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Synthesis of Peptide Derivatives with Actinomycin D Sequence[1]

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2-Nitro-3-benzyloxy-4-methylbenzoyl-L-threonyl-D-valyl-L-prolylsarcosyl-L-N-methylvaline has been synthesized by stepwise elongation of the peptide chain starting from the C terminal, and has been converted into actinomycin D acid.

Actinomycin D (C_1)[2] (Figure 1) has been synthesized by Brockmann and Lackner;[3-5] however few experimental details have been published. The protected tetrapeptide For-val-Pro-Sar-MeVal-OBZL[6] prepared starting from For-val-Pro-OH, was after deformylation with 10% HCl in benzylalcohol condensed with 2-nitro-3-benzyloxy-4-methylbenzoylthreonine.[5] The ensuing pentapeptide derivative was hydrogenated and subsequently oxidized to give actinomycin D acid which was lactonized in 28% yield with the use of a mixture of acetylimidazole and acetylchloride to give actinomycin D. This communication describes the synthesis of 2-nitro-3-benzyloxy-4-methylbenzoyl-L-threonyl-D-valyl-L-prolylsarcosyl-L-N-methylvaline (VIII) by a different approach.[7] Starting with C-terminal L-N-methylvaline *t*-butyl ester (I) the peptide chain was synthesized *via* the stepwise elongation procedure[8] (Scheme I). Compound VIII was obtained in an overall yield of 53% based on I. Owing to the extremely weak tendency of N-methylamino acid derivatives to crystallize, all peptide derivatives were obtained as oils or amorphous powders and all purifications had to be carried out by countercurrent distribution (ccd). The purity of a compound was judged by the degree of coincidence of the experimental distribution curve with the theoretical plot, and was verified by elemental analysis of the oils. The purity of benzyloxycarbonyl-L-threonyl-D-valyl-L-prolylsarcosyl-L-N-methylvaline (VI) was demonstrated in addition by a correct amino acid analysis. The optical purity of VI was corroborated by microbiological assays for threonine, valine, and proline.

Actinomycin acid (IX) was prepared by catalytic

SCHEME I

SCHEMATIC OUTLINE OF THE SYNTHETIC PATHWAY

H-MeVal-*t*-OBu
I

96% ↓ DCCI

Z-Sar-MeVal-*t*-OBu
II

82% ↓ a. Z-Pro-OSu
91% ↓ b. Cl·COO-*t*-Bu

Z-Pro-Sar-MeVal-*t*-OBu
III

95% ↓ a. Z-val-ONP
95% ↓ b. DCCI

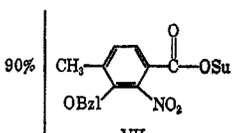
Z-val-Pro-Sar-MeVal-*t*-OBu
IV

83% ↓ azide method

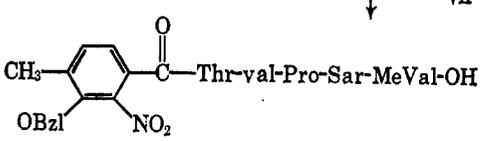
Z-Thr-val-Pro-Sar-MeVal-*t*-OBu
V

85% ↓ trifluoroacetic acid

Z-Thr-val-Pro-Sar-MeVal-OH
VI

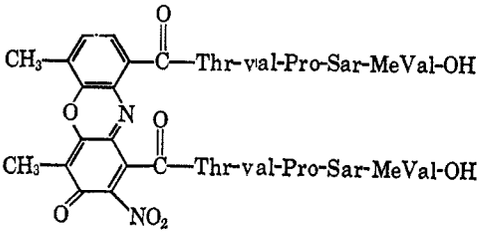
90% ↓ 

VII



VIII

76% ↓ a. catalytic hydrogenation
b. $K_3[Fe(CN)_6]$ at pH 7.3



IX
actinomycin D acid

(1) Supported in part by Public Health Service Research Grant C-6516 from the National Cancer Institute, National Institutes of Health.

(2) L. C. Vining and S. A. Waksman [*Science*, **120**, 389 (1954)] marked one of their actinomycin preparations with the letter D. This was found to be identical with the crystalline actinomycin C_1, isolated by H. Brockmann and H. Gröne, *Naturwiss.*, **41**, 65 (1954).

