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Isolation, X-Ray Analysis, and Synthesis of a Metabolite of (−)-3-Hydroxy-*N*-allylmorphinan

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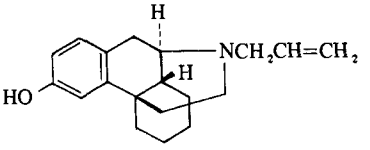
The structure of a metabolite (2) of (−)-3-hydroxy-*N*-allylmorphinan (1, levallorphan) isolated from urine of rats was established by single-crystal X-ray analysis of the HBr salt to be (−)-*N*-allyl-3,6β-dihydroxymorphinan (2). Compound 2 was synthesized from (−)-3-methoxy-6-oxo-*N*-methylmorphinan (3). No analgesia was observed for 1 or 2 in the tail flick, hot plate, and Nilsen tests. The two compounds were approximately equal in their antagonism to morphine in the tail flick and Nilsen methods.

Previous studies[1] on the *in vivo* and *in vitro* metabolism of levallorphan (1), a potent morphine antagonist, demonstrated the formation of two metabolites. One metabolite (metabolite II) was found to be identical with (−)-3-hydroxymorphinan. The other metabolite (metabolite I) was isolated from rat urine and rat liver incubation mixtures, but the structure was not elucidated.



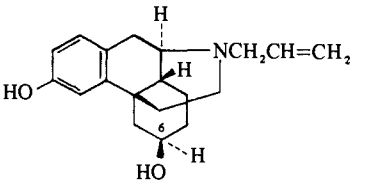
1

Elemental analysis[1] and mass spectral data† indicated that metabolite I had been formed by the addition of one oxygen to levallorphan (1). Chemical and spectral studies were unable to ascertain the exact position of the oxygen.

For further characterization of this metabolite, urine from rats treated with 17.6 g of levallorphan tartrate was collected. After hydrolysis of the urine with HCl, the metabolite was isolated by a series of extractions and column chromatography procedures described in the Experimental Section. After repeated crystallizations, 33 mg of crystals was obtained with a melting point which compared favorably to that reported for sublimed metabolite I.[1]

A single-crystal X-ray analysis of 2·HBr revealed that 1 had been oxidized at the 6β position. The structure and configuration of the metabolite are shown in the stereodrawing (Figure 1).

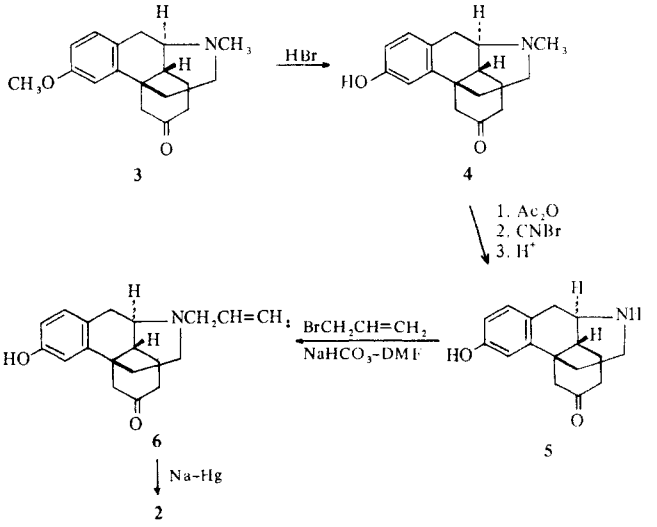
For comparison of the biological activity of 1 and 2, compound 2 was synthesized according to Scheme I. Treatment of 3 with 48% hydrobromic acid at reflux temperature gave the phenol 4. The *O*-acetyl derivative of 4 on treatment with cyanogen bromide in chloroform yielded, after acid hydrolysis, the secondary amine 5. Alkylation of 5 with allyl bromide in dimethylformamide in the presence of sodium bicarbonate gave the *N*-allylmorphinan 6. Sodium amalgam reduction of 6 afforded the desired 6β-alcohol 2, which was purified by fractional crystallization. The nmr spectrum of the crude reduction product indicated the presence of a minor amount of the epimeric 6α-alcohol. No attempt was made to isolate the epimer.



