SYNTHESIS OF POSSIBLE ACTINOMYCIN D PRECURSORS[1]

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ABSTRACT

The preparation of a number of polypeptides whose sequences are found in the actinomycin D molecule is described. The 4-methyl-3-hydroxyanthranoyl derivatives of these peptides were also prepared as possible substrates for an enzyme that forms the phenoxazinone system of the antibiotic.

An enzyme that has been named phenoxazinone synthetase (1) catalyzes the oxidative condensation of 2 moles of 4-methyl-3-hydroxyanthranilic acid to 1 mole of actinocin, the chromophoric unit of the actinomycins;[2] this enzyme is believed to play an important role in actinomycin biosynthesis. In order to try to elucidate the steps in the biosynthesis of this family of antibiotics, a group of polypeptides whose sequences appear in actinomycin D has been attached to the 4-methyl-3-hydroxyanthranoyl unit for study as possible substrates for the oxidative enzyme;[3] the preparation of these compounds is the subject of this manuscript.

Because we were interested in obtaining both the free polypeptides and the anthranoyl peptides, a stepwise addition of a N-carbobenzyloxyamino acid to a *t*-butyl aminoester was generally employed to give a blocked peptide that could serve as a common intermediate. Catalytic hydrogenation of the blocked peptide prepared in this way yielded an aminoester suitable for coupling with 3-(benzyloxy)-2-nitro-*p*-toluic acid (XXVIII) (3) to prepare the anthranoyl peptides, whereas a two-step process of hydrogenation and cleavage of the tertiary ester with trifluoroacetic acid yielded the free peptide, usually as a trifluoroacetic acid salt.

The phosphoryl chloride coupling method (4, p. 1006), because of its convenience, was used mostly in preparing the peptides (see Reaction Schemes 1 and 2). The blocked tripeptide (VII) was also prepared by the azide method (4, p. 1949), which is known to proceed without racemization, and by the carbodiimide technique (4, p. 1016); the products from the three preparations had identical optical rotations. The blocked pentapeptide (XXIV) was more conveniently prepared by coupling of the azide derived from V with the tripeptide ester (XXI); the alternative preparation of XXIV by the stepwise route using phosphoryl chloride couplings, however, gave an essentially identical product.

Hydrogenolysis of the blocked dipeptide (XI) proceeded normally, but the product, L-ProSarO*t*-Bu, cyclized to the diketopiperazine (XXVII) very readily at room temperature; special reaction conditions were required to obtain useful yields of the blocked tripeptide (XII). Ordinarily the *t*-butyl esters of dipeptides are quite stable to diketopiperazine cyclization and are thus advantageous in synthetic work. For example, Anderson (5) noted that *t*-butyl glycylphenylalaninate showed no evidence of diketopiperazine when held at room temperature for 23 days. The present work shows that, at least in

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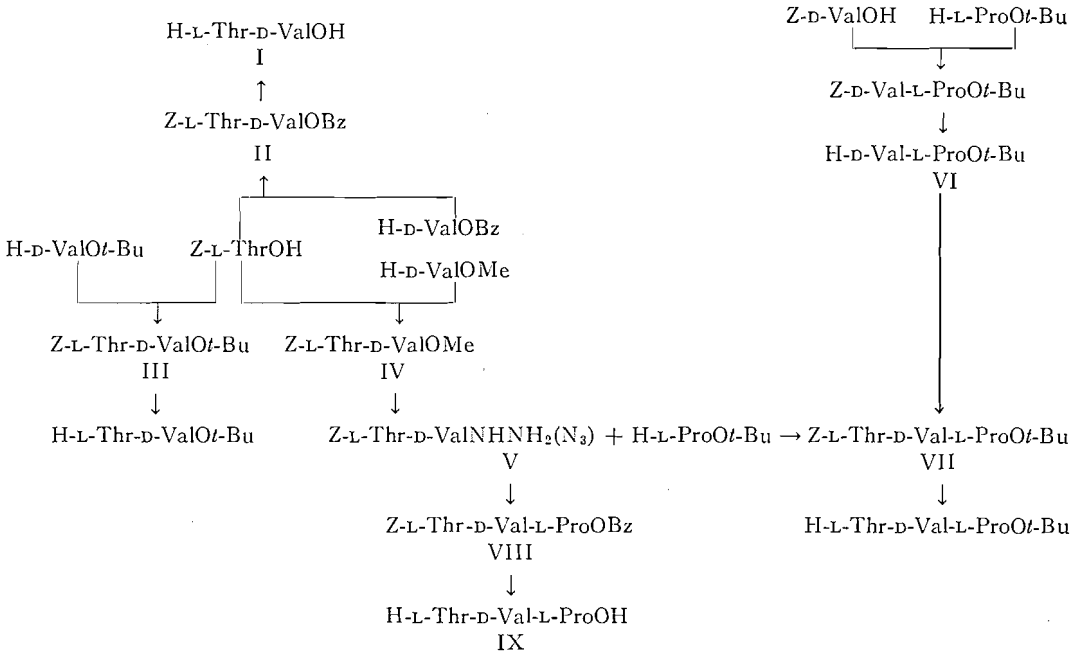
[2]*For pertinent reviews see ref. 2.*

[3]*The enzymatic studies will be reported separately by Dr. Katz and his co-workers.*



REACTION SCHEME 1.

certain cases, *t*-butyl esters of dipeptides are not stable to intramolecular cyclization. The ease of cyclization of L-ProSarO*t*-Bu may be attributable to the presence of the proline moiety; recently, the enhanced tendency of proline dipeptides to undergo intramolecular cyclization has been noted in some mass spectral work (6). However, in our own work SarSarO*t*-Bu also cyclizes readily, and the presence of the sarcosine moiety in the dipeptide ester derived from XI may be the dominant factor.

