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Synthesis and Evaluation of Bis-Dipeptide and Bis-Tripeptide Analogues of Actinomycin D

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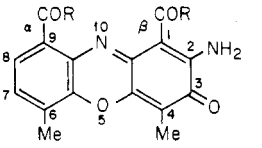
Six bis-dipeptide analogues of actinomycin D, all containing two threonyl-D-valine side chains, were prepared. Also two bis-tripeptide analogues containing an additional proline or oxoproline residue were synthesized. None of the compounds bound to DNA in a manner similar to actinomycin D. This lack of strong intercalative binding emphasizes the importance of the pentapeptidolactone side chains in the binding of actinomycin D to DNA and also highlights the deficiencies inherent in using only small nucleotide sequences in investigating drug–DNA binding. None of the analogues tested showed any antitumor activity, although actinocylbis(threonyl-D-valine methyl ester) did show 10% of the antibacterial activity of actinomycin D vs. *Bacillus subtilis*.

Actinomycin D (**1a**) is an antitumor drug of high potency, but it also shows high toxicity;[1,2] consequently, there is interest in the development of analogues with an improved therapeutic index. The high potency of the parent drug means that analogues showing some diminution in activity could be acceptable if there were a correspondingly greater reduction in host toxicity. Actinomycin D exerts its cytotoxic effect by interaction with guanine residues in DNA,[3,4] and the current detailed knowledge of this interaction can be used as an aid in design of analogues. Although it was initially suggested that actinomycin D binds on the outside of the helix,[5,6] it is now accepted that the chromophoric group intercalates adjacent to a guanine residue and that the cyclic pentapeptide side chains bind externally in the minor groove of the DNA helix, almost certainly inducing a conformational change.[7,8] This model is based on studies of the interaction of actinomycin D with mono-, di-, and oligonucleotides,[9-12] and the data from studies of the interaction with DNA are, on the whole, consistent with this mode of interaction.[2] Sobell and co-workers have proposed a detailed model for the interaction based on elegant X-ray diffraction studies of the actinomycin–(deoxyguanosine)$_2$ complex,[13] and this model exemplifies the current understanding of the actinomycin D–DNA interaction.[8] In the model, the planar ring system of the drug intercalates adjacent to the 3′ face of a guanine–cytosine base pair, the π complexing of this stacking interaction providing the binding force between the phenoxazinone ring system and the adjacent base pairs. The binding is significantly stabilized by H bonding between the 2-amino group of the guanine and the amide carbonyl of one of the threonine residues and between the N-3 of guanine and the NH of the same threonine residue. Furthermore, the amino group on the actinomycin chromophore is postulated to hydrogen bond to the sugar phosphate backbone and hydrogen bonding occurs within the actinomycin D molecule itself, between the NH of one D-valine residue and the CO of the other D-valine residue. Some further stabilization can arise from van der Waals contacts and hydrophobic bonding. On the basis of studies of the interaction of ethidium with ribonucleotides equivalent to two base pairs, Sobell and co-workers have recently suggested that kinking of the DNA and dislocation of the DNA helix axis occur at the point of intercalation of a ligand.[14] Revision of the actinomycin D–DNA model in line with this suggestion[14] gives a model in which the above-mentioned interactions are still implicated but in which there is no longer twofold symmetry and, by inference, asymmetric actinomycin analogues could be accommodated. If the model is a valid representation of the interaction of actinomycin D with DNA, then compounds containing the chromophore of actinomycin D and two Thr-D-Val side chains possess the features required for binding according to this model. Analogues of this type (**1b–g**) have been prepared in this work and tested for



1a, R = Thr-D-Val-Pro-Sar-Me-Val (cyclic, lactone to Thr O)

b, R = Thr-D-Val-OMe
c, R = Thr-D-Val-OH
d, R = Thr(OAc)-D-Val-OMe
e, R = Thr-D-Val-NH(CH$_2$)$_2$OH
f, R = Thr-D-Val-NHCH$_3$
g, R = Thr-D-Val-NHNH$_2$
h, R = Thr-D-Val-Pro-OMe
i, R = Thr-D-Val-4-oxo-Pro-OMe

antitumor and antibacterial activity. The binding to DNA has also been evaluated. To increase the potential for the external interaction, tripeptide derivatives containing an extra proline or oxoproline residue have also been prepared and tested (**1h** and **1c**). It should be stressed, however, that the Sobell model can only reliably predict actinomycin analogues which will bind in the system used as a basis to prepare the model; the model gives, for example, no information on the interaction of the total pentapeptidolactone rings with DNA.