2

The mass spectrum of synthetic 2 shows the molecular ion as required at *m/e* 299. The nmr spectrum features a

Scheme I



†H. M. Fales, unpublished results.

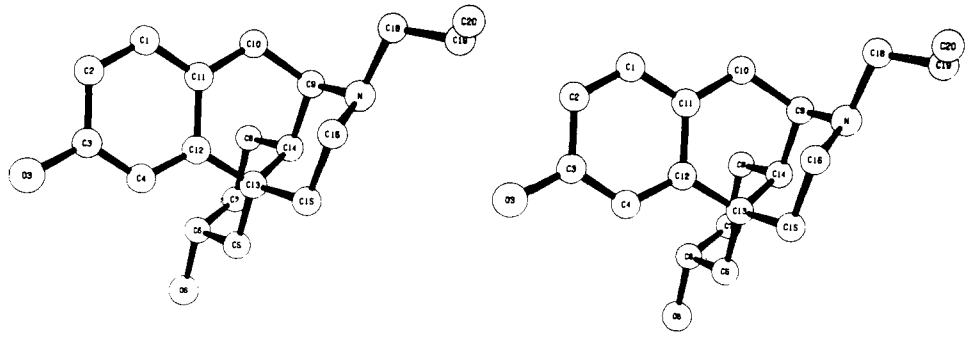


Figure 1. The stereoview of **2** as established from the X-ray analysis of **2** · HBr.

characteristic pattern for the three aromatic protons, namely two doublets centered at δ 6.85 (J = 8 Hz) and 6.69 (J = 2 Hz), and a doublet of doublets at δ 6.50 (J = 2 and 8 Hz). A one-proton singlet for the aromatic hydroxyl proton appears at δ 8.94. The one-proton doublet at δ 4.46 (J = 5 Hz) was assigned to the hydroxyl proton arising from spin-spin coupling between the hydroxyl proton and the proton attached to the hydroxyl-bearing carbon and is exchangeable by deuterium oxide. Since no methine proton was found at a field lower than δ 3.30, the proton attached to the hydroxyl-bearing carbon (C_6) must be axial.[2,3] The axial 6α proton is subjected to two large diaxial and two small axial-equatorial couplings. This combination of splittings results in a very broad signal (half-band width 26 Hz) at δ 3.24. Furthermore, it is known that an equatorial proton attached to an oxygen-bearing carbon appears as a narrow singlet at about δ 4 in substituted cyclohexanols.[3]

The HBr salt of synthetic **2** has the same unit cell and X-ray pattern as the HBr salt of the metabolite **2**.

The analgesic and morphine antagonistic activites of synthetic **2** were compared to levallorphan (Table I). No analgesia was observed for **1** or **2** in the tail flick, hot plate, or Nilsen tests. Analgesia was observed in the phenylquinone-induced writhing test; however, it has been demonstrated[4-6] that some morphine antagonists show analgesia in this test. The two compounds were approximately equal in their antagonism to morphine in the tail flick and Nilsen methods.

Table I. Activity of Levallorphan (**1**) and Metabolite 2 as Analgesics and Morphine Antagonists in Mice

	ED_{50}, mg/kg	
	1 · tartrate	**2** · HBr
Analgesia		
Tail flick test	>200 sc, >100 po	>100 sc
Hot plate test	>25 sc, >20 po	20 sc (53% increase)
Nilsen method	>20 ip	>20 ip
Writhing test	0.29 sc	1.9 sc
Morphine antagonism		
Tail flick test	0.94 sc	1.62 sc
Nilsen method	1.4 ip	1.5 ip

