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Synthetic Studies of Bacitracin. II.[1] Synthesis of a Cyclic Dodecapeptide, an Analogue of Bacitracin A

Yasuo ARIYOSHI,*1 Tetsuo SHIBA and Takeo KANEKO

Department of Chemistry, Faculty of Science, Osaka University, Toyonaka, Osaka

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For the purpose of making a total synthesis of bacitracin A, a protected heptapeptide with a ring structure, N^{α}-(formyl-L-isoleucyl)-*cyclo*-(L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-α-methyl-β-L-aspartyl) was first prepared by a high dilution method. After deformylation, it was coupled with a tetrapeptide corresponding to the *N*-terminal part of bacitracin A, the benzyloxycarbonyl-L-isoleucyl-*S*-benzyl-L-cysteinyl-L-leucyl-D-glutamic acid (α-azide)-γ-*t*-butyl ester, to afford an undecapeptide derivative. This was converted to a hydrazide, which was then coupled with β-benzyl-D-isoasparaginate to give the N^{α}-(benzyloxycarbonyl-L-isoleucyl-*S*-benzyl-L-cysteinyl-L-leucyl-γ-*t*-butyl-D-glutamyl-L-isoleucyl)-*cyclo*-(L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-β-L-aspartyl)-D-isoasparagine β-benzyl ester. The removal of all protecting groups from it gave a free dodecapeptide, which has the same amino acid sequence, including optical properties, as that of the structure of bacitracin A proposed by Stoffel and Craig; it is different from the latter only in having the thiazoline ring replaced with the cysteine residue. The synthetic dodecapeptide was tested for biological activity and for the formation reaction of the thiazoline ring.

In the preceding paper,[1] the synthesis of an intermediate heptapeptide (I) for the total synthesis of the antibiotic bacitracin A was described. The cyclization and elongation of it, presented in this paper, lead to a dodecapeptide (XIX) possessing the same amino acid sequence as that of the structure of bacitracin A proposed by Stoffel and Craig,[2] but its cysteine residue must be cyclized to the thiazoline ring for the completion of the total synthesis of the antibiotic. It is known that a cysteine derivative or a cysteine peptide can be cyclized to the thiazoline derivative in a strong acidic solution.[2-9] For the purpose of investigating the possibility of the formation of the thiazoline ring from the cysteine residue in the dodecapeptide and the relationship between the chemical structure and the biological activity of the antibiotic, a synthesis of the dodecapeptide was attempted and carried out according to the scheme shown in Fig. 1.

A coupling of the benzyloxycarbonyl-*S*-benzyl-L-cysteine *p*-nitrophenyl ester[10] with ethyl L-leucinate[11] gave the benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-L-leucine ethyl ester (II), which was then treated with hydrogen bromide in acetic acid to give *S*-benzyl-L-cysteinyl-L-leucine ethyl ester hydrobromide (III). After the treatment of III with triethylamine, it was coupled with benzyloxycarbonyl-L-isoleucine by *N*, *N'*-dicyclohexylcarbodiimide to yield the benzyloxycarbonyl-L-isoleucyl-*S*-benzyl-L-cysteinyl-L-leucine ethyl ester (IV). This was converted to the corresponding hydrazide (V) by hydrazine hydrate in methanol, or to the carboxylic acid derivative (VI) by saponification. α-Methyl-γ-*t*-butyl D-glutamate was coupled with an azide obtained from the hydrazide (V) to give the benzyloxycarbonyl-L-isoleucyl-*S*-benzyl-L-cysteinyl-L-leucyl-D-glutamic acid α-methyl-γ-butyl ester (VIIa), from which the hydrazoic acid formed as a by-product could not be removed completely. A condensation of the carboxylic acid derivative (VI) with α-methyl-γ-*t*-butyl D-glutamate by *N*, *N'*-dicyclohexylcarbodiimide gave the same tetrapeptide derivative (VIIb).

The fact that the IR spectrum of VIIa was identical with that of VIIb, except for the presence

*1 Present address: Central Research Laboratories, Ajinomoto Co., Inc., Kawasaki, Kanagawa.

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11) F. Röhman, *Ber.*, **30**, 1978 (1897).