Syntheses. The chromophore of actinomycin can be synthesized by oxidative coupling of 2-amino-3-hydroxy-4-methylbenzoic acid using potassium ferricyanide.[15] This has been the basis of the synthetic schemes used in this work, the peptide chains being assembled sequentially before oxidative dimerization, similar to previously reported syntheses.[16] The synthetic scheme is shown in Scheme I. The starting point for all the compounds prepared was (2-nitro-3-benzyloxy-4-methylbenzoyl)-threonine (**2**).[17] Addition of D-valine methyl ester (active ester synthesis using DCCI and *N*-hydroxysuccinimide) gave (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine methyl ester (**3**) which was reduced (5% palladium on charcoal) and oxidatively dimerized to yield **1b**. The product **1b** could not be directly hydrolyzed to yield **1c** and so, as an alternative route to **1c**, the peptide synthesis was repeated using D-valine *p*-nitrophenyl ester in place of D-valine methyl ester. Catalytic reduction of the product **4**, followed by oxidative coupling, yielded **1c**. The other bis-dipeptide analogues **1d–g** and the bis-tripeptide analogue **1h** were all prepared via intermediate **3** as follows. Acetylation of **3**, then reduction, and oxidative coupling gave **1d**; similarly, aminolysis of **3** with ethanolamine or methylamine, followed by reduction and dimerization, gave **1e** and **1f**, respectively. Hydrazinolysis of **3** gave (2-

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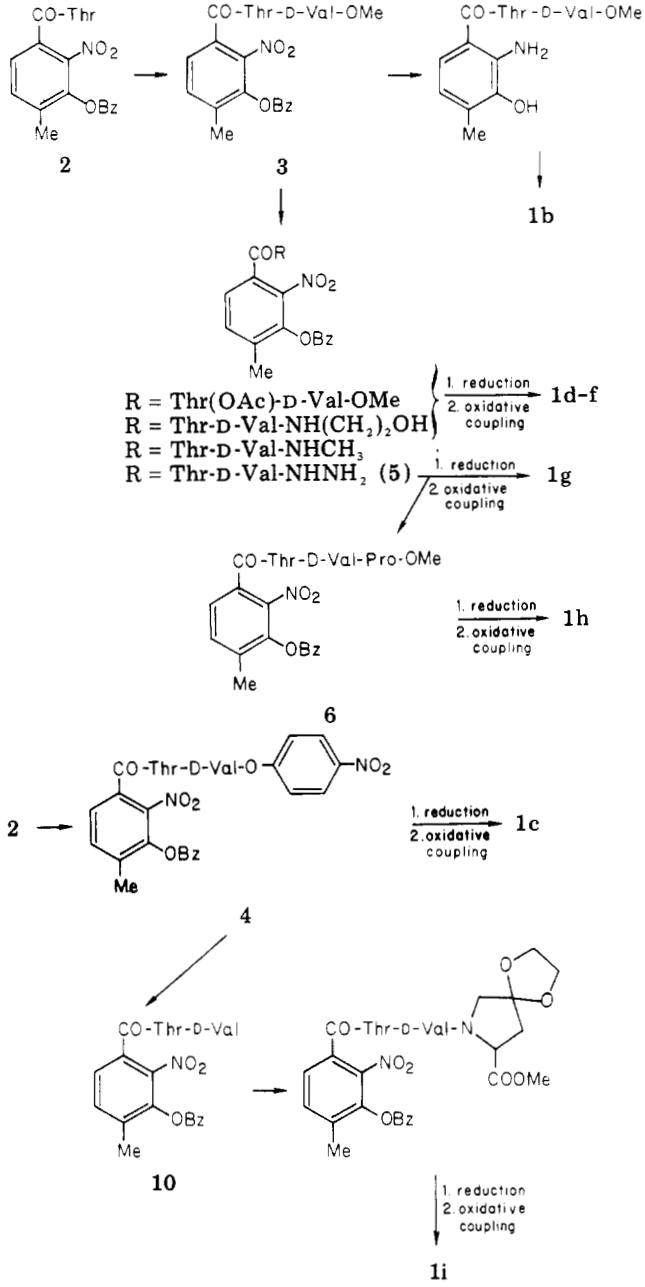
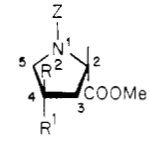
Scheme I. Synthesis of Actinomycin Analogues 1c-i



nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine hydrazide (**5**) which yielded **1g** on reduction and oxidative dimerization. This hydrazide **5** was also the precursor for the bis-tripeptide analogue **1h**. The azide generated from **5** was coupled with proline methyl ester to give the tripeptide **6** which was reduced and oxidatively coupled to give **1h**. In order to prepare the final bis-tripeptide analogue **1i**, it was first necessary to prepare a suitably protected oxoproline derivative. Accordingly, *N*-benzyloxycarbonyl-4-hydroxyproline methyl ester (**7**) (prepared from the acid[18] using methanolic HCl) was oxidized (chromic acid) to *N*-benzyloxycarbonyl-4-oxoproline methyl ester (**8**). This was heated under reflux in eth-