The nuclear magnetic resonance (n.m.r.) spectra of all the peptides containing sarcosine and N-methyl-L-valine showed the double resonances for the N-methyl protons that result from amide *cis–trans* isomerism.

To prepare the anthranoyl peptides (XXXI) the respective *t*-butyl peptide esters were coupled with the acid (XXVIII) (3) by the phosphoryl chloride method (4, p. 1006) to form XXIX (Reaction Scheme 3). Reaction of XXIX with trifluoroacetic acid cleaved both the ester group and the aromatic O-benzyl group (7) to give XXX. Catalytic hydrogenation of XXX yielded the anthranoyl peptides that were stable in the form of their hydrochlorides (XXXI).

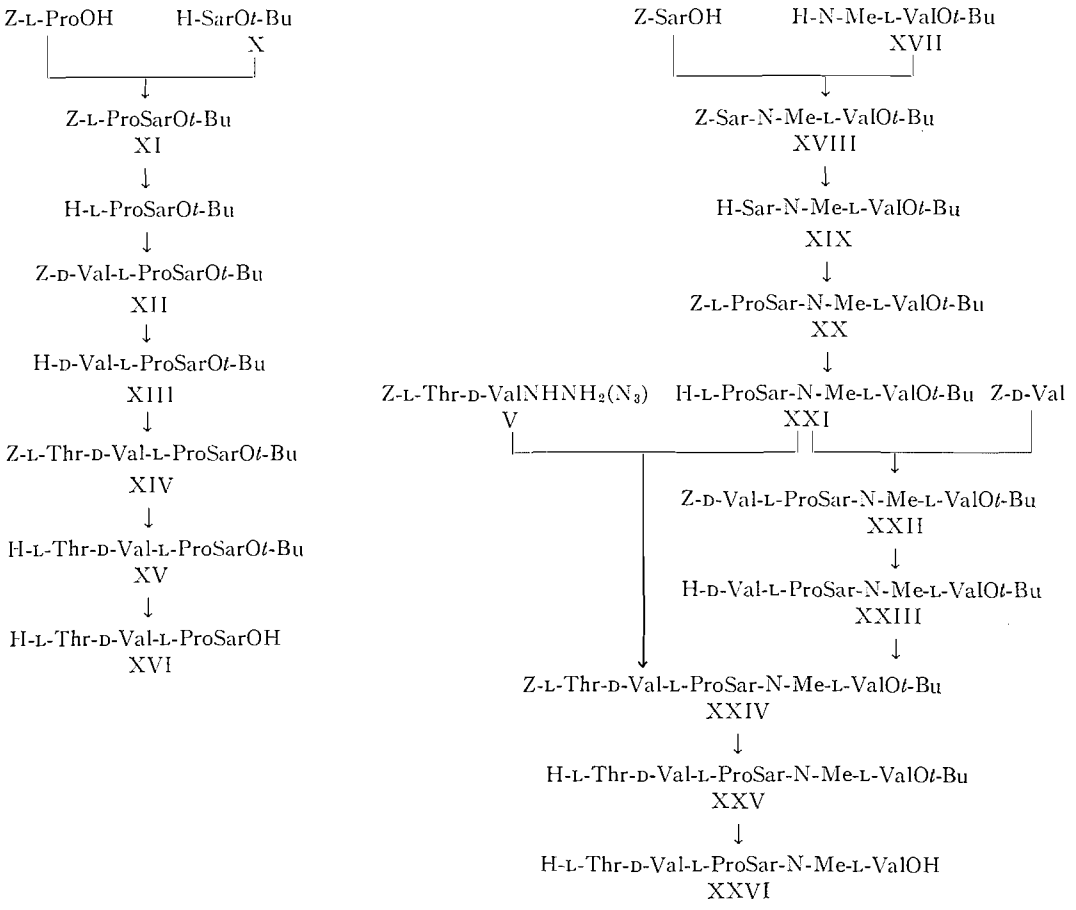
EXPERIMENTAL[4]

t-Butyl N-(Carbobenzoxy)-L-threonyl-D-valyl-L-prolinate (VII)

Phosphoryl Chloride Method

A solution of 1.319 g (4.76 mmoles) of *t*-butyl D-valyl-L-prolinate (VI) and 1.206 g (4.76 mmoles) of N-(carbobenzoxy)-L-threonine in 32 ml of dry, freshly distilled tetrahydrofuran was prepared and cooled