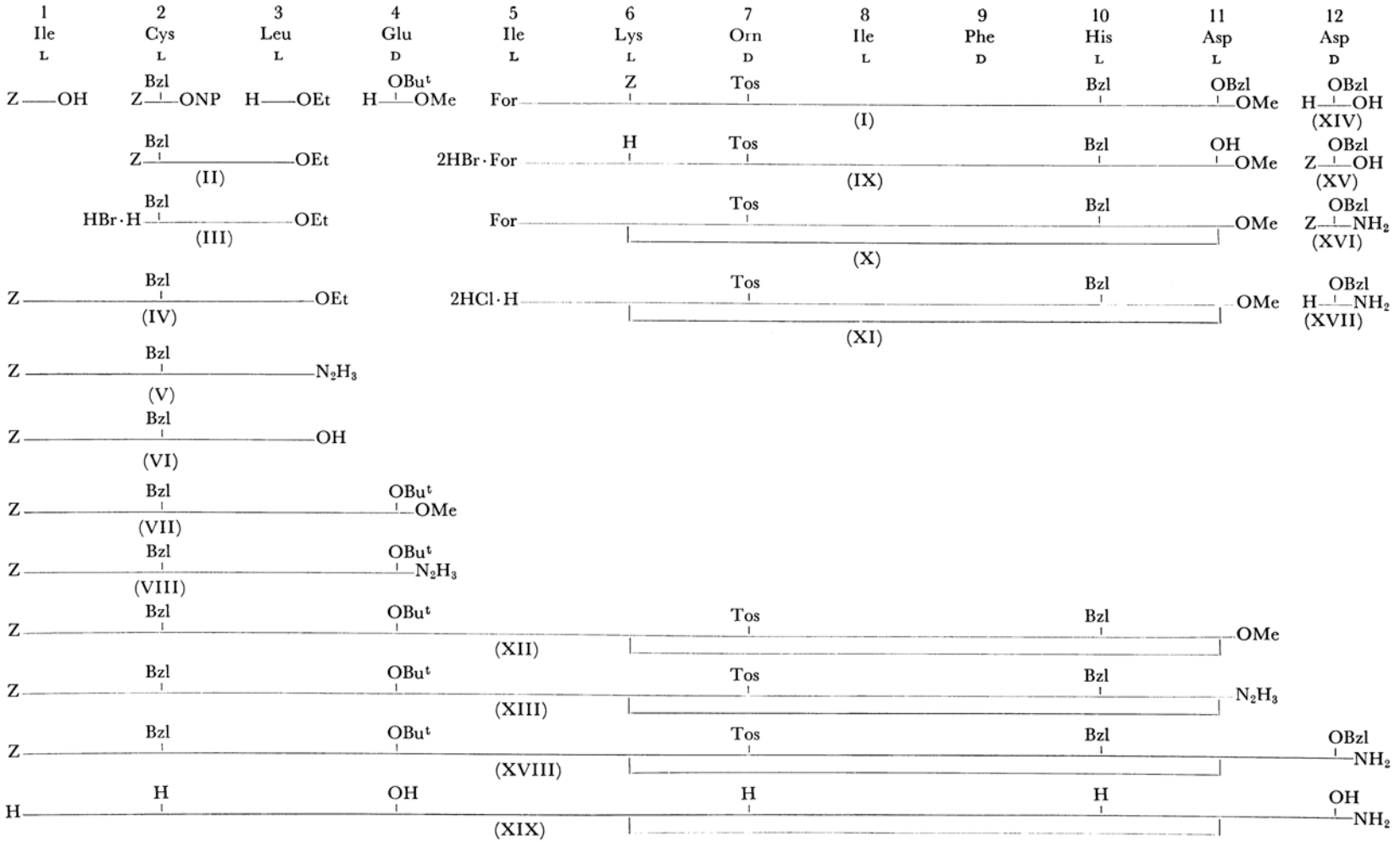

Fig. 1. Scheme for the synthesis of the dodecapeptide with the cyclic structure.
Z: $C_6H_5CH_2OCO$-, Bzl: $C_6H_5CH_2$-, NP: *p*-NO_2-C_6H_4-, For: HCO-, N_2H_3: -$NHNH_2$, Bu^t: -$C(CH_3)_3$, Tos: *p*-CH_3-C_6H_4-SO_2-

of a band due to the hydrazoic acid in the former, showed that VIIa was so pure that it could be used in the following synthetic process without further purification, and that it may be more likely than VIIb to avoid the danger of a partial racemization. Some difficulty in converting the tetrapeptide ester (VII) to the corresponding hydrazide (VIII) with hydrazine hydrate in ethanol was overcome by a repetition of the reaction.

On the other hand, the selective removal of the ε-benzyloxycarbonyl group of the lysine residue and of the β-benzyl group of the aspartic acid residue from 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[1] (I) was carried out by treatment with hydrogen bromide in acetic acid for a short time, followed by hydrogenolysis using palladium on charcoal. The heptapeptide (IX) thus obtained was cyclized to a ring peptide (X) with *N*, *N'*-dicyclohexylcarbodiimide using a high dilution method. The deformylation of X by treatment with hydrochloric acid in methanol gave a peptide (XI), which was then condensed with an azide derived from the hydrazide (VIII) to afford an undecapeptide (XII). This was converted to the corresponding hydrazide (XIII) by hydrazine hydrate in dimethylformamide.