7, $R^1 = H$; $R^2 = OH$
8, $R^1 = R^2 = =O$
9, $R^1 = R^2 = -OCH_2CH_2O-$

anediol containing a catalytic amount of *p*-toluenesulfonic acid to give the ethylene ketal **9**. This ketal was then coupled to (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine (**10**) [prepared by hydrolysis of the intermediate **4** using NaOH–$(CH_3)_2CO$]. Reduction, followed by oxidative coupling and deprotection of the keto group, yielded the bis-tripeptide derivative **1i**.

Table I

Compd	Concn, μg/mL	Mean zone of inhibn, mm	Rel antibacterial act.
Actinomycin D	5.00	18.2	100
	2.50	14.5	
	1.25	11.1	
1b	40.00	16.6[a]	9.6
1c	40.00	14.3[b]	5.3
1f	40.00	8.5[c]	1.1

[a] 3.85 μg/mL of actinomycin D gives an equivalent zone of inhibition. [b] 2.35 μg/mL of actinomycin D gives an equivalent zone of inhibition. [c] 0.65 μg/mL of actinomycin D gives an equivalent zone of inhibition.

Antibacterial Activity. The antibacterial activities of the actinomycins are approximately related to their cytotoxic effects;[2] consequently, the analogues **1b–i** were assayed for antibacterial activity vs. *Bacillus subtilis* according to published procedures.[19] Preliminary studies showed that only actinocylbis(threonyl-D-valine methyl ester) (**1b**), actinocylbis(threonyl-D-valine) (**1c**), and actinocylbis(threonyl-D-valine hydrazide) (**1f**) showed antibacterial activity. The analogues **1d** and **1g–i** showed no activity even at 30 times the concentration of the actinomycin D standard. To obtain an accurate measure of the activities of **1b**, **1c**, and **1f**, the assay was repeated and the concentrations were adjusted so that the zones of inhibition were approximately the same size as those given by the actinomycin D standards. The results are shown in Table I. The most potent analogue is the actinocylbis(threonyl-D-valine methyl ester) (**1b**) which shows about 10% of the potency of actinomycin D.

Antitumor Activity. The bis-dipeptide analogues were also evaluated, through the Division of Cancer Treatment, NCI, for antitumor activity vs. P-388 leukemia in CD_2F_1 mice. None of the compounds gave an increased life span (ILS) of >25%, the minimum level for indication of activity, even at doses of 25 mg/kg per injection (daily injections for 9 days).

Binding to DNA. The lack of in vivo activity of the actinomycin analogues could be due to distributional effects and, since actinomycin D is thought to exert its cytotoxic effects by interaction with DNA, the binding of the analogues to DNA was next evaluated. On binding to DNA, actinomycin D shows a hypochromic and bathochromic shift in the visible region of the spectrum.[7] Solutions containing a constant amount of actinomycin D and increasing amounts of DNA show an isosbestic point, and the absorbance at 440 nm is reduced by about 30%. However, it was found that none of the analogues **1b–i** showed a shift in λ_{max} in the presence of DNA and, consequently, none showed an isosbestic point. Only actinocylbis(threonyl-D-valine methyl ester) (**1b**) showed a measurable decrease (10%) in absorbance at 440 nm in the presence of DNA. This lack of spectral shifts is highly significant since all intercalating agents show marked hypochromic and bathochromic shifts on binding to DNA. The results suggest that the analogues do not mimic the intercalation properties of actinomycin D even though they contain the same chromophore.