[4]*Boiling points and melting points are uncorrected; the latter were obtained with the Thomas–Hoover apparatus. Paper chromatography was run by the descending technique on Whatman No. 1 paper with the solvent system n-butanol – acetic acid – water (5:2:3); ninhydrin was used for detection. Magnesium sulfate was used to dry organic solutions unless otherwise noted. The n.m.r. spectra were run in deuteriochloroform on the Varian A-60 spectrometer with tetramethylsilane as the internal standard unless otherwise noted. Thin-layer chromatography was carried out on Brinkman silica gel HF, 0.25 mm thick. Detection was accomplished by ultraviolet and by 20% H_2SO_4 (v/v) spray. Silicic acid used for chromatography was a reagent grade, 90–200 mesh material. Optical rotations were run in chloroform at the D line unless otherwise noted. Gas chromatography was run in a 5 ft × $\frac{3}{8}$ inch phenyldiethanolamine succinate column, helium being used as a carrier gas.*



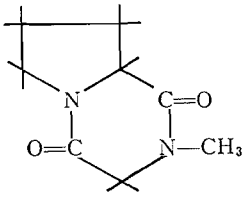
REACTION SCHEME 2.

to −15 to −20°. Phosphoryl chloride (0.436 ml (4.76 mmoles)) was added rapidly followed immediately by 1.32 ml (9.52 mmoles) of triethylamine. The mixture was stirred for 1 h at −15 to −20°, water (20 ml) was added, and then the tetrahydrofuran was evaporated *in vacuo* at room temperature. Water (20 ml) was added to the residue and the mixture was extracted with 25 ml of dichloromethane. The organic solution was washed with two 20 ml portions of water, two 20 ml portions of saturated aqueous sodium bicarbonate solution, and finally 20 ml of water; then the solution was dried and evaporated *in vacuo* to give 1.808 g (75%) of an oil that showed a major single spot on thin-layer chromatography (t.l.c.), with a trace spot at the origin. The oil was chromatographed on 60 g of silicic acid, and VII was obtained, by elution with ethyl acetate – chloroform (1:9), as an oil with $[\alpha]^{23}$ −33.3 ± 1.0° (*c*, 1.89).

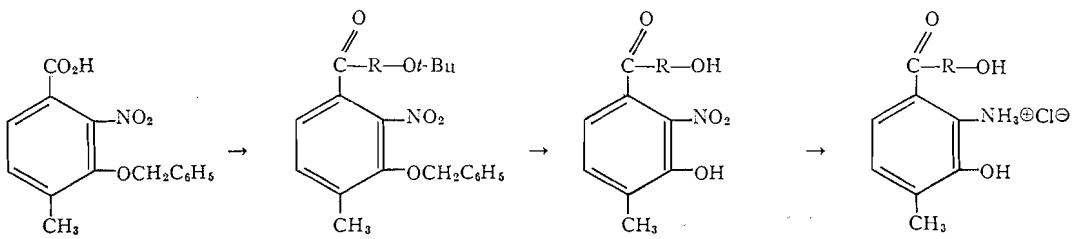
Anal. Calcd. for $C_{26}H_{39}N_3O_7$: N, 8.31. Found: N, 7.99.

Carbodiimide Method

A mixture of 1.455 g (5.25 mmoles) of VI, 1.364 g (5.38 mmoles) of N-(carbobenzoxy)-L-threonine, and 1.11 g (5.38 mmoles) of dicyclohexylcarbodiimide in 25 ml of dichloromethane was prepared, stirred for 18 h at room temperature, and then cooled to 0°. Glacial acetic acid (1 ml) was added, the mixture was filtered, and the filtrate was washed successively with 10 ml portions of water, 2 *N* hydrochloric acid, water, saturated



XXVII



XXVIII XXIX XXX XXXI

a series, R = L-Thr-D-Val
b series, R = L-Thr-D-Val-L-Pro
c series, R = L-Thr-D-Val-L-ProSar
d series, R = L-Thr-D-Val-L-ProSar-N-Me-L-Val

REACTION SCHEME 3.

aqueous sodium bicarbonate, and water; then the filtrate was dried and evaporated. The residue, which contained some solid, was dissolved in 25 ml of cyclohexane, filtered through Celite, and evaporated *in vacuo* to give 2.26 g of an oil that showed several spots on t.l.c. The oil was chromatographed on 76 g of silicic acid to yield, by elution with chloroform and ethyl acetate – chloroform (1:9), 1.819 g (67%) of an oil that showed a single spot on t.l.c. and had $[\alpha]^{25}$ $-33.1 \pm 0.9°$ (*c*, 1.91).

Azide Method

To a stirred solution at 0° of 4.20 g (11.5 mmoles) of N-(carbobenzoxy)-L-threonyl-D-valyl hydrazide (V) in a mixture of 42 ml of glacial acetic acid and 28 ml of 2 *N* hydrochloric acid was added 0.79 g (11.5 mmoles) of sodium nitrite dissolved in 4 ml of cold water. The mixture was diluted with 3 volumes of ice water and was extracted with 40 ml of cold ethyl acetate. The organic layer was washed with two 20 ml portions of ice water and two 20 ml portions of cold saturated aqueous sodium bicarbonate, dried over sodium sulfate, and decanted into a solution of 2.07 g (12.1 mmoles) of *t*-butyl L-prolinate in 10 ml of cold ethyl acetate. All operations were carried out rapidly in a cold room maintained at 4°. The mixture was stirred at 4° for 48 h; washed successively with 25 ml portions of water, saturated aqueous sodium bicarbonate, water, 2 *N* hydrochloric acid, and water; dried; and evaporated *in vacuo* to leave 4.44 g of a glass. This residue showed several spots on t.l.c. A portion of it, 3.218 g, was chromatographed on 76 g of silicic acid and the material that was eluted with ethyl acetate – chloroform (1:9) (1.971 g (45% from V)) had $[\alpha]^{23}$ $-33.1 \pm 1.0°$ (*c*, 1.94) and showed a single spot on t.l.c.

