ligand.[46] The formation of a shorter, more stable, axial bond in the zinc porphyrin is favored by the superior capacity of the Zn^{2+} ion for covalent binding of the pyridine molecule and, in minor degree, by the lessened importance of nonbonding repulsions that is associated with the larger out-of-plane displacement of the cation. The recently published structure determination for crystalline $H_2OMgTPP$[47] leads to a five-coordination group that, with the substitution of a water for a pyridine molecule as the axial ligand, follows the anticipated pattern.

(46) Reversal in the magnitudes of the Zn–X and Mg–X bond lengths, depending on whether the ligands are neutral or carry negative charge, is readily identified in isostructural zinc and magnesium compounds that crystallize in particularly simple structural types. In the fluorides and chlorides the Mg–X bonds are significantly shorter, whereas in the hexaaquo cations in the $M(OH_2)_6SiF_6$ structural type it is the $Zn–OH_2$ bonds that are shorter. Thus the less covalently (or more ionically) inclined Mg^{2+} ion allows itself to be "squeezed" into plane within a not very stable six-coordinate porphyrin ($Py_2MgEtio$) whereas Zn^{2+} does not.

(47) R. Timkovich and A. Tulinsky, *J. Amer. Chem. Soc.*, **91**, 4430 (1969). The precision of parameter determination was limited by the fact that $H_2OMgTPP$ crystallizes in the same disordered structural type as do ClFeTPP[6] and $H_2OZnTPP$.[7]

Syntheses of Actinomycin and Analogs. III. A Total Synthesis of Actinomycin D (C_1) *via* Peptide Cyclization between Proline and Sarcosine[1–3]

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Abstract: A synthesis of actinomycin D (C_1) is described in which the key reaction, cyclization of the pentapeptide lactone, was carried out by peptide bond formation between proline and sarcosine using the *p*-nitrophenyl ester activation. The ester bond between the carboxyl group of N-methylvaline and the hydroxyl group of threonine was formed by a reaction of *t*-butyloxycarbonyl-L-threonine with the mixed anhydride from benzyloxycarbonyl-L-N-methylvaline and isobutyl chloroformate. Several protected peptide intermediates were readily purified by chromatography on Sephadex LH-20 in methanol which fractionated complex reaction mixtures efficiently. The synthetic actinomycin was indistinguishable from natural material in its physical properties and its biological activities.

Actinomycins are a group of closely related peptide antibiotics.[4] Actinomycins D (C_1)[5] and C_3, of known structure[6] (Figure 1), are important as highly effective chemotherapeutics in the treatment of Wilms' tumor,[7] trophoblastic tumors,[8] and rhabdomyosarcoma.[9] Their very high cytotoxicity[10] and their inactivity toward some tumors have prompted the search for modified actinomycins with improved therapeutic indices and with broader antitumor activities. However, none of the naturally occurring variants[4] and none of the many derivatives which have been prepared by substitution at the 2 and 7 positions[11,12] of the phenoxazinone moiety have shown promise.[13] Since the peptide moieties play important roles in the action of actinomycins[14] and control the unusual solubility characteristics,[15] possibly important in cell membrane permeation, we decided to prepare peptide analogs by total synthesis. An efficient pathway was required for this program by which analogs could be prepared in sufficient quantities for comprehensive biological testing.

(1) A preliminary communication has been published: J. Meienhofer, *Experientia*, **24**, 776 (1968), part II in this series.
(2) Part I: J. Meienhofer, *J. Org. Chem.*, **32**, 1143 (1967).
(3) Abbreviations follow the rules of the IUPAC–IUB Commission on Biochemical Nomenclature, in *Biochemistry*, **5**, 1445, 2485 (1966); **6**, 362 (1967); *J. Biol. Chem.*, **241**, 2491 (1966). Threonine, proline, and N-methylvaline are of L configuration; valine is of D configuration. This work was supported by Public Health Service Research Grants (No. C-6516 from the National Cancer Institute, No. FR-05526 from the Division of Research Facilities and Resources), National Institutes of Health, by A. and M. Lasker Foundation, New York, and A. T. and V. D. Fuller Cancer Research Unit Grant, American Cancer Society (Massachusetts Division), Inc.
(4) See H. Umezawa, Ed., "Index of Antibiotics from Actinomycetes," University Park Press, State College, Pa., 1967, pp 91–101.
(5) Designation D is according to L. C. Vining and S. A. Waksman, *Science*, **120**, 389 (1954); designation C_1 is according to H. Brockmann and H. Gröne, *Naturwissenschaften*, **41**, 65 (1954).
(6) H. Brockmann, G. Bohnsack, B. Franck, H. Gröne, H. Muxfeldt, and C. Süling, *Angew. Chem.*, **68**, 70 (1956); E. Bullock and A. W. Johnson, *J. Chem. Soc.*, 3280 (1957).
(7) S. Farber, *J. Amer. Med. Assoc.*, **198**, 826 (1966); S. Farber, R. Toch, E. M. Sears, and D. Pinkel, *Advan. Cancer Res.*, **4**, 1 (1956).
(8) R. Hertz, G. T. Ross, and M. B. Lipsett, *Ann. N. Y. Acad. Sci.*, **114**, 881 (1964); G. T. Ross, L. L. Stolbach, and R. Hertz, *Cancer Res.*, **22**, 1015 (1962).
(9) E. O. Burgert, Jr., and S. D. Mills, *Mayo Clinic Proc.*, **41**, 361 (1966); D. Pinkel and J. Pickren, *J. Amer. Med. Assoc.*, **175**, 293 (1961); C. T. C. Tan, R. B. Golbey, C. L. Yap, N. Wollner, C. A. Hackethal, L. M. Murphy, H. W. Dargeon, and J. H. Burchenal, *Ann. N. Y. Acad. Sci.*, **89**, 426 (1960).
(10) H. S. Schwartz, S. S. Sternberg, and F. S. Philips in "Actinomycin," S. A. Waksman, Ed., Interscience Publishers, New York, N. Y., 1968, pp 101–120.
(11) H. Brockmann, P. Hocks, and W. Müller, *Chem. Ber.*, **100**, 1051 (1967).
(12) H. Brockmann, J. Ammann, and W. Müller, *Tetrahedron Lett.*, 3595 (1966); H. Brockmann, W. Müller, and H. Peterssen-Borstel, *ibid.*, 3531 (1966).
(13) H. Brockmann and H. Lackner, *Chem. Ber.*, **101**, 1312 (1968).
(14) W. Müller and D. M. Crothers, *J. Mol. Biol.*, **35**, 251 (1968); E. Reich and I. H. Goldberg in "Progress in Nucleic Acid Research," Vol. 3, J. N. Davidson and W. E. Cohn, Ed., Academic Press, New York, N. Y., 1964, pp 183–234.
(15) Actinomycin D is very soluble in water at 1° (128 mg/ml) but only slightly soluble at 20° (0.8 mg/ml). It is very soluble in most organic solvents and traces are soluble in cyclohexane or hexane (J. Meienhofer and R. Cotton, unpublished).