Ultracentrifugation of drug–DNA mixtures was used to assess the degree of binding to DNA. After centrifugation, the concentration of drug in the supernatant is equivalent to the free drug concentration, and the bound drug concentration can be calculated by extraction of drug from the DNA pellet with 50% DMF. In the presence of actinomycin D, the DNA pellet was highly colored, and a Scatchard plot of the results yielded $K = 1.56 \times 10^6$ M^{-1} and $n = 0.070$, in good agreement with previously reported values.[4,7,20,21] However, with the analogues, only actinocylbis(threonyl-D-valine methyl ester) (**1b**) and actinocylbis(threonyl-D-valine hydrazide) (**1f**) gave coloration of the DNA pellet, and this was only a weak coloration. There was insufficient binding to derive reliable K and n values. Even at a DNA/drug ratio of 25:1, only 30% of **1b** was bound to DNA (compared to 85% of actinomycin D); similarly, at a DNA/drug ratio of 12:1, only 17% of **1f** was bound to DNA (compared to 70% of actinomycin D). The combined results show that most of the analogues do not bind at all to DNA. Compound **1b**, and probably **1f**, interacts weakly with DNA, presumably with the exterior of the helix.

Conclusions

None of the compounds prepared bind to DNA in a similar manner to actinomycin D. The chromophore of the analogues does not show the spectral shifts, in the presence of DNA, shown by the same chromophore when present in actinomycin D. The chromophore in the analogues, therefore, cannot be intercalating and this implies that the pentapeptidolactone side chains are fundamental to the interaction of actinomycin D with DNA. This is consistent with structure–activity studies which show that changes in the pentapeptidolactone units usually lead to a reduction in activity or to inactivation.[2] Of the compounds reported to date, enhanced activity is only found where the proline residue of actinomycin D is substituted by oxoproline;[22] however, only antibacterial activity is enhanced; antitumor activity is in fact reduced.[2] It appears, therefore, that there is a highly specific interaction of the peptide side chain of actinomycin D with the exterior of the DNA, and this cannot be predicted by the model studies of Sobell. If the chromophore is assumed to intercalate, then the inability of the chromophore of the simplified analogues to intercalate suggests that the binding of the pentapeptidolactone side chains of actinomycin D causes a change in DNA conformation which allows insertion of the chromophore. This importance of the pentapeptidolactone side chains is emphasized in the theory previously proposed by Müller and Crothers;[7] implicit in this theory is the dependence for potency on the slow dissociation of the peptide–helix complex. Also, hydrolysis of the lactone rings of actinomycin D leads to a drastic reduction in activity,[2] implying that a specific conformation of the peptidolactone rings is required for binding to DNA. The fundamental role of the pentapeptidolactone rings cannot be predicted from the studies of the actinomycin–$(deoxyguanosine)_2$ complex and this reveals the limitations of predicting drug–DNA binding properties from studies using bases, base pairs, or small nucleotide sequences. In view of the specificity residing in the pentapeptidolactone units, it would appear to be of value to next investigate the role of the chromophore by substitution with other ring skeletons, while retaining the pentapeptidolactone units of actinomycin D.

Experimental Section

Melting points were recorded on a Kofler hot-stage apparatus and are uncorrected. Spectra were recorded on the following instruments: NMR on a Perkin-Elmer R12B (60 MHz) or Varian HA 100 (100 MHz) spectrometer; mass spectra on an AEI MS9 mass spectrometer.

Actinocylbis(threonyl-D-valine methyl ester) (1b). To a stirred solution of (2-nitro-3-benzyloxy-4-methylbenzoyl)threonine (**2**)[17] (7.85 g, 20.2 mmol) in dichloromethane (30 mL) at 0 °C, *N*-hydroxysuccinimide (3.99 g, 43.8 mmol), *N,N'*-dicyclohexylcarbodiimide (4.16 g, 20.2 mmol), D-valine methyl ester (3.40 g, 26.0 mmol), and triethylamine (2.04 g, 20.2 mmol) were added serially. Stirring was continued for an additional 2 h at 0 °C and 16 h at room temperature with the exclusion of light. The *N,N'*-dicyclohexylurea formed was removed by filtration and the filtrate washed with 0.1 N hydrochloric acid (2 × 40 mL), 0.1 N sodium bicarbonate (2 × 40 mL), and saturated sodium chloride solutions (2 × 40 mL), respectively. The organic phase was dried over magnesium sulfate and evaporated to dryness in vacuo to yield a white solid which on chromatographic examination revealed a major component and a minor component [R_f 0.58 and 0.85, respectively, TLC (silica gel) ethyl acetate–benzene, 2:1]. The major component was separated and purified by column chromatography (3 × 50 cm) on silica gel with the same solvent to yield white crystals which were recrystallized from ethyl acetate–petroleum ether (bp 40–60 °C) to afford white needles of (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine methyl ester: 8.52 g (84%); mp 104–106 °C; IR (Nujol) 3300–3250 (OH, NH), 1740 (ester C=O), 1660–1630 (amide C=O), 1550–1535 (amide C=O, NO_2), 750 and 700 cm^{-1} (substituted benzene); UV λ_{max} (CH_3OH) 210 nm (log ϵ 4.479); ^1H NMR ($CDCl_3$) δ 7.20–7.50 (9 H, m, Thr NH, D-Val NH, 6-CH, 5-CH, C_6H_5), 4.94 (2 H, s, CH_2), 4.30–4.70 (3 H, m, Thr 1'-CH, 2'-CH, and OH), 3.90–3.99 (1 H, d, D-Val 1'-CH), 3.65 (3 H, s, OCH_3), 2.30 (3 H, s, 4-CCH_3), 2.01–2.30 (1 H, m, D-Val 2'-CH), 1.15–1.25 (3 H, d, J = 6.1 Hz, Thr 2'-CCH_3), 0.90–1.00 [6 H, d, J = 6.5 Hz, D-Val 2'-$C(CH_3)_2$]. Anal. Calcd for $C_{25}H_{31}N_3O_8$: C, 59.9; H, 6.2; N, 8.4. Found: C, 59.9; H, 6.2; N, 8.1.