For the preparation of the *C*-terminal residue, β-benzyl benzyloxycarbonyl-D-isoasparaginate (XVI) was synthesized by the amidation, using the mixed anhydride method of β-benzyl benzyloxycarbonyl-D-aspartate (XV), which had been prepared in a way similar to that used for the L-isomer.[1] In the selective removal of the benzyloxycarbonyl group from XVI by treatment with hydrogen bromide in acetic acid for a short time, some unfavorable cleavage of the β-benzyl ester group was also accompanied, therefore, the purification of the product, β-benzyl D-isoasparaginate hydrobromide (XVII), was not easy; however, an analytically pure XVII was secured by suspending the crude product in methylene chloride and by washing it with an aqueous sodium bicarbonate solution to remove the by-product, the β-carboxylic acid derivative. The compound, XVII, thus obtained was coupled with an azide derivative from XIII to give the N^{α}-(benzyloxycarbonyl-L-isoleucyl-*S*-benzyl-L-cysteinyl-L-leucyl-γ-*t*-butyl-D-glutamyl-L-isoleucyl)-*cyclo*-(L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-β-L-aspartyl)-D-isoasparaginate β-benzyl ester (XVIII).

Out of this protected dodecapeptide (XVIII), the *t*-butyl group was first removed by trifluoroacetic acid, and then all the remaining protecting groups were removed by sodium in liquid ammonia. The product thus obtained was lyophilized to give a crude free dodecapeptide (XIX), which showed positive reactions to ninhydrin, sodium nitroprusside, and a Pauly reagent. However, its further purification was difficult because of its hygroscopic nature and the presence of a free sulfhydryl group in the molecule.

The ultraviolet spectrum of a solution of the compound XIX in concentrated hydrochloric acid did not show the absorption band in the 260—270 mμ region to be expected from the formation of the thiazoline ring. It is known that the cysteine residue can be easily cyclized to the thiazoline ring when the α-amino group of the *N*-terminal amino acid residue adjacent to the cysteine residue is protected by either an acetyl[2] or a benzyloxycarbonyl group,[9] while it is impossible when the amino group is free.[2] If it should occur that a cyclol structure can be spontaneously formed from the dodecapeptide (XIX) between an amino group of the *N*-terminal isoleucine and a carbonyl group of phenylalanine by means of an arrangement of the same amino acid sequence in XIX to that of natural bacitracin A (Craig's formula), and if it serves as a protected form of the *N*-terminal amino group, a successive formation of the thiazoline ring from the cyclol dodecapeptide could also be expected under such acidic conditions. However, the fact that this is not the case with the synthetic peptide (XIX) may mean that the previous presence of the thiazoline ring is rather necessary for the formation of the cyclol structure in the molecule of the dodecapeptide.

The antibacterial activity of the synthetic dodecapeptide (XIX) was tested in a synthetic medium by a dilution method using *Staphylococcus aureus* F. D. A. 209p. No activity was shown even at 2000 γ/m*l*. This indicates that the biological activity could be predominantly related to the thiazoline ring rather than to the ring peptide structure in it if Craig's formula is correct.

Experimental

All melting points are uncorrected. The infrared absorption spectra were obtained in Nujol mull with a Nihon Bunko IR-S spectrophotometer. Thin-layer chromatography was carried out by the ascending method on silica gel G.

Benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-L-leucine Ethyl Ester (II). Into a cold solution of 10.8 g (0.055 mol) of ethyl L-leucinate hydrochloride[11] and 5.6 g of triethylamine in 200 m*l* of chloroform, there was added 23.3 g (0.05 mol) of the benzyloxycarbonyl-*S*-benzyl-L-cysteine *p*-nitrophenyl ester.[10] After having been allowed to stand for 2 days at room temperature, the reaction mixture was washed successively with water, aqueous N ammonia (twice), water, N hydrochloric acid, water, a 5% sodium bicarbonate solution, and water. After the solution had been dried over anhydrous magnesium sulfate, the solvent was removed *in vacuo*. The oily residue thus obtained was crystallized from anhydrous ether-petroleum ether to give 20.8 g (85.6%) of II as crystals with a mp of 66.5—68.0°C.

Recrystallization from the same solvent gave needles; mp 67.0—68.5°C, $[\alpha]_D^{17}$ −40.9° (*c* 3.0, dimethylformamide).

Found: C, 64.27; H, 7.09; N, 5.71; S, 6.62%. Calcd for $C_{26}H_{34}O_5N_2S$: C, 64.17; H, 7.04; N, 5.76; S, 6.59%.

S-Benzyl-L-cysteinyl-L-leucine Ethyl Ester Hydrobromide (III). A solution of 40.0 g of II in 200 m*l* of 28% (w/w) hydrogen bromide in acetic acid was shaken occasionally at room temperature for 30 min. The reaction mixture was then concentrated *in vacuo* to an oily residue. Crystallization from ether-petroleum ether gave III as a semisolid; wt, 39.0 g.