Anal. Calcd. for $C_{26}H_{39}N_3O_7$: C, 61.8; H, 7.77; N, 8.31. Found: C, 61.0; H, 7.51; N, 7.94.

Benzyl N-(carbobenzoxy)-L-threonyl-D-valinate (II) was prepared by the carbodiimide route to give an 80% yield of II, m.p. 90.1–90.6° (from methanol–water), that had $[\alpha]^{24}$ $-19.3 \pm 0.4°$ (*c*, 4.63).

Anal. Calcd. for $C_{24}H_{30}N_2O_6$: C, 65.2; H, 6.83; N, 6.33. Found: C, 65.1; H, 6.81; N, 6.53.

L-Threonyl-D-valine (I) was prepared by catalytic hydrogenolysis of II in 80% aqueous acetic acid over 5% palladium on carbon to give a 56% yield of I, m.p. 238.2–239.5° (from water–ethanol), that had $[\alpha]^{22}$ $+47.1 \pm 1.0°$ (*c*, 2.20 in water). On paper chromatography the compound showed a single spot with R_f 0.62.

Anal. Calcd. for $C_9H_{18}N_2O_4$: C, 49.5; H, 8.31; N, 12.8. Found: C, 49.6; H, 8.19; N, 12.6.

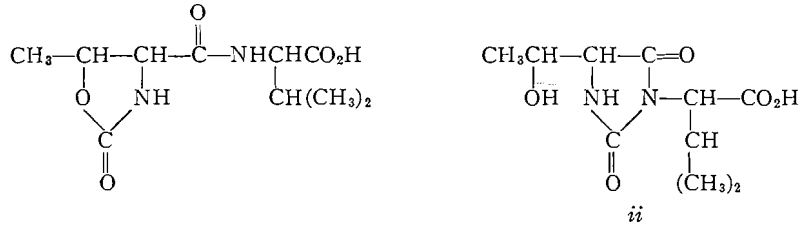
Methyl N-(carbobenzoxy)-L-threonyl-D-valinate (IV) was prepared by the carbodiimide route to give a 45% yield of IV, m.p. 121–122.5° (from benzene – petroleum ether (30–60°)), that had $[\alpha]^{22}$ $-33.7 \pm 0.5°$ (*c*, 4.97).

Anal. Calcd. for $C_{18}H_{26}N_2O_6$: C, 59.0; H, 7.16; N, 7.84. Found: C, 59.3; H, 7.23; N, 7.84.

N-(Carbobenzoxy)-L-threonyl-D-valine hydrazide (V) was prepared conventionally in 60% yield from IV[5] and had m.p. 244.8–246.5° (decomp.) (from absolute ethanol).

Anal. Calcd. for $C_{17}H_{26}N_4O_5$: C, 55.7; H, 7.16; N, 15.3. Found: C, 55.2; H, 7.22; N, 15.0.

[5]*An attempt to saponify IV with dilute base in aqueous dioxane gave a low yield of a solid, m.p. 225–228° (decomp.), whose elemental analysis and spectral data are compatible with the cyclic urethan i. As a referee has pointed out, these data do not eliminate the isomeric hydantoin ii as a possible structure.*



ii

*t-Butyl N-(carbobenzoxy)-*L*-threonyl-*D*-valinate (III)* was prepared by the carbodiimide method to give a 68% yield of solid, m.p. 112.8–115.0° (from cyclohexane–hexane), that had $[\alpha]^{24}$ −27.1 ± 0.5° (*c*, 5.07).

Anal. Calcd. for $C_{21}H_{32}N_2O_6$: C, 61.8; H, 7.90; N, 6.86. Found: C, 61.8; H, 7.87; N, 6.95.

t-Butyl D*-valyl-*L*-prolinate (VI)* was prepared by catalytic hydrogenation over 5% palladium on carbon in 95% ethanol of crude *t*-butyl N-(carbobenzoxy)-D-valyl-L-prolinate that had been prepared as an oil in 77% yield by the phosphoryl chloride method. The dipeptide ester (VI) was an oil with $[\alpha]^{23}$ −107 ± 2° (*c*, 1.56).

Anal. Calcd. for $C_{13}H_{26}N_2O_3$: C, 60.7; H, 9.79; N, 10.9. Found: C, 60.1; H, 9.67; N, 10.6.

*Benzyl N-(carbobenzoxy)-*L*-threonyl-*D*-valyl-*L*-prolinate (VIII)* was prepared from V in 68% yield by the azide method to give an oil that was purified by silicic acid chromatography and which had $[\alpha]^{23}$ −37.4 ± 1.7° (*c*, 1.12).

Anal. Calcd. for $C_{29}H_{37}N_3O_7$: C, 64.5; H, 6.91; N, 7.79. Found: C, 63.4; H, 7.00; N, 7.60.

L*-Threonyl-*D*-valyl-*L*-proline (IX)* was prepared by catalytic hydrogenolysis of VIII in 80% aqueous acetic acid over 5% palladium on charcoal. The tripeptide (IX) had m.p. 180.0–181.5° (decomp.) (from water–ethanol) and $[\alpha]^{25}$ −2.8 ± 0.7° (*c*, 1.48 in water). On paper chromatography the compound showed a single spot with R_f 0.64.