The benzyloxy group was reductively cleaved and the resulting aminophenol oxidatively coupled as follows. A solution of the above (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine methyl ester (200 mg, 0.4 mmol) in methanol (10 mL) was hydrogenated over 5% palladium on charcoal (80 mg) in the dark at room temperature and atmospheric pressure for 4 h. The solution was filtered and added to a stirred solution of potassium ferricyanide (378 mg, 1.16 mmol) in pH 7.2 phosphate buffer (10 mL). Stirring was continued for 30 min in the dark after which time the red product was extracted into ethyl acetate (3 × 40 mL). The bulked ethyl acetate extracts were washed with 1 N hydrochloric acid (2 × 30 mL), 1 M sodium bicarbonate (2 × 30 mL), and saturated sodium chloride solutions (2 × 30 mL) and dried over magnesium sulfate. The dried ethyl acetate was evaporated to dryness in vacuo to yield a red powder which was crystallized from ethyl acetate–petroleum ether (bp 40–60 °C) to afford red microcrystals of **1b**: 120 mg (80%); mp 108–110 °C; IR (KBr) 3460–3250 (NH_2, NH, OH), 1745 (ester C=O), 1670–1630 (amide C=O), 1565 cm^{-1} (phenoxazone); UV λ_{max} (CH_3OH) 241 nm (log ϵ 4.567), 443 (4.381); ^1H NMR ($CDCl_3$) δ 11.0 (1 H, d, J = 5.7 Hz, D-Val NH), 7.35–7.60 (5 H, m, 3 × NH, 8-CH, 7-CH), 4.30–5.05 (10 H, m, 2 × Thr 1'-CH and 2'-CH, 2 × Thr OH, 2 × D-Val 1'-CH, NH_2), 3.75 (3 H, s, OCH_3), 3.65 (3 H, s, OCH_3), 2.55 (3 H, s, 6-CCH_3), 2.24 (3 H, s, 4-CCH_3), 2.15–2.35 (2 H, m, 2 × D-Val 2'-CH), 1.25 and 1.33 (6 H, dd, J = 6.1, 6.61 Hz, respectively, 2 × Thr CCH_3), 0.80–1.08 [12 H, m, 2 × D-Val 2'-$C(CH_3)_2$]. Anal. Calcd for $C_{36}H_{48}N_6O_{12}$: C, 57.1; H, 6.4; N, 11.1. Found: C, 57.4; H, 6.4; N, 10.9.

Actinocylbis(threonyl-D-valine) (1c). To a stirred solution of (2-nitro-3-benzyloxy-4-methylbenzoyl)threonine (**2**) (1 g, 2.6 mmol), *N*-hydroxysuccinimide (0.59 g, 5.1 mmol), and *N,N'*-dicyclohexylcarbodiimide (0.58 g, 2.8 mmol) in tetrahydrofuran (20 mL), triethylamine (0.25 g, 2.57 mmol) and the hydrobromide salt of D-valine *p*-nitrobenzyl ester were added. Stirring was continued overnight with the exclusion of moisture and light. The triethylamine hydrobromide salt formed was removed from the reaction mixture by filtration and the solution was evaporated in vacuo. The resulting gum was dissolved in dichloromethane (30 mL), the insoluble dicyclohexylurea was removed by filtration, and the solution was washed with 1 M hydrochloric acid (2 × 50 mL), 1 M sodium bicarbonate (2 × 50 mL), and saturated sodium chloride solutions (2 × 50 mL) and dried over magnesium sulfate.