Benzyloxycarbonyl-L-isoleucyl-S-benzyl-L-cysteinyl-L-leucine Ethyl Ester (IV). To a cold solution of 39.0 g (0.08 mol) of III and 9.1 g of triethylamine in 100 m*l* of chloroform, there was added 300 m*l* of anhydrous ether. After the filtration of triethylamine hydrobromide, the filtrate was concentrated *in vacuo*; the oily residue thus obtained was dissolved in 100 m*l* of dioxane. Into this, a solution of 23.9 g (0.09 mol) of benzyloxycarbonyl-L-isoleucine in 150 m*l* of dioxane and 18.6 g of *N, N'*-dicyclohexylcarbodiimide were added with stirring on ice-cooling. After the reaction mixture had stood at room temperature overnight, 2 m*l* of acetic acid was added to it, stirring was then continued for 1 hr. The *N, N'*-dicyclohexylurea thus formed was removed by filtration, and the filtrate was concentrated *in vacuo*. The residue was taken up in 600 m*l* of ethyl acetate and washed with N hydrochloric acid, a 5% sodium bicarbonate solution, and water successively, and finally dried over anhydrous magnesium sulfate. The solvent was removed *in vacuo*, and the gelatinous residue thus obtained was crystallized from ethyl acetate-petroleum ether to give 32.1 g (59.5%) of IV. Recrystallization from anhydrous ethanol gave colorless needles; mp 161—162°C, $[\alpha]_D^{17}$ −41.1° (*c* 3.3, dimethylformamide).

Found: C, 64.17; H, 7.64; N, 6.87; S, 5.39%. Calcd for $C_{32}H_{45}O_6N_3S$: C, 64.08; H, 7.56; N, 7.01; S, 5.35%.

Benzyloxycarbonyl-L-isoleucyl-S-benzyl-L-cysteinyl-L-leucine Hydrazide (V). A solution of 12.0 g (0.02 mol) of IV and 11.0 m*l* of 90% hydrazine hydrate in 120 m*l* of anhydrous methanol was boiled under reflux for 1 hr and then allowed to stand overnight at room temperature. The needlelike crystals thus formed were collected after the addition of 240 m*l* of water to complete the precipitation; yield, 11.6 g (99.2%), mp 230—231°C. Recrystallization from dimethylformamide-ethanol raised the melting point to 231.5—232.5°C. $[\alpha]_D^{17}$ −27.9° (*c* 3.4, dimethylformamide).

Found: C, 61.44; H, 7.39; N, 11.83; S, 5.46%. Calcd for $C_{30}H_{43}O_5N_5S$: C, 61.51; H, 7.39; N, 11.96; S, 5.48%.

Benzyloxycarbonyl-L-isoleucyl-S-benzyl-L-cysteinyl-L-leucine (VI). Into a suspension of 7.8 g (0.013 mol) of IV in 200 m*l* of anhydrous ethanol, 14.3 m*l* of N sodium hydroxide was added with stirring at room temperature. After stirring for 1.5 hr, the clear reaction mixture was acidified with N hydrochloric acid to pH 3, and then diluted with water. The white precipitate thus formed was collected; yield 6.5 g (87.8%), mp 126—129°C. Recrystallization from ethanol gave VI; mp 144.0—145.5°C, $[\alpha]_D^{17}$ −39.6° (*c* 3.3, dimethylformamide). The sample for analysis was dried *in vacuo* at 95°C over phosphorus pentoxide.

Found: C, 63.02; H, 7.40; N, 7.20; S, 5.37%. Calcd for $C_{30}H_{41}O_6N_3S$: C, 63.03; H, 7.23; N, 7.52; S, 5.61%.

Benzyloxycarbonyl-L-isoleucyl-S-benzyl-L-cysteinyl-L-leucyl-D-glutamic Acid α-Methyl-γ-t-butyl Ester (VIIa, b). *VIIa) By the Azide Method.* All the procedures for a coupling reaction in this experiment were carried out at 1—2°C unless otherwise stated. In a mixture of 100 m*l* of acetic acid and 25.5 m*l* of N hydrochloric acid 5.00 g (8.5 mmol) of V were added, and the solution was cooled to −5°C. A cold concentrated solution of 0.65 g (9.4 mmol) of sodium nitrite was added, portion by portion, to the above solution with shaking. After the reaction mixture had stood for 10 min, 150 m*l* of a cold saturated aqueous solution of sodium chloride was added to it. The resulting precipitate (ν_{max} 2170 cm^{-1}) was collected by filtration, and then dissolved in 150 m*l* of cold chloroform. The solution was washed with an ice-cold 3% sodium bicarbonate solution (twice) and water, dried for a short time over anhydrous sodium sulfate, and then filtered. To a cold solution of 2.54 g (0.01 mol) of α-methyl-γ-*t*-butyl D-glutamate hydrochloride[12] and 1.02 g of triethylamine in 10 m*l* of chloroform, 50 m*l* of anhydrous ether were added. After the reaction mixture had stood for 10 min, the triethylamine hydrochloride formed was filtered off, and the filtrate was concentrated *in vacuo*. The oily residue was dissolved in 100 m*l* of chloroform. The solution was mixed with the azide solution prepared above. The mixture was allowed to stand overnight at 1°C, and then at room temperature for 24 hr. After the addition of another 150 m*l* of chloroform, the reaction mixture was washed successively with a 5% aqueous citric acid solution, water, a 5% sodium bicarbonate solution (twice), and water, and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* to give a gelatinous material. Crystallization from ethyl acetate gave the gelatinous VIIa again; mp 171—173°C, yield 4.55 g (69.5%).