Anal. Calcd. for $C_{14}H_{25}N_3O_5 \cdot 0.8H_2O$: C, 51.0; H, 8.13; N, 12.7. Found: C, 50.9; H, 8.01; N, 13.0.

t-Butyl sarcosinate (X) was prepared by catalytic hydrogenolysis of crude *t*-butyl N-(carbobenzoxy)-sarcosinate (prepared from N-(carbobenzoxy)-sarcosine by Anderson's (5) procedure) in 95% ethanol over 5% palladium on carbon. The hydrogenolysis product was purified by distillation to give material that had b.p. 76.5–78° at 40 mm and which showed a single peak on vapor phase chromatography with a retention time of 1.80 min, a column temperature of 130° and a flow rate of 51 ml/min being used. The analytical sample had n_D^{23} 1.4148.

Anal. Calcd. for $C_7H_{15}NO_2$: C, 57.9; H, 10.4; N, 9.65. Found: C, 57.7; H, 10.4; N, 9.42.

*t-Butyl N-(carbobenzoxy)-*L*-prolylsarcosinate (XI)* was prepared in 85% yield by the phosphoryl chloride procedure to give the blocked dipeptide as an oil that showed a trace impurity on t.l.c. and had $[\alpha]^{22.5}$ −52.1 ± 0.9° (*c*, 2.85 in ethanol).

Anal. Calcd. for $C_{20}H_{28}N_2O_6$: C, 63.8; H, 7.49; N, 7.44. Found: C, 63.8; H, 7.47; N, 7.39.

2,5-Dioxo-1-methyl-3,4-trimethylenepiperazine (XXVII)

The blocked dipeptide (XI, 2.92 g) was dissolved in 30 ml of 95% ethanol and was hydrogenated conventionally with 5% palladium on carbon. After evaporation there was an oil (1.80 g) whose n.m.r. spectrum indicated loss of the *t*-butyl group by the reduced integrated intensity of the *t*-butyl protons relative to the rest of the molecule. Distillation (short path) of the material gave 0.68 g (bath temperature 125° at 0.008 mm) of a semisolid which was crystallized from ethyl acetate – cyclohexane to give the hygroscopic product, m.p. 46.2–47.5°, $[\alpha]^{22}$ −131.8 ± 2.9° (*c*, 0.613 in ethanol).

Anal. Calcd. for $C_8H_{12}N_2O_2$: C, 57.1; H, 7.20; N, 16.7. Found: C, 56.7; H, 7.20; N, 16.3.

*t-Butyl N-(carbobenzoxy)-*D*-valyl-*L*-prolylsarcosinate (XII)* was prepared in 71% yield by the phosphoryl chloride method from *t*-butyl L-prolylsarcosinate to give XII as an oil that showed two trace contaminants on t.l.c. The *t*-butyl dipeptide used in the condensation was prepared from XI by hydrogenation at 0°, rapid removal of the solvent at −10 to 0° in a high vacuum, and immediate use of the crude product in the coupling.

Anal. Calcd. for $C_{25}H_{37}N_3O_6$: C, 63.1; H, 7.84; N, 8.83. Found: C, 63.0; H, 7.91; N, 8.81.

t-Butyl D*-valyl-*L*-prolylsarcosinate (XIII)* was obtained in 93% yield as a solid with no definite melting point, by conventional hydrogenolysis in 95% ethanol. It had $[\alpha]^{23}$ −103 ± 1.3° (*c*, 1.55 in ethanol).

Anal. Calcd. for $C_{17}H_{31}N_3O_4$: C, 59.8; H, 9.16; N, 12.3. Found: C, 59.3; H, 9.11; N, 12.3.

*t-Butyl N-(carbobenzoxy)-*L*-threonyl-*D*-valyl-*L*-prolylsarcosinate (XIV)* was prepared in 67% yield by the phosphoryl chloride method to give the blocked tetrapeptide as a foam that showed a single spot on t.l.c. and had $[\alpha]^{23}$ −37.8 ± 1.4° (*c*, 0.923 in ethanol).

Anal. Calcd. for $C_{29}H_{44}N_4O_8$: C, 60.4; H, 7.69; N, 9.72. Found: C, 59.9; H, 7.80; N, 9.79.

t-Butyl L*-threonyl-*D*-valyl-*L*-prolylsarcosinate (XV)* was obtained in 92% yield as a white solid with no definite melting point, by conventional catalytic hydrogenolysis of XIV. It had $[\alpha]^{23}$ −28.1 ± 5.0° (*c*, 0.354 in ethanol).

Anal. Calcd. for $C_{21}H_{38}N_4O_6 \cdot H_2O$: C, 54.8; H, 8.75; N, 12.2. Found: C, 54.5; H, 8.40; N, 11.8.