Evaporation of the solution in vacuo gave an oil which gave one major spot [R_f 0.54, TLC (silica gel) ethyl acetate–benzene, 2:1] and a minor spot (R_f 0.65, same system as above). Repeated attempts to purify the major component by column chromatography on silica gel with the same solvent failed. The reaction product was chromatographed on alumina (3 × 50 cm) with the solvent above, followed by ethyl acetate. The ethyl acetate fraction gave a single spot [R_f 0.54, TLC (silica gel) ethyl acetate–benzene, 2:1]. The ethyl acetate eluent was evaporated in vacuo to yield an oil which was crystallized from ethyl acetate–ether to give (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine *p*-nitrobenzyl ester. Recrystallization from the same solvent afforded white microcrystals: 0.8 g (50%); mp 82–84 °C; IR (Nujol) 3400–3300 (NH, OH), 1745 (ester C=O), 1670–1650 (amide C=O), 1550–1530 (amide C=O, NO_2), 850, 740, and 710 cm^{-1} (substituted benzene); UV λ_{max} (CH_3OH) 208 nm (log ϵ 4.718); ^1H NMR ($CDCl_3$) δ 8.00–9.15 (1 H, d, NH), 7.30–7.70 (12 H, NH, 4 × *p*-nitrobenzyl H, 6-CH, 5-CH, C_6H_5), 5.20 (2 H, s, benzylic CH_2), 4.90 (2 H, s, *p*-nitrobenzylic CH_2), 4.70 (1 H, s, OH), 4.40–4.60 (2 H, m, Thr 1′-CH and 2′-CH), 4.00–4.10 (1 H, d, D-Val 1′-CH), 2.90–3.15 (1 H, m, D-Val 2′-CH), 2.35 (3 H, s, 4-CCH_3), 1.25 (3 H, d, J = 6 Hz, Thr 2′-CCH_3), 0.95 [6 H, d, J = 6.5 Hz, D-Val 2′-C(CH_3)$_2$]. Anal. Calcd for $C_{31}H_{34}N_4O_{10}$: C, 58.9; H, 5.5; N, 9.0. Found: C, 59.2; H, 5.5; N, 9.0.

Reduction and oxidative coupling of this compound to yield **1c** was carried out as follows. A solution of this (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine *p*-nitrobenzyl ester (85 mg, 0.14 mmol) in methanol (7 mL) was hydrogenated over 10% palladium on charcoal (50 mg) for 50 min in the dark at room temperature and pressure. The mixture was filtered and added to a stirred solution of potassium ferricyanide (142 mg, 0.44 mmol) in pH 7.2 phosphate buffer. Stirring was continued for 30 min in the absence of light after which time the solution was acidified with 5% citric acid solution and the red compound was extracted into ethyl acetate (3 × 20 mL). The combined ethyl acetate extract was washed with 1 N hydrochloric acid (3 × 20 mL) and saturated sodium chloride solutions (2 × 20 mL), respectively, dried over magnesium sulfate, and concentrated in vacuo. Compound **1c** was obtained as red microcrystals from ethyl acetate solution with the addition of petroleum ether (bp 40–60 °C): 40 mg (80%); mp 230–235 °C; IR (KBr) 3400–3200 (NH_2, NH, OH), 1720–1700 (acid C=O), 1660–1640 (amide C=O), 1575–1520 cm^{-1} (phenoxazone); UV λ_{max} (CH_3OH) 240 nm (log ϵ 4.669), 448 (4.367); ^1H NMR [(CD_3)$_2$SO] δ 10.25 (1 H, d, J = 5.7 Hz, D-Val NH), 9.55 (1 H, d, J = 6.0 Hz, D-Val NH), 7.87–8.17 (2 H, m, 2 × Thr NH), 7.40–7.75 (2 H, dd, 8-CH and 7-CH), 4.49–4.63 (4 H, m, 2 × Thr 1′-CH and 2′-CH), 4.09–4.41 (4 H, m, 2 × D-Val 1′-CH and 2 × Thr OH), 3.40–4.00 (4 H, br s, 2 × COOH and NH_2), 2.54 (3 H, s, 6-CCH_3), 2.19 (3 H, s, 4-CH_3), 2.10–2.20 (2 H, m, 2 × D-Val 2′-CH), 1.20 (6 H, d, J = 5.9 Hz, 2 × Thr 2′-CCH_3), 0.85–1.00 [12 H, m, 2 × D-Val 2′-C(CH_3)$_2$]. Anal. Calcd for $C_{34}H_{44}N_6O_{12}$: C, 56.0; H, 6.0; N, 11.4. Found: C, 55.8; H, 6.0; N, 11.4.