Experimental Section

Animal Studies. Male Sprague–Dawley strain rats (225–350 g) were housed in metabolism cages and injected subcutaneously at two sites daily (except on Saturday and Sunday) with 5 ml/kg of a 3% aqueous solution of levallorphan tartrate. Water was supplied *ad libitum* but to reduce contamination of the urine, the animals were exposed to food for periods of only 3–4 hr each day. Rats were fed *ad libitum* on Saturdays and Sundays, but urine was not collected on those days. A total of 17.6 g of levallorphan tartrate was injected. Urine was collected daily in bottles containing 1 ml of concentrated HCl, which served as a preservative. The cages were rinsed with 0.1 N HCl and the daily urine collections and washings were pooled and refrigerated until processed. The metabolite content of the various fractions obtained throughout the isolation procedure was monitored using paper chromatography as described previously.[1]

Metabolite Isolation Procedure. A volume of concentrated HCl equal to 10% of the volume of the collected urine was added and the mixture was autoclaved for 30 min at 15 lb of pressure to hydrolyze conjugates of the metabolite.[1] After cooling, the mixture was extracted three times with 10% EtOH in $CHCl_3$ (v/v). The solvent phase was filtered and evaporated to dryness on a steam bath.

The residue was dissolved in 10 ml of EtOH with warming, 20 ml of water was added, and the mixture was poured through a Dowex 2X8 column (1 × 30 cm) which had been washed previously with 0.1 N NaOH until free of Cl^- and then with water until the washings were essentially neutral. The column was washed successively with 60 ml of water, 100 ml of 5% NH_4OH solution (v/v), and 60 ml of water. AcOH (150 ml, 0.15 N) was poured through the column. The metabolite, which was observable as a white band traveling down the column, was collected with 110 ml of the eluate. The eluate was made alkaline with NaOH and extracted with equal volumes of 10% EtOH in $CHCl_3$. Removal of the solvent on a steam bath yielded 210 mg of dry residue.

The residue was dissolved in 10 ml of EtOH with warming, diluted to 60 ml with water, and rechromatographed on a second Dowex 2X8 column in the same manner as described previously except that 100 rather than 150 ml of 0.15 N AcOH was used for elution.

The eluate was poured through a column of Dowex 50WX8 which had been washed previously with 50 ml of water. The column was washed with 75 ml of water and then eluted with 50 ml of 0.05 N NaOH followed by 250 ml of 0.2 N NaOH. The first 125 ml of the colorless eluate was discarded. The next 60 ml of highly colored eluate was collected, made acidic with HCl, and extracted with an equal volume of $CHCl_3$. The $CHCl_3$ extract contained both metabolite and levallorphan, as determined by paper chromatography, and was discarded. The aqueous phase, which contained most of the metabolite, was made acidic with HCl, and an excess of solid $NaHCO_3$ was added carefully. The mixture was extracted three times with an equal volume of 10% EtOH in $CHCl_3$. Removal of the solvent on a steam bath yielded 100 mg of dry residue.

The residue was suspended in hot water and acetone was added while heating was continued until the residue dissolved. Crystals that formed on cooling were collected and recrystallized from hot acetone–water. The 48 mg of crystals obtained in this manner was dissolved in 2–3 ml of hot EtOH and 2 ml of water and a few milligrams of Darco were added. The mixture was filtered and the filtrate placed on a steam bath. Crystals appeared as the EtOH evaporated. Filtration yielded 33 mg of crystals with a slight brownish tinge (mp 240–243°).

Preparation of the HBr Salt of the Metabolite. Anhydrous HBr in EtOAc was added dropwise to *ca.* 10 mg of the crude metabolite crystals (**2**) in 0.5 ml of EtOH until the solution was acidic. Concentration of the solution gave a brownish-white residue. Recrystallization from EtOH–*i*-PrOH (1:1) and twice from EtOH yielded a few minute, needle-like crystals.

Crystallography. A crystal of **2** · HBr which was approximately 0.01 × 0.02 × 0.12 mm in size was selected for the structure analysis; it was the largest crystal obtained by the above recrystallization procedure. Crystals of **2** · HBr are monoclinic, space group $P2_1$, with a = 9.294 (8), b = 11.717 (15), c = 9.555 (6) Å; β = 120.21 (4)°; Z = 2; d_{calcd} = 1.408 g cm^{-3}.