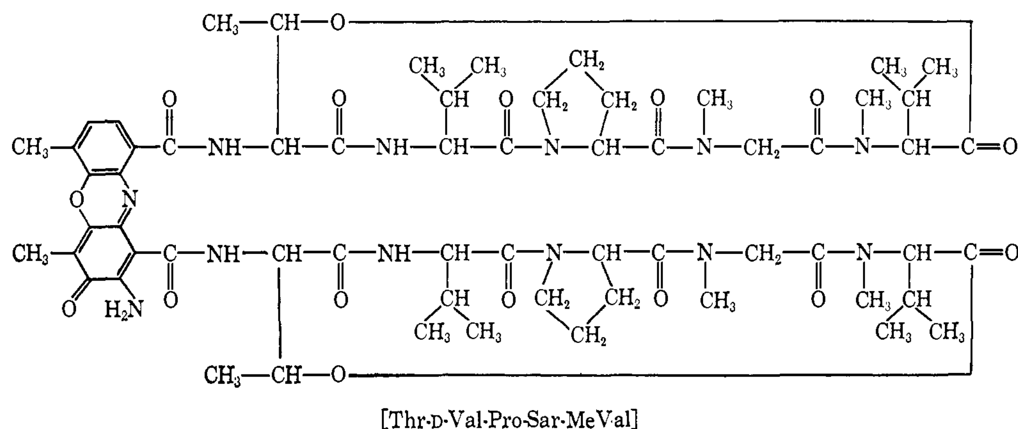


Figure 1. Actinomycin D (C_1). In actinomycin C_3 both D-valine residues are replaced by D-alloisoleucine.

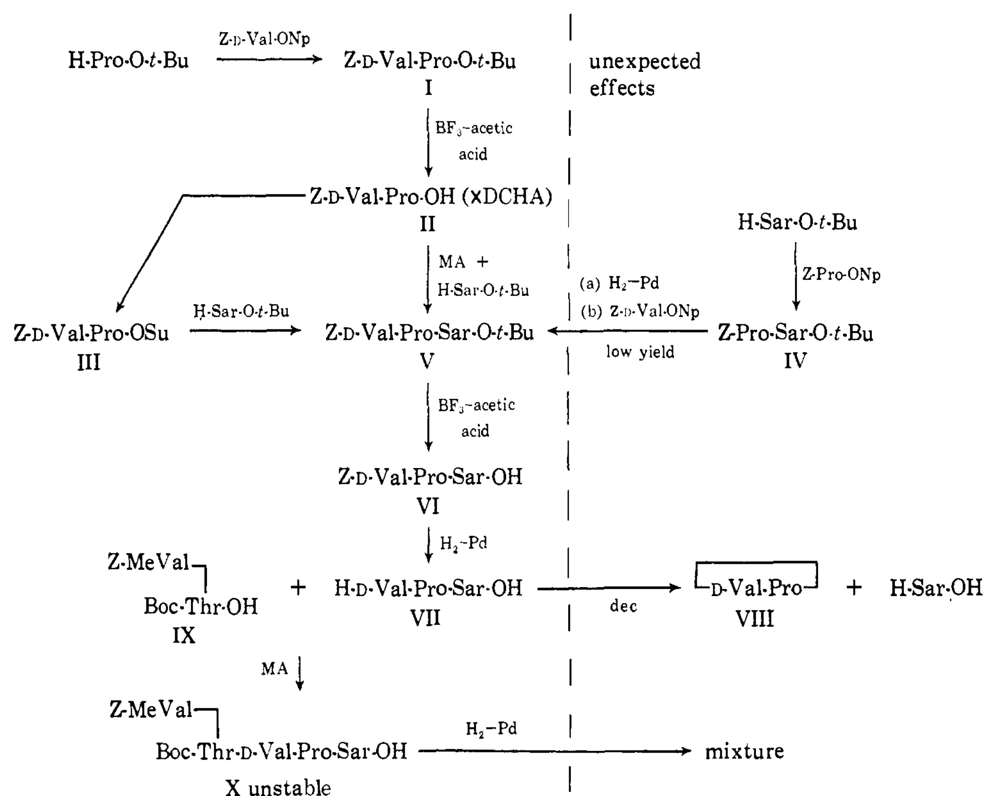


Figure 2. Preparation of intermediates in an attempted actinomycin synthesis *via* peptide cyclization between sarcosine and N-methylvaline (MA, mixed anhydride; DCHA, dicyclohexylamine).

Therefore the synthesis of actinomycin D was undertaken. Its known physical and biological properties permitted evaluation of each synthetic avenue.

Actinomycins D and C_3 have been synthesized[13,16,17] by two different pathways. The key reaction, cyclization to form the pentapeptide lactones, was performed either by ester bond formation (lactonization) or by peptide bond formation between sarcosine and N-methylvaline. The latter procedure gave low yields (about 4%).[17]

We reinvestigated both approaches. The lactonization reaction,[13,16] carried out with a mixture of imidazole and acetyl chloride (in 1000-fold excess) in tetrahydrofuran at 55°, is not fully understood.[13] Our attempts to repeat it were not very successful.[2,18] Other reagents, including carbonyldiimidazole, isobutyl chloroformate, *p*-toluenesulfonyl chloride, polyphosphoric acid ester, and N,N-dimethylformamide dineopentyl acetal, gave only 1–3% cyclization.[2] Extreme steric hindrance, due to the sequence of three imino acids[19] (-Pro-Sar-MeVal-), certainly causes considerable difficulties in forming the tightly packed 16-membered lactones.[20] A scheme describing the preparation of

(16) H. Brockmann and H. Lackner, *Naturwissenschaften*, **51**, 435, 384 (1964).

(17) H. Brockmann and H. Lackner, *Chem. Ber.*, **100**, 353 (1967); *Naturwissenschaften*, **48**, 555 (1961); **47**, 230 (1960).

(18) We reported previously failures to reproduce this cyclization procedure with useful yields.[2] Detailed information (H. Brockmann, personal communication, and ref 13) has since enabled us to carry out lactonizations with acceptable yields.

(19) Of all other peptide antibiotics with known structure only those of the mikamycin groups possess a sequence of three imino acids; they are, however, part of 19-membered hexapeptide lactones.

intermediates required for cyclization between sarcosine and N-methylvaline is shown in Figure 2. Some peptides were unexpectedly unstable at room temperature.[21] For example, D-valyl-L-prolylsarcosine (VII) decomposed spontaneously to give sarcosine and *trans*-1,6-trimethylene-3-isopropyl-2,5-piperazinedione (VIII), the diketopiperazine of D-valine and L-proline.[21,22] Thus, neither pathway provided a sufficiently effective synthesis for our program.