Actinocylbis[threonyl(*O*-acetyl)-D-valine methyl ester] (1d). (2-Nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine methyl ester (**3**) (0.4 g, 0.80 mmol) was dissolved in dry pyridine (2 mL) and acetic anhydride (0.1 mL, 0.9 mmol) added. The solution was left for 72 h with the exclusion of moisture and light at room temperature. The solution was diluted with ethyl acetate (30 mL) and washed with 1 N hydrochloric acid (3 × 30 mL), 1 M sodium bicarbonate (3 × 30 mL), and saturated sodium chloride solutions (3 × 30 mL), respectively. The organic phase was dried over magnesium sulfate and evaporated to dryness in vacuo to yield an oil [R_f 0.65, TLC (silica gel) ethyl acetate–benzene, 10:3]. Repeated evaporation with ether in vacuo yielded (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl(*O*-acetyl)-D-valine methyl ester as a white solid: 0.3 g (70%); mp 64–66 °C; IR (Nujol) 3310–3275 (NH), 1745 (ester C=O), 1660–1645 (amide C=O, NO_2), 740 and 705 cm^{-1} (substituted benzene); UV λ_{max} (CH_3OH) 213 nm (log ϵ 4.638); ^1H NMR ($CDCl_3$) δ 7.15–7.50 (9 H, m, 2 × NH, 5-CH, 6-CH, C_6H_5), 5.40–5.50 (2 H, t, Thr 1′-CH), 4.80–5.05 (3 H, m, benzylic CH_2 and Thr 2′-CH), 4.30–4.70 (1 H, m, D-Val 1′-CH), 3.60 (3 H, s, OCH_3), 2.30 (3 H, s, 4-CCH_3), 2.05–2.20 (1 H, m, D-Val 2′-CH), 2.00 (3 H, s, -$COCH_3$), 1.30–1.40 (3 H, d, Thr 2′-CCH_3), 0.85–0.95 [6 H, d, D-Val 2′-C(CH_3)$_2$]. Anal. Calcd for $C_{27}H_{33}N_3O_9$: C, 59.7; H, 6.0; N, 7.7. Found: C, 59.6; H, 6.0; N, 7.4.

This "monomer" unit was reduced and oxidatively dimerized in an identical manner to the formation of **1a**, yielding **1c** as red microcrystals which were recrystallized from ethyl acetate–petroleum ether (bp 40–60 °C): 55 mg (51%); mp 104–106 °C; IR (Nujol) 3425–3375 (NH_2, NH), 1745 (ester C=O), 1670–1650 (amide C=O), 1580 cm^{-1} (phenoxazone); UV λ_{max} (CH_3OH) 240 nm (log ϵ 4.698), 446 (4.296); ^1H NMR δ 10.88 (1 H, d, J = 6.8 Hz, Thr NH), 8.14 (1 H, J = 6.7 Hz, Thr NH), 7.20–7.70 (4 H, m, 8-CH, 7-CH, 2 × D-Val NH), 5.20–5.62 (4 H, m, NH_2 and 2 × Thr 1′-CH), 4.50–4.90 (3 H, m, 2 × Thr 2′-CH and D-Val 1′-CH), 3.79–3.90 (1 H, m, D-Val 1′-CH), 3.77 (3 H, s, OCH_3), 3.70 (3 H, s, OCH_3), 2.24–2.70 (2 H, m, 2 × D-Val 2′-CH), 2.50 (3 H, s, 6-CCH_3), 2.19 (3 H, s, $COOCH_3$), 2.08 (3 H, s, $COOCH_3$), 1.89 (3 H, s, 4-CCH_3), 1.38 and 1.32 (6 H, dd, J = 6.0 Hz each, 2 × Thr 2′-CCH_3), 0.98 and 0.82 [12 H, dd, J = 6.5 Hz each, 2 × D-Val 2′-C(CH_3)$_2$]. Anal. Calcd for $C_{40}H_{52}N_6O_{14}$: C, 57.1; H, 6.2; N, 10.0. Found: C, 57.0; H, 6.3; N, 10.1.