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(6) Abbreviations used for amino acid residues and protecting groups are standard and described in "tentative rules" of the IUPAC-IUB Commission on Biochemical Nomenclature in Biochemistry **5**, 2485 (1966). For = formyl. Amino acid abbreviations starting with a small letter denote the D configuration.

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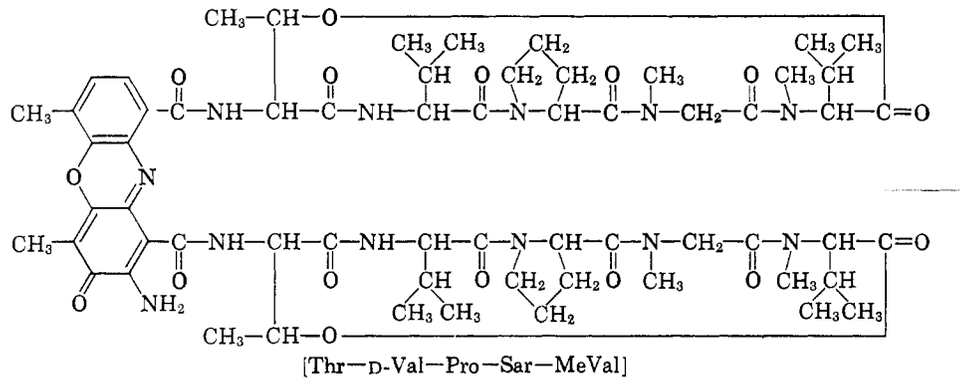

[Thr—D-Val—Pro—Sar—MeVal]

Figure 1.—Actinomycin D (C_1).

hydrogenation of VIII and subsequent oxidation.[3] Attempts at cyclization *via* formation of the lactone rings with various reagents such as acetylimidazole–acetyl chloride,[9] carbonyldiimidazole,[10] isobutyl chloroformate,[11] *p*-toluenesulfonyl chloride,[12] polyphosphoric acid ester,[13] or N,N-dimethylformamide–dineopentyl acetal[14] yielded only 1–3% actinomycin D and hence other synthetic routes are under investigation.

Experimental Section

The synthesis of the peptides was carried out under standard conditions by the methods indicated. The general isolation and washing procedure of the peptide derivatives was carried out as follows: evaporation of the reaction mixture *in vacuo;* solution of the residue in ethyl acetate; successive washing with 1 *M* $NaHCO_3$ or 0.5 *N* NH_4OH, with 1 *M* citric acid or 0.5 *N* H_2SO_4, and with water or saturated NaCl solution; drying over $MgSO_4$; evaporation *in vacuo*. The resulting crude oils were purified by countercurrent distribution (ccd) in the solvent system toluene–chloroform–methanol–water (5:5:8:2).[15] The distribution patterns were determined by the optical density at 570 mμ of the ninhydrin reaction after alkaline hydrolysis.[16] The distribution coefficients (K) are mean values calculated from several ccd values of the crude products (analytically pure products usually exhibit somewhat lower K values). The theoretical curves were calculated according to the formula

$$V = V_{max} e^{-x^2(K+1)^2/2nK}$$

where V_{max} = plot value of peak tube, x = tube numbers in both directions from peak tube (=0), K = distribution coefficient, and n = number of transfers.

Catalytic hydrogenations for the removal of the N-protecting benzyloxycarbonyl group were carried out in methanol with the use of freshly prepared palladium black.[17] Hydrogen was continuously passed through the solution which was stirred by a vibromixer. Thin layer chromatography (tlc) on silica gel G was carried out in two systems: A (acidic), *sec*-butyl alcohol–formic acid–water (75:13.5:11.5) and B (basic), *sec*-butyl alcohol–10% NH_4OH (85:15).

L-N-Methylvaline *t*-Butyl Ester (I). A. *Via* Direct Esterification.—A suspension of L-N-methylvaline (11.9 g, 90 mmoles, prepared according to Quitt, *et al.*[18]) in methylene chloride (300 ml) was cooled to −10°, and isobutylene (300 ml) and *concentrated* H_2SO_4 (3 ml) were added. After shaking for 72 hr in a closed flask the solution was neutralized with $NaHCO_3$ and concentrated to 300 ml. Ethyl acetate (1000 ml) was added and the organic phase was washed twice each with 1 *M* $NaHCO_3$ and water, and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* yielded an oil (R_f 0.6, tlc–A) which upon distillation [bp 82–88° (20 mm)] gave 13.2 g (78%) of a colorless oil, $[\alpha]^{20}D$ +5.3° (*c* 1, chloroform) [lit.[7] bp 70–73° (12 mm), $[\alpha]^{20}D$ +4.7° (*c* 0.95, chloroform)]. For further characterization the oil was converted in 80% yield to the crystalline *p*-toluenesulfonate: mp 109–110°, $[\alpha]^{20}D$ +4.8° (*c* 1.1, water).

