig. 4.

Transformation of One Crystalline Form of VII to Another. In 30 m*l* of hot water there was dissolved 0.50 g of VII with a mp of 234.5—235.5°C (decomp.). After filtration, a small amount of the crystal with a mp of 232—233°C (decomp.) was added to the warm filtrate. The crystals deposited were collected after the filtrate had been cooled for 1 hr in a refrigerator: yield 0.30 g (60%), fine plates, mp 231—233°C (decomp.), $[\alpha]_D^{17}$ −20.3° (*c* 2.1, 6 N hydrochloric acid). The infrared spectrum of this crystal is shown in Fig. 4.

Found: C, 50.50; H, 6.34; N, 9.65; S, 11.18%.

Benzyloxycarbonyl-δ-tosyl-D-ornithine (VIII). To a solution of 37.2 g (0.13 mol) of VII in 156 m*l* of N sodium hydroxide, there were added 26.6 g of benzyl chloroformate and 130 m*l* of N sodium hydroxide with vigorous stirring over a period of 1.5 hr while the mixture was being cooled in an ice bath. After it had been stirred for an additional 1.5 hr at room temperature, the reaction mixture was diluted with 400 m*l* of water and then extracted twice with ether. The aqueous layer was acidified with 6 N hydrochloric acid, and the oil which separated was extracted with ethyl acetate. The extract was washed with water and dried over anhydrous sodium sulfate. Concentration *in vacuo* gave oily VIII, which did not crystallize; yield 49.0 g (89.6%).

Benzyloxycarbonyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanine Ethyl Ester (IX). To a cold solution of 44.9 g (0.116 mol) of VI and 12.5 g of triethylamine in 400 m*l* of chloroform there was added 1 *l* of anhydrous ether on ice cooling. After the mixture had been cooled for 10 min, the triethylamine hydrochloride which had formed was filtered off. The filtrate was concentrated *in vacuo*, and the residual oil was dissolved in 400 m*l* of tetrahydrofuran. To this solution there were added a solution of 49.0 g (0.116 mol) of VIII in a mixture of 400 m*l* of tetrahydrofuran and 50 m*l* of dimethylformamide and then a solution of 24.8 g of *N*,*N*′-dicyclohexylcarbodiimide in 100 m*l* of tetrahydrofuran with stirring while the mixture was being cooled in an ice bath. The resulting solution was allowed to stand overnight at room temperature, after which time 50 m*l* of dimethylformamide and 1 m*l* of acetic acid were stirred in. After the mixture had been stood for 1 hr, a precipitate of *N*,*N*′-dicyclohexylurea was filtered off and washed with 100 m*l* of dimethylformamide. The filtrate and washings were combined and concentrated *in vacuo*. The residue was dissolved in 150 m*l* of dimethylformamide. After cooling and filtration, the filtrate was concentrated to dryness *in vacuo*. The crystallization of the residue from dimethylformamide - chloroform afforded 61.5 g (74.8%) of a gelatinous mass, mp 175—177°C. Recrystallization from ethanol or dimethylformamide gave colorless needles with a mp of 177.5—179.0°C, $[\alpha]_D^{17}$ +13.6° (*c* 3.3, dimethylformamide). A sample for analysis was dried *in vacuo* at 110°C over phosphorus pentoxide.

Found: C, 62.54; H, 6.84; N, 7.88; S, 4.30%. Calcd for $C_{37}H_{48}O_8N_4S$: C, 62.69; H, 6.82; N, 7.89; S, 4.53%.

δ-Tosyl-D-ornithyl-L-isoleucyl-D-phenylalanine Ethyl Ester Hydrobromide (X). A suspension of 53.5 g of IX in 268 m*l* of 26% (w/w) hydrogen bromide in acetic acid was shaken occasionally at room temperature for 40 min. When 2.8 *l* of anhydrous ether were then added, a semisolid was precipitated; this was separated by decantation and washed with ether. It was crystallized from a mixture of 200 m*l* of anhydrous ethanol and 200 m*l* of anhydrous ether to give 36.3 g (73.3%) of colorless crystals, mp 211—212°C. Further recrystallization from the same solvent gave fine colorless needles with a mp of 212.5—213.5°C, $[\alpha]_D^{17}$ +6.6° (*c* 3.7, dimethylformamide).