The IR spectrum of this product showed the absorption band of hydrazoic acid at 2170 cm^{-1}. The hydrazoic acid could not be removed completely by washing with a bicarbonate solution, followed by drying *in vacuo* at 70°C over sodium hydroxide. This product was used for the next reaction without further purification, because a pure hydrazide derivative of this compound was available as a crystalline material in the following experimental step.

VIIb) By the Carbodiimide Method. To a solution of 1.27 g (5 mmol) of α-methyl-γ-*t*-butyl D-glutamate hydrochloride in 10 m*l* of dimethylformamide, 0.51 g of triethylamine was added on cooling in an ice bath. After the mixture had stood for 10 min, the triethylamine hydrochloride formed was removed by filtration.

12) This compound was prepared according to the directions for the preparation of L-isomer. Mp 125.5—126.5°C, $[\alpha]_D^{25}$ −24.6° (*c* 1.2, methanol).

Found: C, 47.41; H, 7.84; N, 5.49; Cl, 14.10%. Calcd for $C_{10}H_{20}O_4NCl$: C, 47.33; H, 7.95; N, 5.52; Cl, 13.98%.

Lit.: E. Klieger and H. Gibian, *Ann.*, **655**, 195 (1962). L-Isomer; mp 125—126°C, $[\alpha]_D^{25}$ +23.9° (*c* 1.02, methanol).

Into the filtrate, a solution of 2.86 g (5 mmol) of VI in 30 m*l* of dimethylformamide and 1.03 g of *N, N'*-dicyclohexylcarbodiimide was added with stirring on an ice-cooling. After the reaction mixture had stood overnight at room temperature, the *N, N'*-dicyclohexylurea thus formed was removed by filtration and the filtrate was concentrated to dryness *in vacuo*. The residue was crystallized from ethyl acetate - petroleum ether to give a gelatinous material; yield 1.90 g (49.1%), mp 180—182°C (sintered at 169°C). Recrystallization from ethyl acetate did not raise the melting point. $[\alpha]_D^{17}$ −14.4° (*c* 3.1, dimethylformamide). The sample for analysis was dried *in vacuo* at 85—90°C over phosphorous pentoxide.

Found: C, 62.56; H, 7.62; N, 7.22; S, 4.38%. Calcd for $C_{40}H_{58}O_9N_4S$: C, 62.31; H, 7.58; N, 7.27; S, 4.16%.

The IR spectrum of this compound is identical with that obtained by the azide method (VIIa), except for the absence of the absorption band of the hydrazoic acid. This compound was prepared only in order to compare the purity of VIIa with it, it was not used for the following synthetic path, because the azide method gives a more reliable material than the carbodiimide method in the sense of optical purity.

Benzyloxycarbonyl - L - isoleucyl - *S* - benzyl - L - cysteinyl-L-leucyl-D-glutamic Acid (*α*-Hydrazide)-*γ*-*t*-butyl Ester (VIII). A solution of 4.3 g (5.5 mmol) of VIIa prepared by the azide method and 3.0 g of 90% hydrazine hydrate in 150 m*l* of anhydrous ethanol was boiled under reflux for 1 hr, and then allowed to stand overnight at room temperature. The mixture of crystals and gelatinous material was collected by filtration, and washed with ethanol. Yield, 3.6 g; mp 214—216°C (decomp.). Recrystallization from ethanol gave 2.8 g of crystals, which was found still to contain the unreacted ester. It was dissolved in 100 m*l* of anhydrous ethanol containing 2.0 g of 90% hydrazine hydrate, and refluxed again for 80 min. After the reaction mixture had stood overnight at room temperature, the crystals formed were collected; yield 2.3 g. A second crop was obtained from the mother liquor; 0.4 g. Total yield 2.7 g (62.8%) mp 224—226°C (decomp.). $[\alpha]_D^{31}$ −15.8° (*c* 2.8, acetic acid).

Found: C, 60.74; H, 7.63; N, 10.84; S, 4.24%. Calcd for $C_{39}H_{58}O_8N_6S$: C, 60.75; H, 7.58; N, 10.90; S, 4.16%.

Formyl-L-isoleucyl-L-lysyl-*δ*-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-L-aspartic Acid *α*-Methyl Ester Dihydrobromide (IX). A suspension of 3.00 g (2.12 mmol) of 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 (I)[12] in 30 m*l* of 28% (w/w) hydrogen bromide in acetic acid was shaken occasionally at room temperature for 30 min. To the reaction mixture, 400 m*l* of anhydrous ether was added to give a white precipitate, this precipitate was collected by filtration, and then subjected to hydrogenolysis in 60 m*l* of 85% methanol in the presence of 1.0 g of 5% palladium on charcoal for 4.5 hr at room temperature. The filtrate from the catalyst was concentrated *in vacuo*, The residue obtained was crystallized from methanol-ether to give 2.02 g (70.4%) of IX. Recrystallization from the same solvent gave 1.80 g of a hygroscopic IX; mp 154—156°C (decomp., sintered at 145°C), $[\alpha]_D^{16}$ −2.6° (*c* 3.1, dimethylformamide). It gave no Pauly reaction. R_f=0.83 (detected by ninhydrin spraying or exposure in iodine vapor) on thin-layer chromatography with a solvent system of *n*-butanol - acetic acid - water (3 : 1 : 1, v/v).[13]

Found: C, 51.72; H, 6.73; N, 11.04%. Calcd for $C_{58}H_{83}O_{13}N_{11}SBr_2 \cdot H_2O$: C, 51.51; H, 6.34; N, 11.39%.