Anal. Calcd for $C_{17}H_{29}N_1O_5S_1$ (tosylate) (359.5): C, 56.8; H, 8.13; N, 3.92; O, 22.3; S, 8.92. Found: C, 57.0; H, 8.15; N, 3.92; O, 22.3; S, 8.92.

B. *Via* Benzyloxycarbonyl-L-N-methylvaline *t*-Butyl Ester.—A solution of benzyloxycarbonyl-L-N-methylvaline[19] (30 g, 113 mmoles) in methylene chloride (400 ml) was treated as described in A and an oil was obtained (36.4 g, 100%) which upon catalytic hydrogenation in ether (200 ml) gave I (19.1 g, 93.6%).

Benzyloxycarbonylsarcosyl-L-N-methylvaline *t*-Butyl Ester (II).—Compound II was prepared from I (7.5 g, 40 mmoles) and benzyloxycarbonylsarcosine[20] (11.2 g, 50 mmoles) in acetonitrile (100 ml) with the use of dicyclohexylcarbodiimide (10.3, 50 mmoles). The isolation and washing procedure gave a yellow oil (16.3 g). Two successive countercurrent distributions (ccd) over 100 and 200 transfers, respectively, showed a peak (K = 0.11) which closely coincided with the theoretical curve. Evaporation *in vacuo* of the peak tubes gave a colorless oil (15 g, 96%), $[\alpha]^{23}D$ −83° (*c* 1, methanol) [lit.[7] $[\alpha]^{23}D$ −78° (*c* 1.07, ethanol)].

Anal. Calcd for $C_{21}H_{32}N_2O_5$ (392.5): C, 64.3; H, 8.22; N, 7.14. Found: C, 64.3; H, 8.21; N, 7.04.

Benzyloxycarbonyl-L-prolylsarcosyl-L-N-methylvaline *t*-Butyl Ester (III). A. *Via* the N-Hydroxysuccinimid Ester Method.[21]—Compound II (27.5 g, 70 mmoles) was hydrogenated (R_f 0.35, tlc–A) and was treated with benzyloxycarbonyl-L-proline N-hydroxysuccinimide ester[21] (27 g, 78 mmoles) in dimethylformamide (100 ml). Ccd over 200 transfers (K = 0.23) yielded a colorless oil (28.1 g, 82%): R_f 0.67, tlc–A; $[\alpha]^{23}D$ −111° (*c* 1, methanol). An analytically pure sample showed K = 0.16 upon ccd [lit.[7] $[\alpha]$ ^1D −102.8° (*c* 0.864, ethanol)].

Anal. Calcd for $C_{26}H_{39}N_3O_6$ (489.6): C, 63.8; H, 8.03; N, 8.58. Found: C, 64.0; H, 8.15; N, 8.42.

B. *Via* the Mixed Anhydride Method.[22]—The mixed anhydride from benzyloxycarbonyl-L-proline[23] (8.75 g, 35 mmoles) in peroxide-free tetrahydrofuran (100 ml), triethylamine (4.9 ml), and isobutyl chloroformate (4.62 ml, 35 mmoles) was combined with hydrogenated II (prepared from 11.7 g, 30 mmoles). Ccd over 200 transfers (K = 0.3) yielded a colorless oil (13.4 g, 91.2%), $[\alpha]^{22}D$ −103° (*c* 1, methanol).

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Benzyloxycarbonyl-D-valyl-L-prolylsarcosyl-L-N-methylvaline *t*-Butyl Ester (IV). A. *Via* Nitrophenyl Ester Method.[24]—Hydrogenated III (prepared from 19.6 g, 40 mmoles, R_f 0.3, tlc–A) was dissolved in ethyl acetate (150 ml) along with benzyloxycarbonyl-D-valine *p*-nitrophenyl ester[25] (16.7 g, 45 mmoles). Glacial acetic acid (1 ml) was added to accelerate the reaction.[26] Ccd over 200 transfers showed a symmetrical peak (K = 0.2) identical with the theoretical plot and yielded a slightly cream-colored oil (22.4 g, 95.3%), $[\alpha]^{23}D$ −75° (*c* 1, methanol) [lit.[7] $[\alpha]^{21}D$ −75.3° (*c* 1, ethanol)].