Found: C, 52.94; H, 6.72; N, 8.52%. Calcd for $C_{29}H_{43}O_6N_4SBr$: C, 53.11; H, 6.61; N, 8.54%.

The infrared spectrum of this crystal is shown in Fig. 5.

Transformation of One Crystalline Form of X to Another. In 40 m*l* of anhydrous ethanol there were dissolved 1.3 g of X (mp 212.5—213.5°C) on slight warming. Anhydrous ether was then added to this solution until turbidity appeared. After slight warming, the resulting clear solution was immediately cooled in an ice bath. The colorless needles thus formed were collected after the solution had been stored overnight at 0°C; yield 0.90 g (69.2%), mp 171—172°C, $[\alpha]_D^{24}$ +6.1° (*c* 3.6, dimethylformamide).

Found: C, 52.93; H, 6.80; N, 8.41%.

The infrared spectrum of this crystal is shown in Fig. 5. This crystal immediately changed to the original crystalline form above its own melting point (171—

*2 Mp 110—112°C, $[\alpha]_D^{25}$ −31.2° (*c* 2.2, 70% ethanol), −22.7° (*c* 2.6, 70% ethanol+1 eq. of sodium hydroxide).

Found: C, 48.33; H, 4.76; N, 4.69; Br, 26.37%. Calcd for $C_{12}H_{14}O_3NBr$: C, 48.02; H, 4.70; N, 4.67; Br, 26.62%.

24) A. Hunter, *Biochem. J.*, **33**, 27 (1939).

172°C). The resolidified material thus obtained melted above 200°C and was identical in infrared spectrum with the crystal with the higher melting point.

Formyl-L-isoleucyl-ε-benzyloxycarbonyl-L-lysine Methyl Ester (XI). Twelve grams of triethylamine were added to an ice-cooled solution of 36.4 g (0.11 mol) of methyl ε-benzyloxycarbonyl-L-lysinate hydrochloride[17] in 100 m*l* of dimethylformamide, and the triethylamine hydrochloride thus formed was filtered off. The filtrate obtained above and 22.7 g of *N*,*N*′-dicyclohexylcarbodiimide were then added to a solution of 15.9 g (0.1 mol) of formyl-L-isoleucine[16] in 100 m*l* of dimethylformamide which had previously been cooled at −10°C. The mixture was allowed to stand for 48 hr at 0°C. After the addition of 1 m*l* of acetic acid and the filtration of the urea derivative thus formed, the filtrate was concentrated *in vacuo* to the residue, which was then dissolved in chloroform, and washed successively with N hydrochloric acid, water, a 5% sodium bicarbonate solution, and water. The solution was dried over anhydrous sodium sulfate and evaporated *in vacuo* to remove the solvent. The residue was crystallized from ethyl acetate to give 15.5 g (35.6%) of colorless needles, mp 147—149°C. Recrystallization from the same solvent raised the melting point to 152.5—153.5°C. $[\alpha]_D^{16}$ −13.4° (*c* 5.0, dimethylformamide).

Found: C, 60.96; H, 7.76; N, 9.71%. Calcd for $C_{22}H_{33}O_6N_3$: C, 60.67; H, 7.64; N, 9.65%.

Formyl-L-isoleucyl-ε-benzyloxycarbonyl-L-lysine Hydrazide (XII). A solution of 6.0 g (0.014 mol) of XI and 7.5 g of 90% hydrazine hydrate in 60 m*l* of anhydrous methanol was kept at 50°C for 2 hr. The gelatinous material thus formed was collected after the addition of 150 m*l* of water to complete the precipitation; yield 5.9 g (98.3%), mp 186—189°C. Recrystallization from methanol again gave a gelatinous material; mp 210—210.5°C (sintered at 192—193°C), $[\alpha]_D^{17}$ −18.5° (*c* 3.3, dimethylformamide). A sample for analysis was dried *in vacuo* at 90—100°C over phosphorus pentoxide.

Found: C, 58.09; H, 7.90; N, 15.87%. Calcd for $C_{21}H_{33}O_5N_5$: C, 57.91; H, 7.64; N, 16.08%.