N^{α}-(Formyl-L-isoleucyl)-*cyclo*-(L-lysyl-*δ*-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-*α*-methyl-*β*-L-aspartyl) (X). a) To a solution of 1.35 g (1 mmol) of IX in 5 m*l* of dimethylformamide, there was added 0.21 g of triethylamine on cooling. After the triethylamine hydrobromide thus formed had been filtered off, the filtrate was diluted to 30 m*l* with dimethylformamide. This solution was added to 500 m*l* of dimethylformamide containing 10 g of *N, N'*-dicyclohexylcarbodiimide over a period of 9 hr with vigorous stirring at room temperature. After stirring had been continued for 64 hr, 11.1 m*l* of 50% acetic acid was added to the reaction mixture, which was then concentrated *in vacuo* at 55°C; the *N, N'*-dicyclohexylurea formed during concentration was removed by filtration. The residue was reprecipitated from dimethylformamide - ether; wt, 1.37 g. This material was dissolved in 70 m*l* of the upper layer of 2% acetic acid - *s*-butanol (1 : 1 v/v) on warming and diluted with 70 m*l* of ethyl acetate. The solution was washed with 50 m*l* of the lower layer of the above solvent mixture, and then with 50 m*l* of the lower layer of a 5% aqueous sodium bicarbonate solution - *s*-butanol (1 : 1 v/v). The organic layer was concentrated to dryness *in vacuo*, and the residue thus obtained was recrystallized from dimethylformamide - ether. Yield 0.53 g (43.1%). Recrystallization from the same solvent gave X with a mp of 170—172°C, $[\alpha]_D^{24}$ +1.1° (*c* 2.8, dimethylformamide).

Found: C, 60.61; H, 7.45; N, 12.60; S, 2.58%. Calcd for $C_{58}H_{79}O_{12}N_{11}S \cdot C_4H_{10}O$(*s*-butanol): C, 60.61; H, 7.30; N, 12.54; S, 2.61%. Mol wt Found: 1130 (Rast). Calcd: 1230. Hexahydro - *p*-aminobenzoic acid lactam was used as the solvent for the molecular-weight determination by the Rast method.[14]

This compound gave negative reactions to ninhydrin and a Pauly reagent. R_f=0.63 (detected by exposure in iodine vapor) was obtained on thin-layer chromatography with a *n*-butanol - acetic acid - water (7 : 1 : 2) solvent system.[15]

b) Into a solution of 1.35 g (1 mmol) of the free amino peptide obtained from the dihydrobromide (IX) in a mixture of 2.5 *l* of dimethylformamide and 10 *l* of benzene, 78 g of *N, N'*-dicyclohexylcarbodiimide was added with vigorous stirring at 10°C. Stirring was then continued for 5 days at 10°C. After the addition of 86 m*l* of 50% acetic acid, the solvent was removed *in vacuo*. The residue thus obtained was purified by a procedure similar to that of Method (a). Yield 0.61 g

13) M. Brenner and A. Niederwieser, *Experientia*, **16**, 378 (1960).

14) G. Wendt, *Ber.*, **75**, 425 (1942). Among the several cyclic peptides prepared in this work, only the compound XI has enough solubility in an adequate solvent, such as methanol or ethanol, for the molecular weight to be measured by an osmometer, although even with this compound an exact value of the molecular weight could not be obtained because of dissociation.

15) R. A. Boissonnas and R. L. Huguenin, *Helv. Chim. Acta*, **43**, 182 (1960).

(49.6%).

Found: C, 60.42; H, 7.41; N, 12.75; S, 2.54%.

N^{α}-L-Isoleucyl-*cyclo*-(L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-α-methyl-β-L-aspartyl) Dihydrochloride (XI). In 10 m*l* of hot methanol, 1.05 g of X was almost completely dissolved. After the solution had then been cooled to room temperature, 0.5 m*l* of concentrated hydrochloric acid was added to it. The reaction mixture was allowed to stand for 48 hr at room temperature. After filtration, the filtrate was concentrated *in vacuo*. The residue obtained was dissolved in a small amount of methanol, and the solvent was removed by evaporation *in vacuo*. This evaporation process was repeated two more times after the addition of a small amount of methanol each time. The methanolic solution of the final residue obtained was treated with charcoal. On the addition of ether to the filtrate from the charcoal, a white precipitate was separated out. Yield 0.83 g (72.2%). Recrystallization from methanol - ether gave 0.77 g (67.0%) of XI, mp 175—177°C (decomp.), $[\alpha]_D^{29}$ +7.6° (*c* 2.5, dimethylformamide).