The known resistance of proline to racemization encouraged us to examine peptide bond formation between proline and sarcosine for the cyclization. A scheme of the successful synthesis is shown in Figure 3. Treatment of the mixed anhydride[23] from benzyloxycarbonyl-L-N-methylvaline[24] and isobutyl chloroformate with *t*-butyloxycarbonyl-L-threonine[25] followed by countercurrent distribution gave O-(benzyloxycarbonyl-L-N-methylvalyl)-N-*t*-butyloxycarbonyl-L-threonine (IX) as an oil. The dicyclohexylamine salt was crystalline. Catalytic hydrogenation of IX afforded the crystalline β-didepsipeptide derivative, O-(L-N-methylvalyl)-N-*t*-butyloxycarbonyl-L-threonine (XI). It was very stable and could be recrystallized from dimethylformamide at 100°; it was subsequently prepared directly from crude IX without intermediate countercurrent distribution.

Condensation of XI with benzyloxycarbonylsarcosine[26] by the mixed carboxylic–carbonic anhydride method[27] afforded crystalline O-(benzyloxycarbonylsarcosyl-L-N-methylvalyl)-N-*t*-butyloxycarbonyl-L-threonine (XII). Consendation of XII with *t*-butyl D-valyl-L-prolinate by the mixed anhydride method gave O-(benzyloxycarbonylsarcosyl-L-N-methylvalyl)-N-*t*-butyloxycarbonyl-L-threonyl-D-valyl-L-proline *t*-butyl ester (XIV),[28] which was purified by countercurrent distribution. The optical purity of this key intermediate was ascertained by microbiological assay.[29]

Treatment of XIV with a 0.4 *M* solution of boron trifluoride in glacial acetic acid[30] gave chromatographically uniform O-(benzyloxycarbonylsarcosyl-L-N-methylvalyl)-L-threonyl-D-valyl-L-proline (XIVa) in good yields. In contrast, treatment of XIV with anhydrous trifluoroacetic acid[31] or 1–2 *N* hydrogen chlo-

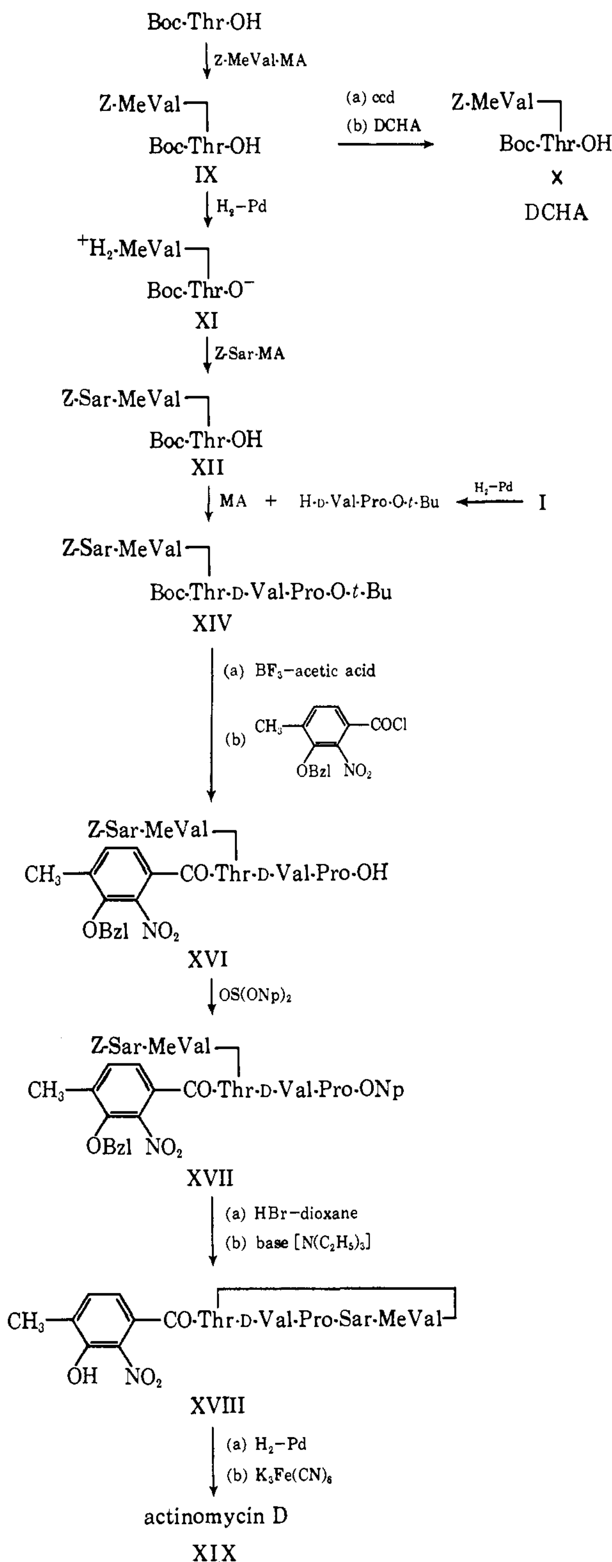


Figure 3. Synthesis of actinomycin D (MA, mixed anhydride; ccd, countercurrent distribution; DCHA, dicyclohexylamine).

(20) A PCK calotte model of actinomycin C_3 is depicted in W. Müller and I. Emme, *Z. Naturforsch.*, **20B**, 835 (1965).

(21) J. Meienhofer, Y. Sano, and R. P. Patel, in "Peptides: Chemistry and Biochemistry," B. Weinstein, Ed., M. Dekker, New York, N. Y., 1970, pp 419–434.

(22) I thank Dr. A. B. Mauger (Washington Hospital Center, Washington, D. C.) for gifts of authentic samples of *cis*- and *trans*-diketopiperazines (of L-valine and L-proline and of D-valine and L-proline, respectively) for comparison, and for gas chromatographic identification of VIII as the *trans*-diketopiperazine.

(23) M. A. Ondetti and P. L. Thomas, *J. Amer. Chem. Soc.*, **87**, 4373 (1965); L. Velluz, G. Amiard, and R. Heymes, *Bull. Soc. Chim. Fr.*, 1283 (1955).

(24) P. A. Plattner, K. Vogler, R. O. Studer, P. Quitt, and W. Keller-Schierlein, *Helv. Chim. Acta*, **46**, 927 (1963).

(25) E. Schnabel, *Ann.*, **702**, 188 (1967); K. Hofmann, R. Schmiechen, R. D. Wells, Y. Wolman, and N. Yanaihara, *J. Amer. Chem. Soc.*, **87**, 611 (1965); E. Wünsch and G. Wendlberger, *Chem. Ber.*, **97**, 2504 (1964).

(26) D. Ben-Ishai and E. Katchalski, *J. Amer. Chem. Soc.*, **74**, 3688 (1952).

(27) T. Wieland and H. Bernhard, *Ann.*, **572**, 190 (1951); R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); J. R. Vaughan, Jr., *J. Amer. Chem. Soc.*, 73, 3547 (1951).

(28) Use of the corresponding benzyl ester derivative, XV, was abandoned when attempts to remove both the benzyloxycarbonyl group and the benzyl ester by catalytic hydrogenation in several solvents gave complex mixtures even after prolonged hydrogenation.

(29) Assays for threonine, valine, and proline were carried out by Shankman Laboratories, Los Angeles, Calif. An organism specific for N-methylvaline was not available; however, the N-benzyloxycarbonyl protecting group employed during its incorporation is known to minimize racemization.