L*-Threonyl-*D*-valyl-*L*-prolylsarcosine (XVI) Trifluoroacetate*

The tetrapeptide ester (XV, 0.700 g) was dissolved in 15 ml of trifluoroacetic acid, and the solution was allowed to stand at room temperature for 45 min and then was evaporated *in vacuo*. The residue was treated with 20 ml of benzene, the mixture evaporated *in vacuo*, and the treatment repeated. The residue was then dissolved in 10 ml of absolute ethanol, the solution filtered, and the filtrate treated with excess dry ether to precipitate the peptide salt. After a second ethanol–ether precipitation the product was isolated by centrifugation to yield 0.415 g (55%) of the salt, m.p. 147–160° (decomp.), $[\alpha]^{23}$ +9.1 ± 1.5° (*c*, 0.667 in ethanol). On paper chromatography the compound showed a single spot with R_f 0.67.

Anal. Calcd. for $C_{17}H_{30}N_4O_6 \cdot CF_3CO_2H$: C, 45.6; H, 6.25; F, 11.4; N, 11.2. Found: C, 45.6; H, 6.65; F, 11.7; N, 11.7.

Amino acid analysis of the tetrapeptide showed a molar ratio for Thr:Val:Pro:Sar of 1.06:1.04:1.01:0.77.

*t-Butyl N-Methyl-*L*-valinate (XVII)*

Liquid isobutylene (25 ml) was added to a solution of 2.9 g of N-methyl-L-valine (8) in 25 ml of dry dioxane containing 2.4 ml of concentrated sulfuric acid. The mixture, in a heavy-walled, tightly stoppered bottle, was stirred vigorously for 18 h at room temperature; then the excess isobutylene was vented and the residue was poured into an ice-cold mixture of 100 ml of water and 200 ml of ether. The water layer was separated, adjusted to pH 10 with 1 *N* sodium hydroxide, and extracted with three 25 ml portions of ether. The ether extract was dried and the ether removed by distillation through a short Vigreux column. Finally some dioxane was removed by application of an aspirator vacuum at room temperature. The residual liquid (3.0 g) was distilled through a spinning-band column to give 1.87 g (77%) of distillate collected in two fractions, b.p. 64–76° at 16 mm and 70–73° at 12 mm. The second fraction was used for analysis and had $[\alpha]^{20}$ +4.7 ± 0.9° (*c*, 0.95). Vapor phase chromatography showed a single peak with a retention time of 1.55 min, a column temperature of 139° and a flow rate of 143 ml/min being used.

Anal. Calcd. for $C_{10}H_{21}NO_2$: C, 64.1; H, 11.3; N, 7.48. Found: C, 63.8; H, 11.5; N, 7.36.

On a larger scale the removal of the dioxane was troublesome and caused difficulty in the isolation of XVII. The conversion of N-benzyl-N-methyl-L-valine (8) into the *t*-butyl ester, isolated as a crystalline hydrochloride, was simpler, but no practical system for debenzylation of the ester could be found.

*t-Butyl N-(carbobenzoxy)sarcosyl-N-methyl-*L*-valinate (XVIII)* was prepared in 71% yield by the phosphoryl chloride method to give the product as an oil that showed several trace spots on t.l.c. and had $[\alpha]^{23}$ −78.0 ± 0.9° (*c*, 1.08 in ethanol). The integration of the n.m.r. spectrum of the product showed the proper ratios for structure XVIII.

Anal. Calcd. for $C_{21}H_{32}N_2O_5$: C, 64.3; H, 8.22; N, 7.14. Found: C, 63.9; H, 7.95; N, 7.41.

*t-Butyl sarcosyl-N-methyl-*L*-valinate (XIX)* was prepared by conventional hydrogenolysis of XVIII to give a 65% yield of an oil that had $[\alpha]^{22}$ −68.1 ± 3.2° (*c*, 1.71 in ethanol) and which showed no signs of cyclization at room temperature.

Anal. Calcd. for $C_{13}H_{26}N_2O_3$: C, 60.4; H, 10.1; N, 10.9. Found: C, 60.4; H, 10.2; N, 11.0.

*t-Butyl N-(carbobenzoxy)-*L*-prolylsarcosyl-N-methyl-*L*-valinate (XX)* was prepared by the phosphoryl chloride method to give a 75% yield of an oil that was purified by silicic acid chromatography. The fraction eluted with chloroform represented a 44% yield and had $[\alpha]^{21}$ −102.8 ± 2.0° (*c*, 0.864 in ethanol).

Anal. Calcd. for $C_{26}H_{39}N_3O_6$: C, 63.8; H, 8.03; N, 8.58. Found: C, 63.7; H, 8.05; N, 8.79.

t-Butyl L*-prolylsarcosyl-N-methyl-*L*-valinate (XXI)* was prepared conventionally from XX to give 95% of an oil that had $[\alpha]^{23}$ −141.2 ± 0.6° (*c*, 0.91 in ethanol).

Anal. Calcd. for $C_{18}H_{33}N_3O_4$: C, 60.8; H, 9.36; N, 11.8. Found: C, 60.7; H, 9.21; N, 11.9.

*t-Butyl N-(carbobenzoxy)-*D*-valyl-*L*-prolylsarcosyl-N-methyl-*L*-valinate (XXII)* was prepared by the phosphoryl chloride procedure to give, after silicic acid chromatography, a 63% yield of an oil that had $[\alpha]^{21}$ −75.3 ± 2.0° (*c*, 1.00 in ethanol).