The intensity data were collected on a Hilger-Watts Model Y290 four-circle diffractometer. Nickel-filtered Cu Kα radiation and pulse height discrimination were used. The peak intensity was measured by a six-step scan for which the total scan range was 0.1° (2θ) and the time was 144 sec. The background was measured at θ (hkl) ±0.4°; each measurement was for 48 sec. These extreme times were necessary due to the small size of the crystal; the peak intensity exceeded 100 counts/sec for only one reflection. Data were collected in the $h \geq 0$ hemisphere for $2\theta \leq 85.3°$. Of the 1510 reflections in the hemisphere, 873 had intensities significantly greater than background (429 observed data in the unique quadrant). An empirical correction was applied to convert the peak top data to integrated scan data, but no absorption correction was made.

The positon of the Br was obtained from a sharpened Patterson function. Five electron density syntheses, based on successively more complete trial structures, were required in order to locate all the lighter atoms except the hydrogens. The absolute configuration of **2**·HBr was assumed to be the same as that established for **1**. The structure was refined by full-matrix least squares. Individual isotropic temperature factors were used for all atoms except Br, which was assigned anisotropic thermal parameters. The Br^- scattering curve was corrected for the real and imaginary parts of the anomalous dispersion. The final difference Fourier has no features greater than 1.0 e $Å^{-3}$ in magnitude. The final unweighted R is 0.088 for the 873 observed reflections.‡ Positional standard deviations for the carbon atoms are on the order of 0.03 Å.

Crystals of the HBr salt of the synthetic metabolite **2** were found to be the same (with respect to the relative configuration) as those of the HBr salt of the metabolite. The agreement of the two sets of data (R = 13.6%) was relatively high due to the fact that the crystals of both samples were of the very smallest size adequate for a structure analysis.

Chemistry. Melting points were taken in capillary tubes with a Thomas-Hoover melting point apparatus and are uncorrected. Uv and ir spectra were measured with Cary Model 14 and Beckman Model IR9 spectrophotometers, respectively. Nmr spectra were measured with a Varian A-60 or HA-100 spectrometer (Me_4Si). The proton signals are designated as s = singlet, d = doublet, dd = doublet of doublets, and m = multiplet. Mass spectra (70 eV, direct inlet system) were determined with a CEC 21-110 spectrometer. Where analyses are indicated only by symbols of the elements, results obtained for those elements were within ±0.4% of the theoretical values.

(−)-3-Hydroxy-6-oxo-*N*-methylmorphinan (4). A solution of 1.90 g (0.006 mol) of (−)-3-methoxy-6-oxo-*N*-methylmorphinan (3)§ in 10 ml of aqueous HBr (48%) was refluxed for 8 min. The mixture was cooled rapidly and the excess HBr was removed by distillation *in vacuo* at 40°. The residue was partitioned between 150 ml of $CHCl_3$ and dilute NH_4OH. The $CHCl_3$ extract was dried ($MgSO_4$) and filtered. The solvent was removed *in vacuo* and the product was allowed to crystallize from acetone. Recrystallization from the same solvent afforded 1.73 g (96%) of the hemihydrate of **4**: mp 227–228° (lit.[7] mp 226–227°); $[\alpha]^{25}D$ −108° (*c* 1.01, EtOH); mass spectrum, molecular ion at *m/e* 271.