(30) R. G. Hiskey and J. B. Adams, Jr., *J. Org. Chem.*, **31**, 2178 (1966).

(31) H. Kappeler and R. Schwyzer, *Helv. Chim. Acta*, **43**, 1453 (1960); **44**, 1136 (1961).

Table I. Physical and Biological Characteristics of Synthetic and Natural Actinomycin D (C_1)

Characteristics[a]	Synthetic actinomycin D (XIX)	Natural actinomycin D[f]	Literature[g] Synthetic	Literature[g] Natural
Melting point (°C)	244–246	241–243	244–246	246–247
Optical rotation ($[\alpha]^{20}D$, deg)[b]	-340 ± 10	-323 ± 10	-334 ± 10	-328 ± 10
Uv absorption [ϵ (λ, mμ)] methanol	24,300 (443)	24,400 (443)	25,400 (443)	25,000 (443)
	33,600 (240)	34,100 (240)	33,000 (240)	34,000 (231)
Ir absorption (cm^{-1}, KCl)	1192 (lactone COC)	1195		1200[c]
	1580 (chromophore)	1580		1585
	1620–1670 (amide)	1620–1670		1620–1670
	1745 (lactone C=O)	1745		1760
Antibacterial activity (ID_{50}, μg/ml)			*d*	*d*
L. arabinosus	0.028–0.03	0.03		
L. fermenti	0.028–0.03	0.03–0.04		
KB cells (ID_{50}, μg/ml)	0.003	0.002		
Toxicity (LD_{50}, μg/kg, mice)	425	425		500[e]
Antitumor activity (MED, μg/kg per day × 4, i.p., mice)	60–70	50–60		

[a] See Experimental Section for methods and references. [b] In methanol at *c* 0.25. [c] H. Brockmann, *Angew. Chem.*, **66**, 1 (1954). [d] Similar activities (*B. subtilis*, *S. aureus*). [e] C. L. Maddock, G. J. D'Angio, S. Farber, and A. H. Handler, *Ann. N. Y. Acad. Sci.*, **89**, 386 (1960). [f] See ref 41. [g] See ref 13.

ride in acetic acid[32] for periods of 0.5–1 hr was not sufficient to cleave the *t*-butyl ester group quantitatively; prolonged treatment (4–5 hr) did result in complete *t*-butyl ester cleavage but also in some undesired fission of the benzyloxycarbonyl group.

After treatment of XIVa with 2-nitro-3-benzyloxy-4-methylbenzoyl chloride,[33] the crude product was purified by column chromatography on Sephadex LH-20[34] in methanol. Two main peaks were observed with similar elemental and amino acid analyses, but different optical rotations. The slower moving material was the desired product, O-(benzyloxycarbonylsarcosyl-L-N-methylvalyl)-N-(2-nitro-3-benzyloxy-4-methylbenzoyl)-L-threonyl-D-valyl-L-proline (XVI),[35] which was treated with di-*p*-nitrophenyl sulfite in pyridine[36] to give the *p*-nitrophenyl ester, XVII.[37] The benzyloxycarbonyl group of XVII was best removed by treatment with 4–5 *N* hydrogen bromide in dioxane.[38]

Cyclization was effected at high dilution (approximately 0.5%) in pyridine for 6 hr at 60°.[39] Purification of the crude product by column chromatography on Sephadex LH-20 in methanol afforded the crystalline cyclic pentapeptide lactone derivative, (2-nitro-3-hydroxy-4-methylbenzoyl)-L-threonyl-D-valyl-L-prolylsarcosyl-L-N-methylvaline (threonine hydroxyl) lactone (XVIII), in yields ranging from 24 to 26%. Crystalline actinomycin D (XIX) was almost quantitatively obtained from XVIII *via* catalytic hydrogenation to give the 3-hydroxy-4-methylanthraniloyl pentapeptide lactone, followed by oxidation with potassium ferricyanide[40] in methanol–phosphate buffer pH 7.1 (1:1). Recrystallization from ethyl acetate–hexane gave red trigonal prisms, possessing physical characteristics which compared favorably with natural actinomycin D.[41] Microbiological assays and toxicological and antitumor assays in mice all showed the synthetic product to be indistinguishable from natural actinomycin D. The physical and biological data are summarized in Table I.

The above described synthesis of actinomycin D resulted from investigations of eight different pathways including two which had been reported by Brockmann and Lackner.[13,17] In order to fractionate complex reaction mixtures these authors had to use successive adsorption chromatography on aluminum oxide and silica gel columns and partition chromatography on cellulose. We obtained excellent and reproducible purifications by chromatography on Sephadex LH-20 in methanol which fractionates by molecular size. This recently developed procedure[2,34] is easily performed and allows rapid purifications of gram amounts of protected peptides in single runs. Our synthesis readily produces crystalline actinomycin in improved yields and in amounts sufficient for comprehensive biological testing.

Experimental Section

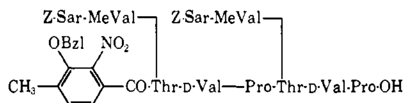
Methods. Freshly prepared palladium black[42] was used for catalytic hydrogenations. Hydrogen was continuously passed

(32) F. C. McKay and N. F. Albertson, *J. Amer. Chem. Soc.*, **79**, 4686 (1957); G. W. Anderson and A. C. McGregor, *ibid.*, **79**, 6180 (1957).

(33) S.-W. Chow, Y.-S. Kao, C.-H. Chou, and B. Hsu, *Sci. Sinica* (Peking), **12**, 49 (1963); H. Brockmann and H. Muxfeldt, *Chem. Ber.*, **91**, 1242 (1958).

(34) E. Nyström and J. Sjövall, *J. Chromatogr.*, **24**, 208 (1966); H. Aoyagi, T. Kato, M. Waki, O. Abe, R. Okawa, S. Makisumi, and N. Izumiya, *Bull. Chem. Soc. Jap.*, **42**, 782 (1969).

(35) The material from the faster moving peak was



which will be discussed elsewhere: R. Cotton and J. Meienhofer, in preparation.

(36) B. Iselin, W. Rittel, P. Sieber, and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957).

(37) Dicyclohexylcarbodiimide and *p*-nitrophenol [M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959); D. F. Elliott and D. W. Russell, *Biochem. J.*, **66**, 49P (1957); M. Rothe and F. W. Kunitz, *Ann.*, **609**, 88 (1957)] gave considerable amounts of the acyl-N,N'-dicyclohexylurea of XVI.

(38) K. Okawa, *Bull. Chem. Soc. Jap.*, **30**, 976 (1957); D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

(39) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **40**, 624 (1957).

(40) W. G. Hanger, W. C. Howell, and A. W. Johnson, *J. Chem. Soc.*, 496 (1958).

(41) Actinomycin D, lot No. 3008A-30B and 1687C-41AII, from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y. 10965.