Found: C, 55.56; H, 7.11; N, 12.05%. Calcd for $C_{57}H_{81}O_{11}N_{11}SCl_2 \cdot 2H_2O$: C, 55.41, H, 6.94; N, 12.47%.

R_f=0.80 (detected by ninhydrin and iodine vapor) on thin-layer chromatography.[13]

N^{α}-(Benzyloxycarbonyl-L-isoleucyl-*S*-benzyl-L-cysteinyl-L-leucyl-γ-*t*-butyl-D-glutamyl-L-isoleucyl)-*cyclo*-(L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-α-methyl-β-L-aspartyl) (XII). To a cold solution of 0.77 g (0.623 mmol) of XI in 5 m*l* of dimethylformamide, there was added 0.175 m*l* of triethylamine; the triethylamine hydrochloride thus formed was then filtered off. A solution of 0.48 g (0.623 mmol) of VIII in a mixture of 6 m*l* of acetic acid and 1 m*l* of water was cooled to −5°C, after which 0.47 m*l* of a cold aqueous solution of sodium nitrite (100 mg/m*l*) was added to the cold solution with stirring at −5°C. After the reaction mixture had been kept at this temperature for 10 min, 30 m*l* of a cold saturated aqueous solution of sodium chloride was added. The precipitate (ν_{max} 2170 cm^{-1}) thus formed was collected by filtration, and washed with a cold 1% potassium carbonate solution and then with ice water. The azide thus obtained was added to the solution of the free amino peptide prepared above, and the solution was diluted to 30 m*l* with dimethylformamide. After the reaction mixture had stood for 2 days at 2°C, 0.5 m*l* of acetic acid was added. This was then concentrated to a small volume *in vacuo*. On the addition of water, a white precipitate was obtained; yield 0.92 g (80.0%). Recrystallization from dimethylformamide - ether gave 0.66 g (57.4%) of XII, mp 204—207°C, $[\alpha]_D^{30}$ −5.3° (*c* 3.2, dimethylformamide).

Found: C, 60.54; H, 7.44; N, 11.05; S, 3.57%. Calcd for $C_{96}H_{133}O_{19}N_{15}S_2 \cdot 2H_2O$: C, 60.64; H, 7.26; N, 11.05; S, 3.37%.

N^{α}-(Benzyloxycarbonyl-L-isoleucyl-*S*-benzyl-L-cysteinyl-L-leucyl-γ-*t*-butyl-D-glutamyl-L-isoleucyl)-*cyclo*-(L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-β-L-aspartyl-α-hydrazide) (XIII). A mixture of 0.60 g of XII and 0.35 m*l* of 90% hydrazine hydrate in 3.5 m*l* of dimethylformamide was allowed to stand at room temperature for 2 days. On the addition of 30 m*l* of water, the precipitate thus formed was collected; yield 0.53 g (87.5%), mp 198—200°C. Recrystallization from dimethylformamide - water gave 0.40 g (66.1%) of XIII, mp 202—203°C, $[\alpha]_D^{30}$ −15.8° (*c* 3.0, acetic acid).

Found: C, 59.49; H, 7.51; N, 12.80; S, 3.45%. Calcd for $C_{95}H_{133}O_{18}N_{17}S_2 \cdot 3H_2O$: C, 59.45; H, 7.30; N, 12.41; S, 3.34%.

β-Benzyl D-Aspartate (XIV). This compound was synthesized by the same procedure as that used in the preparation of the L-isomer,[10] mp 221°C (decomp.), $[\alpha]_D^{17}$ −28.3° (*c* 3.0, N hydrochloric acid).

Found: C, 59.02; H, 6.02; N, 6.19%. Calcd for $C_{11}H_{13}O_4N$: C, 59.18; H, 5.87; N, 6.28%.

β-Benzyl Benzyloxycarbonyl-D-aspartate (XV). This compound was prepared according to the same procedure as that used for the L-isomer,[10] mp 108.5—109.5°C, $[\alpha]_D^{19}$ −12.8° (*c* 10.1, acetic acid).

Found: C, 63.69; H, 5.35; N, 3.95%. Calcd for $C_{19}H_{19}O_6N$: C, 63.86; H, 5.36; N, 3.92%.

β-Benzyl Benzyloxycarbonyl-D-isoasparaginate (XVI). Into a solution of 10.7 g (0.03 mol) of XV and 3.1 g of triethylamine in a mixture of 100 m*l* of chloroform and 375 m*l* of dioxane, 3.6 g of ethyl chloroformate was added with vigorous stirring at −5°C. After stirring for 25 min at this temperature, 6 m*l* of 28% aqueous ammonia was added to the reaction mixture; stirring was then continued for 3 more hours at room temperature. The reaction mixture was washed with 0.5 N hydrochloric acid, and the solvent was evaporated to dryness *in vacuo*. The residue was crystallized from ethyl acetate - petroleum ether to give needles; yield 6.5 g (60.9%), mp 119.0—119.5°C. Recrystallization from the same solvent gave the same melting point. $[\alpha]_D^{19}$ −9.1° (*c* 9.9, acetic acid).