(−)-3-Hydroxy-6-oxomorphinan (5). A solution of 1.42 g (0.005 mol) of **4** in 20 ml of Ac_2O was heated at reflux for 19 hr. The excess of Ac_2O was removed *in vacuo* and to the residue, cooled in an ice bath, was added 15 ml of water. The mixture was made basic with concentrated NH_4OH and extracted with 120 ml of $CHCl_3$. The $CHCl_3$ extract was dried ($MgSO_4$) and filtered, and the solvent was removed *in vacuo* to afford 1.34 g of crude acetyl derivative of **4** which, in 15 ml of $CHCl_3$, was added gradually to 550 mg of CNBr in 30 ml of $CHCl_3$. The solution was refluxed for 4 hr and evaporated to dryness *in vacuo*. The residue and 35 ml of 6% HCl were refluxed for 18 hr, cooled, and made alkaline with NH_4OH. The crude base **5** which crystallized from the aqueous solution was collected by filtration and recrystallized from MeOH to give 0.63 g (48%) of pure **5**: mp 350°; ir (KBr) 3300, 2925, 2500, 1680, 1600, and 1560 cm^{-1}; uv max (95% EtOH) infl 220 mμ (ε 8000), 282 (2390), and infl 290 (2040); mass spectrum, molecular ion at *m/e* 257. *Anal.* ($C_{16}H_{19}NO_2$) C, H, N.

(−)-3-Hydroxy-6-oxo-*N*-allylmorphinan (6). A stirred mixture of 0.56 g (0.002 mol) of **5**, 0.27 g of allyl bromide, 0.38 g of $NaHCO_3$, and 10 ml of DMF was refluxed for 4.5 hr. The reaction mixture was filtered and the filter cake washed with EtOH. The filtrate and the washings were combined and the solvent was removed *in vacuo*. Chromatography of the residual oil on silica gel, using $CHCl_3$ as eluent, gave 0.41 g (64%) of **6**, mp 198–199°. Recrystallization from MeOH raised the melting point to 200–201°; $[\alpha]^{25}D$ −139.0° (*c* 1.01, MeOH); ir (KBr) 3400, 2875, 2650, 2630, 1690, 1590, and 1550 cm^{-1}; uv max (95% EtOH) infl 225 mμ (ε 6300) and 283 (2200); mass spectrum, molecular ion at *m/e* 297. *Anal.* ($C_{19}H_{23}NO_2$) C, H, N.

(−)-*N*-Allyl-3,6β-dihydroxymorphinan (2). To a suspension of 0.35 g (0.001 mol) of **6** in 7 ml of water was added over 30 min 2.8 g of 5% Na–Hg. The mixture was stirred at room temperature for 3 hr and then heated on a steam bath for 0.5 hr. The solution was made acidic with concentrated HCl and then made basic with concentrated NH_4OH. The aqueous suspension was extracted with 150 ml of $CHCl_3$. The $CHCl_3$ extract was dried ($MgSO_4$) and filtered. The solvent was removed *in vacuo* to give 0.18 g of crude **2**, which after recrystallization from EtOH afforded 0.10 g (28%) of pure **2**: mp 254–255°; $[\alpha]^{25}D$ −110.06° (*c* 1.07, MeOH); ir (KBr) 3500, 2940, 2670, 2580, 1622, and 1590 cm^{-1}; uv max (95% EtOH) infl 220 mμ (ε 7850), 282 (2420), and infl 289 (2200); nmr (DMSO-d_6) δ 8.94 (s, 1 H, ArOH), 6.85 (d, 1 H, J = 8 Hz, Ar H), 6.69 (d, 1 H, J = 2 Hz, Ar H), 6.50 (dd, 1 H, J = 2 and 8 Hz, Ar H), 5.73 (m, 1 H, –CH=), 5.17 (m, 2 H, C=CH_2), 4.46 (d, 1 H, J = 5 Hz, CHO*H*), 3.24 (m, 1 H, C*H*OH), and 3.5–1.0 ppm (m, 16 H); mass spectrum, *m/e* (rel intensity) 299 (M^+, 100), 298 (60), 272 (58), 216 (18), 192 (36), 157 (14), 85 (35), 70 (14), 57 (21), 43 (22). *Anal.* ($C_{19}H_{25}NO_2$) C, H, N.