(42) H. Willstätter and E. Waldschmidt-Leitz, *Ber.*, **54**, 113 (1921); H. Wieland, *ibid.*, **45**, 484 (1912).

through the solution which was stirred by a vibromixer.[43] Countercurrent distributions were carried out with the system toluene–chloroform–methanol–water (5:5:8:2).[44] Distribution was determined from the optical density at 570 mμ of ninhydrin treated fractions after alkaline hydrolysis,[45] or from the optical density at 255 mμ after evaporating the fractions to dryness and dissolving them in methanol. The distribution coefficients (*K*) are mean values of crude products (analytically pure products usually exhibit somewhat lower *K* values). Column chromatography on Sephadex LH-20 in methanol was performed on a 5 × 190 cm column, with flow rate at 100 ml/hr, and fraction volume of 15 ml. Separations (elution volumes) with these columns were very reproducible. Solvent systems for thin layer chromatography (tlc) were A (acidic), *sec*-butyl alcohol–formic acid–water (75:13.5:11.5) and B (basic), *sec*-butyl alcohol–10% NH_4OH (85:15). Average yields are estimated from the results of six to twelve experiments. Melting points were done on a Fisher-Johns apparatus and are corrected. Optical rotations were determined with a Rudolph Model 200 manual spectropolarimeter. Infrared absorptions (Perkin-Elmer Infracord spectrophotometer) are given in cm^{-1}. Elemental analyses were performed by Werby Laboratories, Inc., Boston, Mass., and by Galbraith Laboratories, Inc., Knoxville, Tenn. Amino acid analyses[46] were done with a Phoenix analyzer, Model M-6800.

Materials. Several analytical grade solvents were routinely distilled prior to use: tetrahydrofuran from $LiAlH_4$, dioxane from Na or from $LiAlH_4$, pyridine from ninhydrin (1 g/l.) or from KOH pellets, dimethylformamide *in vacuo* from ninhydrin. Hydrogen bromide was purified by bubbling through a 10% solution of resorcinol in anhydrous trifluoroacetic acid.

Microbiological Assays. For antibacterial assays[47] *Lactobacillus arabinosus* (ATCC 8014) and *L. fermenti* (ATCC 9388) pantothenate and thiamine-dependent assay systems, respectively, were employed, and the ID_{50} was determined as described elsewhere.[48] Inhibitory activity in mammalian cell culture was determined with KB cells (human carcinoma) and expressed as the ID_{50}.[49]

Antitumor Activity and Toxicity. Ridgway osteogenic sarcoma were transplanted intramuscularly into AKD_2-F_1 mice.[50] After 14 days, when tumor sizes averaged 5–15 mm (50–300 mg), actinomycin was administered intraperitoneally at daily dose levels of 600, 300, 150, 75, and 37.5 μg/kg for four consecutive days to 10 animals each. The animals were killed on day 28 and the tumors were weighed. The minimum effective dose (MED, the dose required to inhibit tumor growth to 10% of the control tumor weight) was estimated by linear interpolation of the log dose–response curve at doses straddling the required value. Applying the Spearman-Kärber method, the toxicity, LD_{50}, for each treatment was estimated.[51]

Preparations. Syntheses of peptide derivatives which are not direct intermediates of the most successful method are not described in detail but in condensed form in Table II.[52]

Benzyloxycarbonyl-D-valyl-L-proline *t*-Butyl Ester (I). A solution of benzyloxycarbonyl-D-valine *p*-nitrophenyl ester[2] (58 g, 157 mmol) and of L-proline *t*-butyl ester[53] (31 g, 170 mmol) in tetrahydrofuran (250 ml) was kept for 46 hr at 40°. The solvent was then replaced by ethyl acetate (600 ml). Washing with 1 *N* NH_4OH (fifteen 200-ml portions), 1 *M* citric acid (twice), and saturated NaCl (twice), drying ($MgSO_4$), evaporating, and recrystallizing from hexane or isopropyl ether afforded colorless prisms: 57 g (90%); mp 92°; $[\alpha]^{19}D$ −19.6° (*c* 1, CH_3OH); tlc R_f 0.83 (A), 0.8 (B).

Anal. Calcd for $C_{22}H_{32}N_2O_5$ (404.5): C, 65.3; H, 7.98; N, 6.93. Found: C, 65.4; H, 8.05; N, 7.06.

Spontaneous Decomposition of D-Valyl-L-prolylsarcosine. *trans*-1,6-Trimethylene-3-isopropyl-2,5-piperazinedione, Diketopiperazine of D-Valine and L-Proline (VIII). Catalytic hydrogenation of benzyloxycarbonyl-D-valyl-L-prolylsarcosine (VI, 2.2 g, 5.2 mmol) in methanol (100 ml) with Pd black (2 g) for 20 min at room temperature and evaporation afforded an oil, VII (1.5 g, 100%, tlc R_f 0.25–0.35 (A), 0.13 (B); amino acid analysis, valine 1.0, proline, 0.95, sarcosine 1.0), which was kept in a closed flask at room temperature. Spontaneous crystallization of sarcosine occurred after 10 days. After 21 days the mixture was triturated with ethyl acetate. Sarcosine was filtered off, washed with ethyl acetate, and dried: 0.3 g; mp 220–225° dec; tlc R_f 0.13–0.16 (A), identical with authentic sarcosine. Evaporation of the ethyl acetate solution and repeated recrystallization of the residue from ethyl acetate–hexane afforded colorless prisms: 0.5 g; mp 152–155°; $[\alpha]^{20}D$ −92.9° (*c* 0.25, H_2O); −92.9° (*c* 1, CH_3OH); tlc R_f 0.57 (A); ir (KCl) 3260 (NH), 1650–1680 (amide), and 1490, 1450 (NH).

Anal. Calcd for $C_{10}H_{16}N_2O_2$ (196.3): C, 61.2; H, 8.21; N, 14.3. Found: C, 61.4; H, 8.27; N, 14.2.

Authentic diketopiperazine of D-valine and L-proline[20] possessed mp 154–156°; $[\alpha]^{20}D$ −96.4 (*c* 0.25, H_2O); ir (KCl) 3250 (NH), 1650–1680 (amide), and 1485, 1445 (NH); tlc R_f 0.57 (A); diketopiperazine of L-valine and L-proline[20] possessed mp 184–185°; $[\alpha]^{20}D$ −182.8° (*c* 0.25, H_2O); ir (KCl) 3240 (NH), 1560–1580 (amide), 1440 (NH), and 1340 (C–N); tlc R_f 0.6 (A). An equal mixture of both showed well separated spots on tlc (A).

D-Valyl-L-prolylsarcosine hydrochloride (oil) was stable at room temperature.