Found: C, 64.06; H, 5.69; N, 7.89%. Calcd for $C_{19}H_{20}O_5N_2$: C, 64.03; H, 5.66; N, 7.86%.

β-Benzyl D-Isoasparaginate (XVII). A solution of 2.0 g of XVI in 20 m*l* of 28% (w/w) hydrogen bromide in acetic acid was permitted to stand at room temperature with occasional shaking for 7 min. After the addition of 200 m*l* of anhydrous ether, the crystals thus formed were collected; yield 1.6 g (94%), mp 179—181°C. Recrystallization from isopropanol gave 0.9 g of needles of the hydrobromide; mp 186.0—186.5°C.

Found: C, 42.62; H, 4.98; N, 9.30; Br, 26.92%. Calcd for $C_{11}H_{15}O_3N_2Br$: C, 43.58; H, 4.99; N, 9.24; Br, 26.36%.

As has been shown above, the elementary analysis of the product did not agree well with the theoretical values of the hydrobromide because of contamination by the debenzylated derivative, but the free amino ester (XVII) was purified as follows. A suspension of 0.9 g of the crude hydrobromide obtained above in 100 m*l* of methylene chloride was washed with a 5% sodium bicarbonate solution until a washing became clear, and then with water. After drying over anhydrous sodium sulfate, the solution was concentrated *in vacuo* to give a crystalline residue. It was recrystallized from ethyl acetate - petroleum ether to give 0.16 g of needles; mp 86.5—87.5°C, $[\alpha]_D^{30}$ +18.3° (*c* 1.8, ethyl acetate).

Found: C, 59.36; H, 6.23; N, 12.71%. Calcd for $C_{11}H_{14}O_3N_2$: C, 59.45; H, 6.35; N, 12.60%.

R_f=0.74 (detected by ninhydrin) on thin-layer chromatography.[13]

N^{α}-(Benzyloxycarbonyl-L-isoleucyl-*S*-benzyl-L-cysteinyl-L-leucyl-γ-*t*-butyl-D-glutamyl-L-isoleucyl)-*cyclo*-(L-lysyl-δ-tosyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-*im*-benzyl-L-histidyl-β-L-aspartyl)-D-isoasparagine β-Benzyl Ester (XVIII). All the experiments for this reaction were carried out at 2—3°C unless otherwise stated. In a mixture of 1.8 m*l* of acetic acid and 0.5 m*l* of water there was dissolved 0.19 g (0.1 mmol) of XIII. To this solution, 0.16 m*l* of a cold aqueous solution of sodium nitrite (50 mg/m*l*) was added, portion by portion, with shaking at −5°C. After the reaction mixture had been kept for 10 min, 20 m*l* of a cold saturated aqueous solution of sodium chloride was added to it. The resulting precipitate (ν_{max} 2170 cm^{-1}) was collected by filtration, and washed with a 1% cold potassium carbonate solution and then with water. To a solution of this precipitate in 6 m*l* of dimethylformamide, 0.03 g (0.135 mmol) of XVII was added. After the solution had stood for 3 days at 3°C, it was concentrated *in vacuo* to a small volume. On an addition of 0.1 m*l* of acetic acid and 20 m*l* of water, the precipitate thus formed was collected; yield 0.16 g (79.2%), mp 200—203°C. Recrystallization from dimethylformamide - ether gave 0.10 g (49.5%) of XVIII; mp 203—205°C, $[\alpha]_D^{30}$ −6.5° (*c* 2.6, dimethylformamide).

Found: C, 59.57; H, 7.19; N, 11.69; S, 3.36%. Calcd for $C_{106}H_{143}O_{21}N_{17}S_2 \cdot 2H_2O$: C, 59.48; H, 7.34; N, 11.79; S, 3.18%.

Deprotection of XVIII. A solution of 100 mg of XVIII in 1.0 m*l* of trifluoroacetic acid was allowed to stand at room temperature for 45 min. The solution was concentrated to dryness *in vacuo*, and the residue obtained was dried *in vacuo* over sodium hydroxide and phosphorus pentoxide. This material was dissolved in 30 m*l* of liquid ammonia, and small pieces of sodium were added to the mixture with stirring until a permanent blue color remained. The blue color was discharged by the addition of Dowex 50W NH_4^+ form, and the ammonia was allowed to evaporate spontaneously. The residue was dissolved in 10 m*l* of water, and the solution was neutralized with Dowex 50W H^+ form. After the removal of the resin by filtration, the filtrate was washed with ether. The lyophilization of the solution gave a white hygroscopic powder; wt, 75 mg. No further purification of this material could be carried out because of the presence of the free sulfhydryl group in the molecule and because of its extremely hygroscopic character; this reacted positively against ninhydrin, sodium nitroprusside, and a Pauly reagent. A solution of this compound XIX in concentrated hydrochloric acid gave no absorption band in the 260—270 mμ region, even at several different concentrations.

This compound was biologically inactive against *Staphylococcus aureus* F. D. A. 209p, even at a high concentration (2000 γ/m*l*), whereas the commercial bacitracin exhibited a considerable activity against the same organism (dilution method).

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