(−)-*N*-Allyl-3,6β-dihydroxymorphinan Hydrobromide (2·HBr). Hydrogen bromide (anhydrous) in EtOAc was added to a solution of 0.15 g (0.001 mol) of **2** in 4 ml of MeOH. The resulting precipitate was collected by filtration and recrystallized twice from EtOH to give 0.16 g (84%) of 2·HBr: mp 271–273°; $[\alpha]^{25}D$ −70.99° (*c* 0.81, MeOH); ir (KBr) 3350, 3000, 2950, and 1610 cm^{-1}; uv max (95% EtOH) 220 mμ (ε 7040), 282 (2350), and infl 289 (2020); mass spectrum, essentially identical with that of **2**. *Anal.* ($C_{19}H_{25}NO_2$·HBr) C, H, N.

Pharmacology. Five male CF-1S mice (17–30 g) were used per dose level in all tests. Levallorphan tartrate and the synthetic metabolite (HBr salt) were administered in aqueous solutions.

The hot plate test,[8] tail flick test,[9] a modification of the Nilsen test,[10] and the phenylquinone writhing test[11] were used to measure analgesia in mice. Antagonism to morphine was measured by the tail flick test and the Nilsen test.

In the tail flick test for morphine antagonism, the compounds were given sc 10 min prior to administration of morphine sulfate (10 mg/kg sc). The per cent elevation in reaction time was determined during each test. The per cent antagonism was calculated according to the formula of Harris and Pierson[12] using the actual per cent increase in reaction time.

In the Nilsen test for morphine antagonism, the compounds were administered ip 15 min after administration of morphine sulfate (25 mg/kg sc). Analgesia was tested 15 min after the test compounds according to the method of Perrine, *et al.*[10] ED_{50} values were calculated by the method of Miller and Tainter.[13]

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‡A table of the final atomic parameters will appear following these pages in the microfilm edition of this volume of the journal. Single copies may be obtained from the Business Operations Office, Books and Journals Division, American Chemical Society, 1155 Sixteenth St., N.W., Washington, D. C. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number JMED-73-352.

§(−)-3-Methoxy-6-oxo-*N*-methylmorphinan which was generously supplied by Dr. Y. K. Sawa, Shionogi & Co., Osaka, Japan, had been synthesized from desoxythebainone A.[7]

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Synthesis of Actinomycin D Lactam, [1′,1′-Bis(L-*threo*-α,β-diaminobutyric acid)]actinomycin D†

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Actinomycin D lactam has been synthesized as a potential antitumor chemotherapeutic. In this peptide analog both threonine residues of actinomycin D have been replaced by L-*threo*-α,β-diaminobutyric acid. The key intermediate, N^{α}-benzyloxycarbonyl-L-*threo*-α,β-diaminobutyryl-D-valyl-L-prolylsarcosyl-L-*N*-methylvalyl(N^{β}-diaminobutyric acid) lactam (VIII), was obtained by cyclization through a *p*-nitrophenyl ester condensation between the proline and sarcosine residues. The phenoxazinone precursor, *N*-2-nitro-3-benzyloxy-4-methylbenzoyl substituent, was introduced *via* its symmetrical anhydride. [1′,1′-Bis(L-*threo*-α,β-diaminobutyric acid)]actinomycin D exhibited five times lower toxicity and antibacterial and temporary antitumor activity in mice (Ridgway Osteogenic Sarcoma) at *ca.* ten times higher dose levels than actinomycin D.

In a search for actinomycin analogs with improved chemotherapeutic properties, we are engaged in a program aimed at assessing the contribution of the peptide moiety to antitumor activity of the drug. Actinomycin D is used successfully, when combined with radiation therapy, in the treatment of Wilms' tumor.[3] However, its usefulness for other tumors is severely restricted by its toxicity.[4,5] Thus, there is strong incentive for obtaining actinomycins with broader antitumor activities and improved therapeutic indices.