O-(Benzyloxycarbonyl-L-N-methylvalyl)-N-*t*-butyloxycarbonyl-L-threonine (IX). To a stirred solution of benzyloxycarbonyl-L-N-methylvaline[24] (26.53 g, 100 mmol) in tetrahydrofuran (100 ml) at −10° was added triethylamine (14.02 ml, 100 mmol), followed by isobutyl chloroformate (13.18 ml, 100 mmol). The temperature of the mixture was maintained at −10° throughout the addition. After 30 sec a precooled solution (−5°) of N-*t*-butyloxycarbonyl-L-threonine[25] (26.3 g, 120 mmol) and triethylamine (16.83 ml, 120 mmol) in tetrahydrofuran (100 ml) was added and the mixture was stirred for 18 hr at −5°. The mixture was filtered and evaporated to dryness. The residue was taken up in ethyl acetate (300 ml) and washed with 1 *M* citric acid (twice) and with saturated NaCl (four times). The aqueous washings were back-extracted with ethyl acetate. The organic layers were combined, dried ($MgSO_4$), filtered, and evaporated to give a colorless oil, A (63 g), which was subjected to countercurrent distribution for 150 transfers. Three peaks were obtained with *K* 0.44, 1.45, and 3.5. Unchanged *t*-butyloxycarbonyl-L-threonine was isolated from peak *K* 3.5. Total hydrolysis of the material isolated from peak *K* 1.45 showed only N-methylvaline by tlc. The product, IX, was in the peak *K* 0.44. It was obtained as a colorless oil (65–85%): $[\alpha]^{20}D$ −35 ± 2° (*c* 1, CH_3OH); tlc R_f 0.8 (A); COOH titration 448.

Anal. Calcd for $C_{23}H_{34}N_2O_8$ (446.5): C, 59.2; H, 7.35; N, 6.01. Found: C, 59.4; H, 7.49; N, 6.51.

The oil was converted into its crystalline dicyclohexylammonium salt in ether–hexane or ethyl acetate–hexane (56–66%) and recrystallized from acetone–hexane: mp 145–146°; $[\alpha]^{20}D$ −21.7 ± 1° (*c* 1, CH_3OH); ir (KCl) 1725, 1710, 1695 (C=O), 1570 (COO^-), and 1155 (ester COC).

Anal. Calcd for $C_{35}H_{57}N_3O_8$ (647.8): C, 64.9; H, 8.87; N, 6.49; O, 19.8. Found: C, 64.9; H, 9.03; N, 6.56; O, 19.6.

O-(L-N-Methylvalyl)-N-*t*-butyloxycarbonyl-L-threonine (XI). A. From IX. The dicyclohexylammonium salt of IX (1.65 g, 2.5 mmol) was treated in ethyl acetate with 1 *N* H_2SO_4, and the organic phase was washed twice with water, dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The residual oil was dissolved in methanol (50 ml) containing a drop of glacial acetic acid and catalytically hydrogenated for 15 min. Evaporation afforded colorless prisms: 0.83 g (96%); mp 208°; $[\alpha]^{20}D$ +57.1° (*c* 1, CH_3OH); tlc R_f 0.6 (A). A sample for analysis was recrystallized from dimethylformamide or from acetonitrile.

Anal. Calcd for $C_{15}H_{28}N_2O_6$ (332.4): C, 54.2; H, 8.49; N, 8.43; O, 28.9. Found: C, 54.2; H, 8.60; N, 8.41; O, 28.9.

B. Direct Preparation without Intermediate Isolation of IX. Oil A (63 g) from a synthesis of IX, using 100 mmol of benzyloxycarbonyl-L-N-methylvaline[24] and 120 mmol of *t*-butyloxycarbonyl-L-threonine,[25] was subjected to catalytic hydrogenation in absolute

(43) See Figure 2 in J. Meienhofer, *Chimia*, **16**, 385 (1962).
(44) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T.-B. Lo, and J. Ramachandran, *J. Amer. Chem. Soc.*, **83**, 4449 (1961); **82**, 5760 (1960).
(45) C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **219**, 623 (1956); S. Moore and W. H. Stein, *ibid.*, **211**, 907 (1954).
(46) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
(47) G. E. Foley, R. E. McCarthy, and V. M. Binns, *Ann. N. Y. Acad. Sci.*, **76**, 413 (1958).
(48) G. E. Foley, *Antibiot. Annu.*, 432 (1955–1956).
(49) G. E. Foley and H. Eagle, *Cancer Res.*, **18**, 1012 (1958); H. Eagle and G. E. Foley, *ibid.*, **18**, 1017 (1958).
(50) G. J. D'Angio, C. L. Maddock, S. Farber, and B. L. Brown, *ibid.*, **25**, 1002 (1965).
(51) V. M. Rosenoer in "Experimental Chemotherapy," Vol. 4, R. J. Schnitzer and F. Hawking, Ed., Academic Press, New York, N. Y., 1966, pp 9–77.
(52) I thank Dr. C. H. Li, Hormone Research Laboratory, San Francisco, Calif., for the suggestion to condense experimental details into a table.
(53) R. Roeske, *J. Org. Chem.*, **28**, 1251 (1963); G. W. Anderson and F. M. Callahan, *J. Amer. Chem. Soc.*, **82**, 3359 (1960).

Table II. Preparation and Properties of Peptide Derivatives with Actinomycin D Sequence

No.	Derivative	Starting compd	Method[a] or reagent	Solvent[b]	Reaction time, hr	Temp, °C	Purification[c]
II	Z-D-Val-Pro-OH × DCHA	I	CF_3COOH	CF_3COOH	3	20	Cryst, acetone
			BF_3	CH_3COOH	1	20	
III	Z-D-Val-Pro-OSu	II	Dicyclohexylcarbodiimide	CH_3CN	2–3	0–20	Cryst, EtOAc
IV	Z-Pro-Sar-O-*t*-Bu[e]		*p*-Nitrophenyl ester	EtOAc	72	20	Ccd, *K* 0.25 LH-20
V	Z-D-Val-Pro-Sar-O-*t*-Bu[f]	II	MA, isobutyl chloroformate	THF	6	−20–20	Ccd, *K* 0.25 LH-20
VI	Z-D-Val-Pro-Sar-OH	V	BF_3	CH_3COOH	2	20	Ccd, *K* 1.0 LH-20
X	Z-MeVal┐ Boc-Thr-D-Val-Pro-Sar-OH	IX + V	MA, isobutyl chloroformate	DMF + THF	5	−10–20	Ccd, *K* 0.26
XIII	Z-Sar-MeVal┐ Boc-Thr-ONp	XII	$OS(ONp)_2$	Pyridine	20	20	Cryst, ether–hexane
XV	Z-Sar-MeVal┐ Boc-Thr-D-Val-Pro-OBzl	XII	Dicyclohexylcarbodiimide	DMF	5 + 15	0, 20	Ccd, *K* 0.06

[a] BF_3, boron trifluoride etherate; MA, mixed anhydride; $OS(ONp)_2$, di-*p*-nitrophenyl sulfite. [b] EtOAc, ethyl acetate; THF, tetrahydrofuran; DMF, dimethylformamide. [c] Ccd, countercurrent distribution. [d] $[\alpha]^{20}D$ −37.6° (*c* 1, glacial acetic acid), −2.1° (*c* 1, dioxane).

ethanol for 1–3 hr. Precipitated L-N-methylvaline (formed from unchanged benzyloxycarbonyl-N-methylvaline) was removed by filtration along with the catalyst. The amino acid was washed from the catalyst with water and the aqueous solution was evaporated to dryness to give colorless crystalline L-N-methylvaline, 25–35% based on the amount of benzyloxycarbonyl-L-N-methylvaline; sublimes above 270°; $[\alpha]^{20}D$ +34° (*c* 1, 6 *N* HCl) [lit.[54] +32.1°].