Anal. Calcd. for $C_{31}H_{48}N_4O_7$: C, 63.2; H, 8.22; N, 9.53. Found: C, 63.0; H, 8.71; N, 9.43.

t-Butyl D*-valyl-*L*-prolylsarcosyl-N-methyl-*L*-valinate (XXIII)* was prepared from XXII to give a 97% yield of a gum that had $[\alpha]^{25}$ −125.2 ± 2.4° (*c*, 1.15 in methanol).

Anal. Calcd. for $C_{23}H_{42}N_4O_5$: C, 60.8; H, 9.31; N, 12.3. Found: C, 60.9; H, 9.09; N, 12.4.

*t-Butyl N-(carbobenzoxy)-*L*-threonyl-*D*-valyl-*L*-prolylsarcosyl-N-methyl-*L*-valinate (XXIV)* was prepared both by the phosphoryl chloride procedure from XXIII and by the azide coupling of XXI and the azide derived from V. The phosphoryl chloride procedure provided, after chromatography on activated Florosil, 57% of a gum that gave a single spot on t.l.c. and had $[\alpha]^{21}$ −78.3 ± 1.0° (*c*, 0.548 in ethanol).

Anal. Calcd. for $C_{35}H_{55}N_5O_9$: C, 60.9; H, 8.04; N, 10.2. Found: C, 60.5; H, 8.16; N, 9.78.

The product from the azide coupling reaction was obtained in 60% yield after chromatography on Florosil and showed t.l.c. behavior identical with that of the phosphoryl chloride product; it had $[\alpha]^{21}$ −74.4 ± 1.7° (*c*, 1.10 in ethanol).

t-Butyl L*-threonyl-*D*-valyl-*L*-prolylsarcosyl-N-methyl-*L*-valinate (XXV)* was prepared from XXIV to give 92% of a foam that had $[\alpha]^{21}$ −75.4 ± 1.8° (*c*, 0.966 in ethanol).

Anal. Calcd. for $C_{27}H_{49}N_5O_7$: C, 58.4; H, 8.89; N, 12.6. Found: C, 57.8; H, 8.72; N, 12.3.

L*-Threonyl-*D*-valyl-*L*-prolylsarcosyl-N-methyl-*L*-valine (XXVI) trifluoroacetate* was prepared from XXV by the procedure described for XVI to give an 81% yield of a white foam that had $[\alpha]^{22}$ −42.4 ± 1.6° (*c*, 1.05 in ethanol). On paper chromatography the compound showed a single spot with R_f 0.69.

Anal. Calcd. for $C_{23}H_{41}N_5O_7 \cdot CF_3CO_2H$: C, 48.9; H, 6.90; F, 9.29; N, 11.4. Found: C, 48.7; H, 7.05; F, 9.66; N, 11.9.

*t-Butyl N-[3-(benzyloxy)-2-nitro-p-toluoyl]-*L*-threonyl-*D*-valinate (XXIXa)* was prepared by the phosphoryl chloride coupling of XXVIII and *t*-butyl L-threonyl-D-valinate to give a 60% yield of a crystalline solid having an undefined melting point after crystallization from cyclohexane.

Anal. Calcd. for $C_{28}H_{37}N_3O_8$: C, 61.9; H, 6.86; N, 7.73. Found: C, 61.8; H, 6.75; N, 7.53.

*N-(2-Nitro-3,4-cresotoyl)-*L*-threonyl-*D*-valine (XXXa)*

A solution of 3.578 g of XXIX*a* in 25 ml of trifluoroacetic acid was allowed to stand at room temperature for 1.5 h, and then was evaporated *in vacuo*. The residue was freed of acid by the successive addition and

evaporation of three 25 ml portions of benzene. The residue was partitioned between 25 ml each of chloroform and saturated aqueous sodium bicarbonate. The aqueous extract was acidified to pH 1 with 3 *N* hydrochloric acid and was extracted with 25 ml of chloroform. The organic extract was dried and evaporated *in vacuo* to leave 2.59 g of a yellow solid. Recrystallization from benzene yielded three crops of crystalline solid, 1.63 g (62%). A sample that was recrystallized for analysis had m.p. 101.2–104.0°.

Anal. Calcd. for $C_{17}H_{23}N_3O_8$: C, 51.4; H, 5.83; N, 10.6. Found: C, 51.6; H, 6.31; N, 10.3.

N-(2-Amino-3,4-cresotoyl)-L-*threonyl*-D-*valine Hydrochloride (XXXIa)*

A stirred mixture of 1.467 g of XXX*a*, 25 ml of absolute ethanol that contained 5 drops of glacial acetic acid, and 350 mg of 5% palladium on carbon was maintained under a hydrogen atmosphere for 18 h, during which time 343 ml (corrected for catalyst absorption) of hydrogen was absorbed. The mixture was filtered through Celite and the filtrate was treated with 10 ml of hydrogen chloride saturated ether. Dry hydrogen chloride was bubbled through the chilled solution for about 15 min, and then the solution was evaporated *in vacuo*. The residue was dissolved in the minimum amount of absolute ethanol, and the product was precipitated with excess dry ether. The precipitate (1.135 g (76%)) was collected by centrifugation to give a solid that showed no definite melting behavior and had $[\alpha]^{23}$ +13.8 ± 0.4° (*c*, 3.82 in 1 *N* hydrochloric acid), λ_{max}^{pH1} 221 mμ (ε 27 400) and 285 mμ (ε 6 450).