All natural and biosynthetic actinomycins possess two common amino acids, the threonine residue in position 1′ and the sarcosine in position 4′. Because of this, we are interested in preparing analogs in which substitutions have been made in either of these positions and observing the biological activity of these compounds. Following a total synthesis[6] of actinomycin D, the analog [1′,1′-bis(L-α,β-diaminopropionic acid)]actinomycin D has been synthesized.[7]

In designing new peptide analogs certain empirical rules have emerged in the past; *i.e.*, functional analogs (in which chemically reactive groups have been substituted) should be isosteric and steric analogs (in which chemically nonreactive space-filling groups have been replaced) should be isofunctional.[8] Interpretations of differences in the biological properties of analogs are facilitated if minimal structural changes are made.[9]

We wish to report the synthesis of actinomycin D lactam, *i.e.*, [1′,1′-bis(L-*threo*-α,β-diaminobutyric acid)]actinomycin D (Figure 1).[1] Replacement of the L-threonine residues by L-*threo*-α,β-diaminobutyric acid affords an isosteric functional analog in which -NH- groups are substituted for the lactam oxygens of actinomycin D. A common amino acid has therefore been replaced in a natural compound by an unusual amino acid rarely found in nature. Only recently has α,β-diaminobutyric acid been identified as a constituent of amphomycin.[10] The use of unusual residues in preparing analogs of biologically active peptides has provided many interesting results, deamino-oxytocin being a classical example.[11]

The synthetic route (Figure 2) resembles that recorded for the synthesis of the diaminopropionic acid analog.[7] The pentapeptide lactam is obtained by ring closure between the proline and sarcosine residues using a *p*-nitrophenyl ester condensation. N^{α}-Benzyloxycarbonyl-L-*threo*-α,β-diaminobutyric acid methyl ester hydrochloride (I)‡,§ was condensed with *tert*-butyloxycarbonyl-L-*N*-methylvaline[1] by the mixed anhydride method.[12] The β-dipeptide methyl ester (II) was obtained in crystalline form; however, a side product was also isolated from the mother liquor after Sephadex LH-20 chromatography. This compound, characterized by nmr spectroscopy, was N^{α}-benzyloxycarbonyl-N^{β}-isobutyloxycarbonyl-L-*threo*-α,β-diaminobutyric acid methyl ester (IIa). Obviously, it resulted from nucleophilic attack by the β-amino group of the α,β-diaminobutyric acid derivative on the isobutylcarbonyl of the mixed anhydride.[13]

The *tert*-butyloxycarbonyl group of II was removed by the action of trifluoroacetic acid.[14] Mixed anhydride coupling of III with *tert*-butyloxycarbonylsarcosine[15] gave the crystalline tripeptide methyl ester IV. After alkaline saponification the mixed anhydride was generated and coupled to D-valyl-L-proline *p*-nitrophenyl ester[7] to afford N^{α}-benzyloxycarbonyl-N^{β}-(*tert*-butyloxycarbonylsarcosyl-L-*N*-methylvalyl)-L-*threo*-α,β-diaminobutyryl-D-valyl-L-proline *p*-nitrophenyl ester (VI).

Removal of the *tert*-butyloxycarbonyl group from VI by the action of boron trifluoride in glacial acetic acid[6,16] and conversion to the hydrochloride VII was followed by cyclization at 55–60° in pyridine (c ~0.05) in the presence of triethylamine.[6,17] N^{α}-Benzyloxycarbonyl-L-*threo*-α,β-diaminobutyryl-D-valyl-L-prolylsarcosyl-L-*N*-methylvalyl-(N^{β}-diaminobutyric acid) lactam (VIII) was obtained in 38%

†Syntheses of Actinomycin and Analogs. 9. For part 8, see ref 1. Abbreviations follow the rules of the IUPAC-IUB Commission on Biochemical Nomenclature in ref 2. Dbu, L-*threo*-α,β-diaminobutyric acid.

‡E. Atherton and J. Meienhofer, unpublished results.

§Prepared from L-threonine *via* 48-hr treatment of *N,O*-ditosyl-L-threonine methyl ester with NH_3-saturated CH_3OH, followed by ester hydrolysis in 6 *N* HCl, N^{β}-acylation with *tert*-butyloxycarbonyl azide, Na in liquid NH_3 cleavage of the N^{α}-tosyl group, benzyloxycarbonylation of N^{α}, and acidolytic liberation of N^{β}.