The ethanol solution was evaporated to dryness to give a colorless foam (40 g), which was taken up in ethyl acetate and extracted with water (three times). The aqueous extracts were washed once with ethyl acetate and evaporated to dryness *in vacuo* to give a colorless solid. The solid was treated with boiling acetonitrile (35 ml/g) for about 5 min and was then collected, washed with cold acetonitrile, and dried to give colorless crystals, 34–35% based on the amount of benzyloxycarbonyl-L-N-methylvaline used: mp 207–208°; $[\alpha]^{20}D$ +55 ± 2° (*c* 1, methanol).

O-(Benzyloxycarbonylsarcosyl-L-N-methylvalyl)-N-*t*-butyloxycarbonyl-L-threonine (XII). To a stirred solution of benzyloxycarbonylsarcosine[26] (3.21 g, 15 mmol) in tetrahydrofuran (20 ml), at −10°, was added N-methylmorpholine (1.68 ml, 15 mmol) followed by isobutyl chloroformate (1.98 ml, 15 mmol). After 1–2 min N-methylmorpholine (1.34 ml, 12 mmol) was added followed by a solution of XI (4.0 g, 12 mmol) in dimethylformamide (100 ml). The temperature was maintained at −10 to −15° for 1 hr. The mixture was then stirred for 20 hr at −5°. It was worked up as described for IX to give a crude oil which was subjected to countercurrent distribution for 100 transfers. Three peaks were obtained with *K* 0.25, 1.0, and 4.0. The fractions of peak *K* 1.0 contained benzyloxycarbonylsarcosine, and those of peak *K* 4.0 contained unaltered XI. The main product with *K* 0.25 crystallized from ethyl acetate–hexane: colorless prisms; 3.31 g (51%); mp 163°; $[\alpha]^{20}D$ −59.7° (*c* 1, CH_3OH); tlc R_f 0.7 (A). Recrystallized from ethyl acetate.

Anal. Calcd for $C_{26}H_{39}N_3O_{11}$ (537.6): C, 58.1; N, 7.31; N, 7.82; O, 26.8. Found: C, 58.2; H, 7.46; N, 7.69; O, 27.0.

Crystalline XII was subsequently obtained by seeding of the crude oil without intermediate countercurrent distribution, average yields 40–52%.

O-(Benzyloxycarbonylsarcosyl-L-N-methylvalyl)-N-*t*-butyloxycarbonyl-L-threonyl-D-valyl-L-proline *t*-Butyl Ester (XIV). To a stirred solution of XII (10 g, 18.6 mmol) in tetrahydrofuran (100 ml), at −10°, was added N-methylmorpholine (2.1 ml, 18.6 mmol) followed by isobutyl chloroformate (2.46 ml, 18.6 mmol). After 1 min a precooled (−10°) solution of hydrogenated I (20 mmol) in tetrahydrofuran (30 ml) was added dropwise by means of a jacketed dropping funnel. The mixture was stirred for 2 hr at −10° and for 15 hr at room temperature. It was worked up as described for IX (with additional washing with 1 *M* $NaHCO_3$) to give an oil which was subjected to countercurrent distribution for 200 transfers. Evaporation of the fractions from the one main peak (*K* 0.16) afforded an amorphous colorless solid (13.3 g, 91%): $[\alpha]^{20}D$ −42.6 ± 1° (*c* 1, CH_3OH); ir (KCl) 1740 (ester C=O), 1665 (amide), and 1155 (ester COC); tlc R_f 0.84 (A), 0.77 (B). For analysis a sample was reprecipitated from ether–hexane at low temperature to give a colorless powder.

Anal. Calcd for $C_{40}H_{63}N_5O_{11}$ (789.9): C, 60.8; H, 8.04; N, 8.87. Found: C, 60.9; H, 8.08; N, 8.84.

Amino acid analysis (6 *N* HCl, 110°, 24 hr) gave the following molar ratios, valine:sarcosine:proline:threonine:N-methylvaline, 1.0:1.0:1.0:0.9:1.0.[55]

Microbiological assay[29] (5 mg, 6.3 μmol, 24 hr at 110°) employing bacterimetric tests specific for the L forms of amino acids gave L-threonine, 5 μmol; L-proline, 6.3 μmol; L-valine, less than 0.05 μmol.

Preparations of XIV by the *p*-nitrophenyl ester method (using XIII) and by the dicyclohexylcarbodiimide method proceeded with 80 and 85% yields.

O-(Benzyloxycarbonylsarcosyl-L-N-methylvalyl)-N-(2-nitro-3-benzyloxy-4-methylbenzoyl)-L-threonyl-D-valyl-L-proline (XVI). Treatment of thoroughly dried XIV with 0.4 *M* boron trifluoride in glacial acetic acid for 3 hr at 20° gave after evaporation and precipitation from dioxane–ether a colorless amorphous powder, XVIa, in 99% yield: $[\alpha]^{20}D$ −44° (*c* 1, CH_3OH); tlc (A) R_f 0.69. It was thoroughly dried (6 g, 9.4 mmol) and dissolved in dioxane or dimethylformamide (20–30 ml). At 0° N-methylmorpholine (3.16 ml, 28.2 mmol) was added followed by a solution of 2-nitro-3-benzyloxy-4-methylbenzoyl chloride[33] (2.88 g, 9.4 mmol) in tetrahydrofuran (20 ml). The mixture was stirred for 30 min at 0° in the dark. Ethyl acetate (500 ml) was added and the organic phase washed with 1 *N* HCl (three times) and with saturated NaCl solution (four times), dried ($MgSO_4$), filtered, and evaporated to give an oil (8–9 g). The crude product was purified by column chromatography on Sephadex LH-20 in methanol to give from the fractions of the second large peak a colorless amorphous powder (4.7 g, 55%; average yield 50–70%): mp 115–120°; $[\alpha]^{20}D$ −2.8 ± 1° (*c* 0.5, CH_3OH); tlc R_f 0.85 (A); ir (KCl) 1740 (COOH), 1665 (amide), 1550 (NO_2), 1155 (ester COC). A sample for analysis was reprecipitated from dioxane–hexane.

Anal. Calcd for $C_{46}H_{58}N_6O_{13}$ (903.0): C, 61.2; H, 6.47; N, 9.31; O, 23.0. Found: C, 61.2; H, 6.84; N, 9.02; O, 23.0.

O-(Benzyloxycarbonylsarcosyl-L-N-methylvalyl)-N-(2-nitro-3-benzyloxy-4-methylbenzoyl)-L-threonyl-D-valyl-L-proline *p*-Nitro-

(54) P. Quitt, J. Hellerbach, and K. Vogler, *Helv. Chim. Acta*, **46**, 327 (1963).