Anal. Calcd for $C_{31}H_{48}N_4O_7$ (588.7): C, 63.2; H, 8.22; N, 9.52. Found: C, 63.3; H, 8.45; N, 9.61.

B. *Via* **Dicyclohexylcarbodiimide.**[27]—Hydrogenated III (prepared from 21.3 g, 43 mmoles), benzyloxycarbonyl-D-valine[28] (10.9 g, 43 mmoles), and dicyclohexylcarbodiimide (10.5 g, 50 mmoles) were allowed to react in acetonitrile (200 ml). Ccd over 200 transfers (K = 0.16) yielded a colorless oil (24.4 g, 95.3%), $[\alpha]^{24}D$ −77° (*c* 1, methanol).

Benzyloxycarbonyl-L-threonyl-D-valyl-L-prolylsarcosyl-L-N-methylvaline *t*-Butyl Ester (V).—To an ice-cold solution of benzyloxycarbonyl-L-threonine hydrazide[29] (4.4 g, 16.5 mmoles) in 1 *N* HCl (60 ml) was slowly added with stirring an aqueous solution (5 ml) of sodium nitrite (1.2 g). After 1 min ice-cold ethyl acetate (75 ml) was added and stirring was continued for 5 min when the organic phase was separated and the water phase was washed twice with 10 ml each of ice-cold ethyl acetate. The combined organic phase was washed successively twice with 1 *M* $NaHCO_3$ in saturated NaCl and once with saturated NaCl and dried at 0° over Na_2SO_4. It was filtered and added to an ice-cold solution of hydrogenated IV (prepared from 7.4 g, 12.6 mmoles, R_f 0.4, tlc–A) in ethyl acetate (50 ml). After 72 hr of stirring at 5° the product was isolated and washed as usual and gave a yellow oil (8.3 g). Ccd over 100 transfers (K = 0.28) yielded a colorless oil (7.2 g, 83%): $[\alpha]^{22}D$ −86° (*c* 1, methanol); tlc R_f 0.65 (A), 0.7 (B) [lit.[7] $[\alpha]^{21}D$ −74.4° (*c* 1.1, ethanol)].

Anal. Calcd for $C_{35}H_{55}N_5O_9$ (689.8): C, 60.9; H, 8.04; N, 10.2; O, 20.9. Found: C, 60.9; H, 8.62; N, 10.2; O, 21.0.

Benzyloxycarbonyl-L-threonyl-D-valyl-L-prolylsarcosyl-L-N-methylvaline (VI).—Treatment of V (6.7 g, 9.7 mmoles) for 20 min at room temperature with trifluoroacetic acid[30] (70 ml), evaporation of the solvent, two successive treatments with dry benzene (60 ml each), and evaporation, and ccd over 100 transfers (K = 1.1) yielded a colorless oil (5.2 g, 85%): $[\alpha]^{22}D$ −87° (*c* 1, methanol); R_f 0.3, tlc–B.

Anal. Calcd for $C_{31}H_{47}N_5O_9$ (633.7): C, 58.8; H, 7.48; N, 11.1. Found: C, 59.0; H, 7.56; N, 11.3.

Amino acid analysis[31] (1.8 mg, 6 *N* HCl, 24-hr hydrolysis at 110°) gave, based on Val = 1.00, Thr = 0.90, Pro = 0.95, Sar = 1.00. N-Methylvaline showed a skewed peak which could not be integrated accurately.

Microbiological assay[32] (5.2 mg, 6 *N* HCl, 24-hr hydrolysis at 110°) employing bacterimetric tests specific for the L forms of amino acids gave L-threonin = 8.1 μmoles, L-proline = 8.1 μmoles, L-valine = 0.13 μmoles (=1.5%). (An organism specific for N-methylvaline was not available.)