Formyl-L-isoleucyl-ε-benzyloxycarbonyl-L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanine Ethyl Ester (XIII). All the experiments for the preparation of this compound were carried out at 2—3°C. In a mixture of 75 m*l* of acetic acid and 45 m*l* of N hydrochloric acid, 6.53 g (0.015 mol) of XII were dissolved, and the solution was cooled at −5°C. A cold concentrated aqueous solution of 1.14 g (0.0165 mol) of sodium nitrite was added, portion by portion and with shaking, to the above solution. After the solution had stood for 10 min, 400 m*l* of a cold saturated solution of sodium chloride was added to the reaction mixture on cooling. The resulting precipitate (ν_{max} 2170 cm^{-1}) was collected by filtration and dissolved in 300 m*l* of cold chloroform. The solution was washed with an ice-cold 5% sodium bicarbonate solution and then water, dried for a short time over anhydrous sodium sulfate, and filtered. A suspension of 9.84 g (0.015 mol) of X in 500 m*l* of chloroform was mixed with 150 m*l* of a 3% sodium bicarbonate solution until all the solids dissolved. The chloroform layer was washed with water and concentrated to dryness *in vacuo* after a brief drying over anhydrous sodium sulfate. The crystalline residue of the free ester was dissolved in 250 m*l* of dimethylformamide and added to the azide solution prepared above. The mixture was allowed to stand at 3°C overnight, and then at room temperature for two days. The gelatinous mass thus precipitated was collected and dried; wt, 3.2 g. A second crop was obtained from the mother liquor after concentration *in vacuo*; it was reprecipitated from dimethylformamide - chloroform - ethyl acetate to give 5.7 g of a gelatinous material; overall yield 8.9 g (60.7%), mp 215.5—218.5°C. This material was reprecipitated twice from dimethylformamide - ethyl acetate to give 7.8 g (53.2%) of XIII, mp 218—220°C, $[\alpha]_D^{17}$ −2.0° (*c* 2.1, dimethylformamide). A smaple for analysis was dried *in vacuo* at 100°C over phosphorus pentoxide.

Found: C, 61.20; H, 7.40; N, 10.08; S, 3.27%. Calcd for $C_{50}H_{71}O_{11}N_7S$: C, 61.39; H, 7.31; N, 10.03; S, 3.28%.

Formyl-L-isoleucyl-ε-benzyloxycarbonyl-L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanine Hydrazide (XIV). A solution of 6.85 g (0.007 mol) of XIII and 11.8 g of 90% hydrazine hydrate in 100 m*l* of dimethylformamide was allowed to stand at room temperature for five days. The solution was then evaporated to a small volume *in vacuo*. On the addition of 300 m*l* of water, a precipitate was formed; this was collected, washed with water, and then dried; yield 6.60 g (97.8%), mp 196—197°C. This material was recrystallized twice from dimethylformamide - water to give 6.30 g (93.3%) of XIV; mp 198—199°C, $[\alpha]_D^{17}$ −0.8° (*c* 3.0, dimethylformamide). For analysis the compound was dried *in vacuo* at 100°C over phosphorus pentoxide.

Found: C, 59.31; H, 7.45; N, 12.92; S, 3.11%. Calcd for $C_{48}H_{69}O_{10}N_9S \cdot {}^1/_2H_2O$: C, 59,24; H, 7.26; N, 12.96; S. 3.30%.

β-Benzyl L-Aspartate (XV). The method used here was virtually identical with that described for the synthesis of γ-benzyl L-glutamate by Boissonnas *et al.*[18] From a mixture of 500 m*l* of ether, 500 m*l* of benzyl alcohol, and 50 m*l* of concentrated sulfuric acid, an excess of ether was removed *in vacuo* at room temperature, and then 66.6 g (0.5 mol) of L-aspartic acid was stirred, portion by portion, into this solution. The resulting solution was allowed to stand for 48 hr at room temperature, and then 1 *l* of ethanol and 250 m*l* of pyridine were added successively with vigorous stirring. After the mixture had stood overnight at 0°C, the crystals formed were collected and washed with ether. Recrystallization from 1 *l* of water containing 1 m*l* of pyridine gave 47.5 g (42.6%) of colorless plates, mp 216°C (decomp.). Further recrystallization from the same solvent raised the melting point to 221°C (decomp.). $[\alpha]_D^{16}$ +28.5° (*c* 3.0, N hydrochloric acid); lit.,[25,26] mp 222°C (decomp.), $[\alpha]_D^{26}$ +24.2° (*c* 1.18, N hydrochloric acid).