(55) N-Methylvaline produced under standard conditions a very weak ninhydrin reaction [*cf.* M. Ebata, Y. Takahashi, and H. Otsuka, *J. Chromatogr.*, **25**, 1 (1966)] and overlapped with sarcosine. It was, therefore, determined by the method of J. Heilmann, J. Barollier, and E. Watzke, *Hoppe-Seyler's Z. Physiol. Chem.*, **309**, 219 (1957).

Yield, %	Mp, °C	$[\alpha]^{20}D$ ° (c 1, CH_3OH)	Tlc (R_f)	Formula	Calcd, % C	H	N	O	Found, % C	H	N	O
83	159	−18.5	0.73 A 0.3 B	$C_{30}H_{47}N_3O_5$	68.0	8.94	7.93	15.1	68.0	8.86	7.91	15.3
72	158	*d*		$C_{22}H_{27}N_3O_7$	59.3	6.11	9.43	25.1	59.3	6.23	9.71	25.1
87	Oil	−58		$C_{20}H_{28}N_2O_5$	63.8	7.50	7.44	21.3	63.7	7.64	7.32	21.4
80	Oil	−33.5	0.74 A 0.75 B	$C_{25}H_{37}N_3O_6$	63.1	7.84	8.84	20.2	63.2	8.02	8.87	20.3
90	Oil	−27.9	0.7 A 0.25 B	$C_{21}H_{29}N_3O_6$	60.1	6.97	10.0	22.9	59.9	7.23	10.1	23.1
62	Oil	−34.9	0.75 A 0.5 B	$C_{36}H_{55}N_5O_{11}$	58.9	7.55	9.54	24.0	59.0	7.61	9.34	24.2
65	99	−60.1[g]		$C_{32}H_{42}N_4O_{11}$	58.3	6.43	8.51		58.2	6.48	8.20	
92	Oil	−29.7	0.7 A 0.65 B	$C_{43}H_{61}N_5O_{11}$	62.7	7.46	8.50		62.4	7.88	8.91	

[e] J. P. Marsh, Jr., and L. Goodman, *Can. J. Chem.*, **44**, 799 (1966). [f] From III in THF, 96 hr at 20°, 58% yield. [g] *c* 1, ethyl acetate.

phenyl Ester (XVII). To a solution of XVI (4.03 g, 4.5 mmol) in pyridine (20 ml) was added di-*p*-nitrophenyl sulfite (1.62 g, 5 mmol). The mixture was stirred for 4 hr at room temperature, protected from light. Ethyl acetate (500 ml) was added and the crude product was isolated as described for XIV. It was purified by column chromatography on Sephadex LH-20 in ethyl acetate (5 × 20 cm column) to give a colorless amorphous powder: 4.4 g (96%; average yield 86–96%); mp 110–115°; $[\alpha]^{20}D$ −17.9° (*c* 0.5, dimethylformamide); +8.9° (*c* 0.5, ethyl acetate); tlc R_f 0.8 (A). A sample for analysis was reprecipitated from benzene-hexane.

Anal. Calcd for $C_{52}H_{61}N_7O_{15}$ (1024.1): C, 61.0; H, 6.00; N, 9.58. Found: C, 61.3; H, 6.34; N, 9.69.

(2-Nitro-3-hydroxy-4-methylbenzoyl)-L-threonyl-D-valyl-L-prolyl-sarcosyl-L-N-methylvaline (threonine hydroxyl) Lactone (XVIII). Purified hydrogen bromide was introduced, at 0° in darkness, into a solution of XVII (1.28 g, 1.25 mmol) in dioxane (10 ml) until it became 4–6 *N* in HBr as judged by the weight increase. The solution warmed up to room temperature. After 40 min it was added dropwise to stirred ether (200 ml) at −70° to give, after filtration and drying, an amorphous cream colored powder (1.34 g). Thin layer chromatography indicated that the desired product was obtained in approximately 90% yield, R_f 0.5 (A). It was dissolved in dimethylformamide (20 ml) and glacial acetic acid (1 ml) was added. The solution was added dropwise over a period of 2 hr to stirred pyridine (2000 ml), at 60°, containing triethylamine (1 ml). Stirring was continued for an additional 3.5 hr. The pyridine was evaporated *in vacuo* and the residual oil was worked up as described for XIV to give a yellow oil (890 mg). The crude product was purified by column chromatography on Sephadex LH-20 in methanol. Several large peaks were observed. Evaporation of the fractions from the first peak gave a cream colored solid, 240 mg (32%), which crystallized in prisms from ethyl acetate-hexane: 195 mg (26%); mp 190–193°; $[\alpha]^{20}D$ −24.8° (*c* 0.5, CH_3OH); tlc R_f 0.66 (A), 0.44 (B); ir (KCl) 1730 (ester C=O), 1655 (amide), 1185 (ester COC). A sample was recrystallized from ethyl acetate–hexane, mp 192–195°.

Anal. Calcd for $C_{31}H_{44}N_6O_{10}$ (660.7): C, 56.3; H, 6.71; N, 12.7. Found: C, 56.4; H, 6.96; N, 12.6.

Actinomycin D: 2-Amino-4,6-dimethylphenoxazinone(3)-1,9-bis-[carbonyl-L-threonyl-D-valyl-L-prolyl-sarcosyl-L-N-methylvaline (threonine hydroxyl) Lactone] (XIX). A solution of XVIII (0.4 g, 0.6 mmol, thoroughly dried *in vacuo* over P_2O_5–KOH at 90° for 48 hr) in methanol (20 ml) was hydrogenated for 2 hr at room temperature in the dark and then filtered under nitrogen and added to an equal volume of stirred 0.067 *M* phosphate buffer pH 7.1 containing potassium ferricyanide (0.57 g, 1.75 mmol). Excess of oxidizing reagent had to be carefully avoided, because it overoxidizes the phenoxazinone moiety[56] resulting in rapid decrease of yield. The mixture was stirred for 20 min at room temperature. It was then partitioned between ethyl acetate (100 ml) and water (100 ml). The aqueous phase was extracted with ethyl acetate (three 50-ml portions). The combined organic phase was washed with 1 *M* $NaHCO_3$ (twice), with 1 *N* HCl (twice), and with saturated NaCl (twice), dried (Na_2SO_4), and evaporated to afford a red crystalline residue (0.4 g) which was recrystallized from ethyl acetate–hexane to give actinomycin D: red trigonal prisms, 0.36 g (95%); mp 244–246°; $[\alpha]^{20}D$ −340 ± 10° (*c* 0.22, methanol). Spectral, biological, and literature data are listed in Table I. Samples for analysis were dried *in vacuo* at 100° for 48 hr over P_2O_5 and KOH.

Anal. Calcd for $C_{62}H_{86}N_{12}O_{16}$ (1255.4): C, 59.3; N, 6.91; N, 13.4. Found: C, 59.3; H, 6.93; N, 13.4.

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(56) Y. Tsujino, *Tetrahedron Lett.*, 763 (1969); 4111 (1968); J. Gripenberg and J. Martikkala, *Acta Chem. Scand.*, **19**, 1051 (1965).