2-Nitro-3-benzyloxy-4-methylbenzoic Acid N-Hydroxysuccinimide Ester (VII).—To a solution of 2-Nitro-3-benzyloxy-4-methylbenzoic acid[33] (7.3 g, 25.3 mmoles) and N-hydroxysuccinimide (3.5 g, 30 mmoles) in dimethylformamide (50 ml) was added a solution of dicyclohexylcarbodiimide (5.8 g, 28 mmoles) in acetonitrile (25 ml). After 3 hr of stirring at room temperature with exclusion of light, the dicyclohexylurea (5.72 g) was removed by filtration and the solution was evaporated *in vacuo*. The residue was crystallized from ethanol containing a few drops of glacial acetic acid to give colorless crystals (9.35 g 96%), mp 154–156°. A sample was recrystallized from ethanol, mp 155–156°.

Anal. Calcd for $C_{19}H_{16}N_2O_7$ (384.3): C, 59.4; H, 4.20; N, 7.29; O.29.1. Found: C, 59.4; H, 4.18; N, 7.35; O, 29.1.

2-Nitro-3-benzyloxy-4-methylbenzoyl-L-threonyl-D-valyl-L-prolylsarcosyl-L-N-methylvaline (VIII).—To an ice-cold solution of hydrogenated VI (prepared from 4.45 g, 7 mmoles, R_f 0.5, tlc–A) in dimethylformamide (10 ml) was added triethylamine (1.05 ml) and VII (2.9 g, 7.5 mmoles). After 60 hr at room temperature 2 *N* HCl (3.5 ml) was added, the solvent was evaporated *in vacuo*, and the residue was dissolved in ethyl acetate. The organic phase was washed three times each with 1 *N* HCl and water and was dried over $MgSO_4$. Removal of the solvent *in vacuo* gave a cream-colored, solid foam (5.35 g). Precipitation from a solution in chloroform (50 ml) into ether (1000 ml) at −70° gave a colorless powder (4.55 g). A second fraction after evaporation of the ether and reprecipitation weighed 0.3 g, (total yield was 4.85 g, 90%), $[\alpha]^{22}D$ −56° (*c* 1, methanol), sinter point 128° [lit.[3] $[\alpha]^{21}D$ −35° (*c* 1.11, methanol)].

Anal. Calcd for $C_{38}H_{52}N_6O_{11}$ (768.8): C, 59.4; H, 6.82; N, 10.9; O, 22.9. Found: C, 59.5; H, 6.86; N, 11.3; O, 23.2.

Ccd of a sample (0.5 g) over 120 transfers showed one symmetrical peak (K = 0.9) superimposable with the theoretical plot.

Actinomycin D Acid (IX).—Compound VIII (0.4 g, 0.52 mmole) was hydrogenated for 3 hr and the colorless solution was concentrated *in vacuo* to 5 ml and diluted with 0.067 *m* phosphate buffer pH 7.3 (200 ml). A solution of potassium ferricyanide (560 mg, 1.7 mmoles) in phosphate buffer (200 ml) was heated to 60° and added to the solution of hydrogenated VIII. After 30 min the orange-red solution was cooled to 15°, solid citric acid was added to adjust the pH to 2.5, and IX was extracted with chloroform (ten times, 30 ml each). The chloroform phase was twice washed with water, dried over Na_2SO_4, and concentrated to 20 ml, after which it was added to cyclohexane (200 ml) with stirring. The precipitate was collected, washed with cyclohexane, and dried to an orange-red powder (327 mg, 95%), which showed three spots with tlc–A (main spot R_f 0.6, minor spots R_f 0.5 and 0.7). Purification by column chromatography on Sephadex LH 20 in methanol removed the two minor components which formed separate bands in front of and behind the main band. Recovery was 80% of an orange-red powder: $[\alpha]^{23}D$ −117° (*c* 0.2, methanol); λ_{max} (methanol) 237 mμ (ε 41,300) and 445 mμ (ε 25,200), [lit.[3] $[\alpha]^{23}D$ −117 ± 3° (*c* 0.2, methanol)].

Anal. Calcd for $C_{62}H_{90}N_{12}O_{18}$ (1291.5): C, 57.7; H, 7.02; N, 13.0. Found: C, 57.5; H, 7.07; N, 13.0.

Registry No.—I, 5616-87-5; toluenesulfonate, 7635-34-9; II, 5616-88-6; III, 5648-67-9; IV, 6021-08-5; V, 5992-38-1; VI, 7706-44-7; VII, 7646-46-0; VIII, 2478-47-9; IX, 6521-47-7.

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