Found: C, 59.20; H, 5.98; N, 6.26%. Calcd for $C_{11}H_{13}O_4N$: C, 59.18; H, 5.87; N, 6.28%.

β-Benzyl Benzyloxycarbonyl-L-aspartate (XVI). To a solution of 22.3 g (0.1 mol) of XV and 13 g of sodium carbonate in 800 m*l* of water, 17.5 m*l* of benzyl chloroformate were vigorously stirred in on ice cooling

25) T. Hayakawa, H. Nishi, J. Noguchi, K. Ikeda, T. Yamashita and T. Isemura, *Nippon Kagaku Zasshi* (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **82**, 601 (1961).
26) T. Hayakawa, K. Harada and S. W. Fox, This Bulletin, **39**, 391 (1966).

over a period of 1 hr. Stirring was continued for further 3 hr at room temperature. The reaction mixture was extracted three times with ether, and then acidified to Congo red with 6 N hydrochloric acid on cooling in an ice bath. The oil which appeared crystallized slowly when scratched with a glass rod. The crystals were collected, washed with water, and dried; yield 26.0 g (72.8%), mp 104—106°C. Recrystallization from ethyl acetate - petroleum ether gave needles with a mp of 108.5—109.5°C, $[\alpha]_D^{17}$ +13.2° (*c* 10, acetic acid); lit.,[19] mp 108°C, $[\alpha]_D^{25}$ +12.1° (*c* 10, acetic acid).

Found: C, 63.90; H, 5.32; N, 3.91%. Calcd for $C_{19}H_{19}O_6N$: C, 63.86; H, 5.36; N, 3.92%.

β-Benzyl-*N*-carboxy-L-aspartate Anhydride (XVII). This compound was prepared from XVI according to the directions of Katchalski *et al.*,[19] mp 118—119°C (decomp.); lit.,[19] mp 121°C (decomp.).

Found: C, 57.71; H, 4.47; N, 5.72%. Calcd for $C_{12}H_{11}O_5N$: C, 57.83; H, 4.45; N, 5.62%.

α-Methyl-β-benzyl L-Aspartate Hydrochloride (XVIII). To a suspension of 25.0 g (0.1 mol) of XVII in 100 m*l* of anhydrous ether containing 10 g of hydrogen chloride, 100 m*l* of anhydrous methanol were added on cooling in an ice bath. After standing at room temperature for 30 min, the suspension turned to a solution which was then allowed to stand at room temperature for 4 hr. This was concentrated *in vacuo* to the residual oil, which was then poured into 400 m*l* of a cold 5% sodium bicarbonate solution. The oil which separated was taken up in ether, washed with water, dried for a short time over anhydrous magnesium sulfate, and then concentrated *in vacuo*. The oily residue was taken up in chloroform, and the solution was saturated with dry hydrogen chloride on cooling in an ice bath. On the addition of anhydrous ether, XVIII precipitated as crystals; yield 16.7 g (60.8%). Mp 136.5—137.0°C. Recrystallization from anhydrous methanol - anhydrous ether gave colorless needles, mp 137.5—138.0°C, $[\alpha]_D^{17}$ +19.2° (*c* 4.2, water). For analysis the compound was dried *in vacuo* at about 65°C over phosphorus pentoxide.

Found: C, 52.93; H, 5.88; N, 5.39; Cl, 12.87%. Calcd for $C_{12}H_{16}O_4NCl$: C, 52.66; H, 5.90; N, 5.12; Cl, 12.96%.

Benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-aspartic Acid α-Methyl-β-benzyl Ester (XIX). To a cold solution of 5.75 g (0.021 mol) of XVIII and 2.13 g of triethylamine in 50 m*l* of chloroform, 150 m*l* of anhydrous ether were added. After the mixture had stood for 10 min, the triethylamine hydrochloride which had precipitated was filtered off and the filtrate was concentrated *in vacuo*. The oily residue was dissolved in 50 m*l* of dimethylformamide. To a mixture of this solution and a solution of 7.59 g (0.02 mol) of benzyloxycarbonyl-*im*-benzyl-L-histidine[21] in 200 m*l* of dimethylformamide, 4.2 g of *N*,*N*′-dicyclohexylcarbodiimide were stirred in at room temperature. After the mixture had stood overnight, a few drops of acetic acid were added. The precipitate of *N*,*N*′-dicyclohexylurea was filtered off, and the filtrate was concentrated *in vacuo*. An oily residue was taken up in 250 m*l* of methylene chloride, washed successively with N hydrochloric acid, a 5% sodium bicarbonate solution and water, and then dried over anhydrous sodium sulfate. After the solvent had been evaporated *in vacuo*, the resulting oily residue was crystallized from ethyl acetate - petroleum ether to give 10.0 g (83.5%) of XIX, mp 113.5—116.5°C. Recrystallization from the same solvent gave 8.8 g (73.5%) of needles, mp 116—118°C, $[\alpha]_D^{17}$ −8.3° (*c* 3.4, dimethylformamide).

Found: C, 66.22; H, 5.77; N, 9.54%. Calcd for $C_{33}H_{34}O_7N_4$: C, 66.21; H, 5.73; N, 9.36%.

***im*-Benzyl-L-histidyl-L-aspartic Acid α-Methyl-β-benzyl Ester Dihydrobromide (XX).** A suspension of 5.0 g of XIX in 20 m*l* of 28% (w/w) hydrogen bromide in acetic acid was shaken occasionally at room temperature for 10 min. After the addition of 200 m*l* of anhydrous ether and cooling in an ice bath for 10 min, crystals formed which were collected, washed with anhydrous ether, and dried; wt 5.0 g. This compound was used for the next reaction after having been washed with a 5% sodium bicarbonate solution to remove the free carboxylic acid derivative formed by the cleavage of the benzyl ester.

Formyl-L-isoleucyl-ε-benzyloxycarbonyl-L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-L-aspartic Acid α-Methyl-β-benzyl Ester (XXI). All the experiments for this preparation were carried out at 2—3°C. A solution of 3.89 g (0.004 mol) of XIV in 100 m*l* of acetic acid and 12 m*l* of N hydrochloric acid was cooled at −5°C. A cold concentrated aqueous solution of 0.30 g (0.0044 mol) of sodium nitrite was then added portion by portion and with shaking, to the solution. After the mixture had stood for 10 min, 400 m*l* of a cold saturated solution of sodium chloride was added. The resulting precipitate (ν_{max} 2170 cm^{-1}) was collected by filtration and washed with a cold 5% sodium carbonate solution and ice water. It was immediately dissolved in 150 m*l* of dimethylformamide and mixed with a dried solution of the free ester equivalent to 3.76 g (0.006 mol) of XX in 150 m*l* of methylene chloride. The reaction mixture was stirred for 1 hr at 2°C, and then allowed to stand overnight at 10°C. It was concentrated to dryness *in vacuo* at 45°C. The gelatinous residue was triturated with 30 m*l* of hot methylene chloride, and 100 m*l* of anhydrous ether were added. The white amorphous material precipitated was collected and dried; yield 4.40 g (77.7%), mp 174—175°C (sintered at 159°C). Recrystallization from dimethylformamide-methylene chloride-ether gave 3.70 g (65.4%) of an amorphous powder: mp 175—176°C (sintered at 169°C). Recrystallization from dimethylformamide-water did not raise the melting point. $[\alpha]_D^{16}$ −1.8° (*c* 2.2, dimethylformamide). A sample for analysis was dried *in vacuo* at 95—100°C over phosphorus pentoxide.

Found: C, 61.78; H, 6.74; N, 11.15; S, 2.38; H_2O, 1.42%. Calcd for $C_{73}H_{93}O_{15}N_{11}S \cdot H_2O$: C, 61.98; H, 6.79; N, 10.89; S, 2.27; H_2O, 1.29%.

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