Anal. Calcd. for $C_{17}H_{26}ClN_3O_6$: C, 50.6; H, 6.49; Cl, 8.78; N, 10.4. Found: C, 50.8; H, 6.65; Cl, 8.66; N, 10.4.

t-Butyl N-[3-(benzyloxy)-2-nitro-p-toluoyl]-L-*threonyl*-D-*valyl*-L-*prolinate (XXIXb)* was a foam that was chromatographed on silicic acid to give a low yield of an amorphous material that gave a single spot on t.l.c. and whose n.m.r. spectrum was in good agreement with that of the postulated structure.

N-(2-Nitro-3,4-cresotoyl)-L-*threonyl*-D-*valyl*-L-*proline (XXXb)* was prepared from crude XXIX*b* with trifluoroacetic acid to give a 61% yield of solid after crystallization from ethyl acetate – cyclohexane. The material did not have a definite melting point; it had $[\alpha]^{26}$ −19.6 ± 0.8° (*c*, 1.87 in ethanol).

Anal. Calcd. for $C_{22}H_{30}N_4O_9$: C, 53.4; H, 6.14; N, 11.33. Found: C, 53.5; H, 6.73; N, 11.2.

Crystallization of the compound from carbon tetrachloride gave a solvate.

Anal. Calcd. for $C_{22}H_{30}N_4O_9 \cdot 0.4CCl_4$: C, 48.4; H, 5.44; Cl, 10.2; N, 10.1. Found: C, 48.6; H, 5.77; Cl, 9.90; N, 10.2.

N-(2-Amino-3,4-cresotoyl)-L-*threonyl*-D-*valyl*-L-*proline hydrochloride (XXXIb)* was an amorphous powder that was prepared in 66% yield from XXX*b* and which had $[\alpha]^{23}$ −16.9 ± 0.6° (*c*, 2.28 in 1 *N* hydrochloric acid).

Anal. Calcd. for $C_{22}H_{33}ClN_4O_7$: C, 52.8; H, 6.64; Cl, 7.08; N, 11.2. Found: C, 52.6; H, 6.77; Cl, 6.68; N, 10.6.

t-Butyl N-[3-(benzyloxy)-2-nitro-p-toluoyl]-L-*threonyl*-D-*valyl*-L-*prolylsarcosinate (XXIXc)* was obtained as a chromatographically homogeneous foam in 35% yield after chromatography on Florosil. It had $[\alpha]^{23}$ −20.9 ± 1.2° (*c*, 0.94 in ethanol).

Anal. Calcd. for $C_{36}H_{49}N_5O_{10}$: C, 60.8; H, 6.94; N, 9.84. Found: C, 60.9; H, 6.90; N, 9.17.

N-(2-Nitro-3,4-cresotoyl)-L-*threonyl*-D-*valyl*-L-*prolylsarcosine (XXXc)* was obtained from XXIX*c* as an amorphous solid after the usual trifluoroacetic acid treatment and a final precipitation from ethyl acetate with hexane. The material gave poor analytical results.

N-(2-Amino-3,4-cresotoyl)-L-*threonyl*-D-*valyl*-L-*prolylsarcosine hydrochloride (XXXIc)* was obtained as an ethanol solvate from hydrogenation of XXX*c*. It had $[\alpha]^{24}$ −30.3 ± 1.2° (*c*, 0.99 in 1 *N* hydrochloric acid) and λ_{max}^{pH1} 216 mμ (ε 31 200), 222 mμ (shoulder, ε 28 900), and 330 mμ (ε 2 205).

Anal. Calcd. for $C_{25}H_{38}ClN_5O_8 \cdot C_2H_5OH$: C, 52.4; H, 7.17; Cl, 5.74; N, 11.3. Found: C, 52.1; H, 6.85; Cl, 5.71; N, 11.3.

The presence of ethanol was confirmed by mass spectrometry.

t-Butyl N-[3-(benzyloxy)-2-nitro-p-toluoyl]-L-*threonyl*-D-*valyl*-L-*prolylsarcosyl-N-methyl*-L-*valinate (XXIXd)* was obtained in 58% yield as a chromatographically homogeneous foam after chromatography on activated Florosil.

Anal. Calcd. for $C_{42}H_{60}N_6O_{11}$: C, 61.1; H, 7.33; N, 10.2. Found: C, 61.0; H, 7.65; N, 9.45.

N-(2-Nitro-3,4-cresotoyl)-L-*threonyl*-D-*valyl*-L-*prolylsarcosyl-N-methyl*-L-*valine (XXXd)* was obtained in 85% yield from XXIX*d* as a yellow powder that showed a single spot on t.l.c.

Anal. Calcd. for $C_{31}H_{46}N_6O_{11}$: C, 54.9; H, 6.84; N, 12.4. Found: C, 55.3; H, 7.00; N, 12.3.

N-(2-Amino-3,4-cresotoyl)-L-*threonyl*-D-*valyl*-L-*prolylsarcosyl-N-methyl*-L-*valine hydrochloride (XXXId)* was obtained conventionally in 76% yield as an amorphous powder that had $[\alpha]^{22}$ −84.0 ± 2.1° (*c*, 0.61 in ethanol).

Anal. Calcd. for $C_{31}H_{49}ClN_6O_9$: C, 54.3; H, 7.21; Cl, 5.17; N, 12.3. Found: C, 54.1; H, 7.31; Cl, 4.86; N, 12.0.

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