Actinocylbis(threonyl-D-valine ethanolamide) (1e). (2-Nitro-3-benzoyl-4-methylbenzoyl)threonyl-D-valine methyl ester (**2**) (0.4 g, 0.80 mmol) was dissolved in ethanol (2 mL) and 25% aqueous solution of ethanolamine (0.6 mL, 3.3 mmol) added. The solution was left in a stoppered flask for 3 days at room temperature with the exclusion of light. The resulting solid was dissolved in 1-butanol (50 mL) and the solution was washed with 1 N hydrochloric acid (3 × mL), 1 M sodium bicarbonate (3 × 30 mL), and saturated sodium chloride solutions (3 × 30 mL), respectively. The organic phase was dried over magnesium sulfate and evaporated to dryness in vacuo. The residual white solid was crystallized from ethanol–ether to give white needles of (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine ethanolamide: 0.28 g (68%); mp 221–223 °C; IR (Nujol) 3270–3250 (NH, OH), 1660–1640 (amide C=O), 1540 (amide C=O, NO_2), 750 and 700 cm^{-1} (substituted benzene); UV λ_{max} (CH_3OH) 216 nm (log ϵ 4.673); ^1H NMR [(CD_3)$_2$SO] δ 8.35–8.50 (1 H, d, NH), 7.70–7.85 (2 H, m, 2 × NH), 7.35–7.55 (7 H, m, 6-CH, 5-CH, C_6H_5), 4.95 (2 H, s, benzylic CH_2), 4.00–4.60 (4 H, m, Thr 1′-CH, 2′-CH, OH, D-Val 1′-CH), 3.10–3.40 (5 H, m, NCH_2CH_2O, OH), 2.35–2.48 (1 H, m, D-Val 1′-CH), 2.30 (3 H, s, 4-CCH_3), 1.05–1.15 (3 H, d, Thr 2′-CCH_3), 0.75–0.85 [6 H, d, D-Val 2′-C(CH_3)$_2$]. Anal. Calcd for $C_{26}H_{34}N_4O_8$: C, 58.9; H, 6.4; N, 10.6. Found: C, 59.3; H, 6.4; N, 10.5.

This intermediate (0.15 g) was reduced and coupled as previously to yield **1c** which was crystallized from ethanol–ether to give red microcrystals: 0.10 g (87%); IR (Nujol) 3375–3290 (NH_2, NH, OH), 1645 (amide C=O), 1565 cm^{-1} (phenoxazone); UV λ_{max} (CH_3OH) 242 nm (log ϵ 4.568), 443 (4.364); ^1H NMR δ 8.45–8.55 (2 H, m, 2 × D-Val NH), 7.65–8.10 (4 H, m, 2 × Thr NH, 2 × ethanolamide NH), 7.27–7.62 (2 H, dd, 8-CH and 7-CH), 5.05–5.20 (2 H, br s, NH_2), 3.85–3.65 (6 H, m, 2 × Thr 1′-CH and 2′-CH, 2 × Thr OH), 3.00–3.50 (10 H, m, 2 × NCH_2CH_2O, 2 × D-Val 1′-CH), 2.60–2.70 (2 H, m, 2 × D-Val 2′-CH), 2.45 (3 H, s, 6-CCH_3), 2.10 (3 H, s, 4-CCH_3), 2.00 (2 H, br s, 2 × OH), 1.05–1.25 (6 H, m, 2 × Thr 2′-CCH_3), 0.75–0.90 [12 H, m, 2 × D-Val 2′-C(CH_3)$_2$]. Anal. Calcd for $C_{38}H_{54}N_8O_{12}$: C, 56.0; H, 6.6; N, 13.8. Found: C, 55.6; H, 6.5; N, 13.6.

Actinocylbis(threonyl-D-valine methylamide) (1f). This was prepared in an identical manner to **1e** using methylamine in place of ethanolamine. The intermediate (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine methylamide was obtained from ethanol–ether as white needles: 80% from **3**; mp 211–212 °C; IR (Nujol) 3270–3255 (NH, OH), 1660 (amide C=O), 1540 (amide C=O, NO_2), 750 and 700 cm^{-1} (substituted benzene); UV λ_{max} (CH_3OH) 218 nm (log ϵ 4.751); ^1H NMR [(CD_3)$_2$SO] δ 8.39–8.49 (1 H, d, NH), 7.66–7.94 (2 H, m, 2 × NH), 7.54 (2 H, s, 5-CH, 6-CH), 7.33 (5 H, s, C_6H_5), 4.90 (2 H, s, benzylic CH_2), 3.80–4.50 (4 H, m, Thr 1′-CH, 2′-CH, and OH, D-Val 1′-CH), 2.55 and 2.60 (3 H, 2 s, NCH_3), 2.38 (3 H, s, 4-CCH_3), 2.25–2.35 (1 H, m, D-Val 2′-CH), 1.05–1.18 (3 H, d, Thr 2′-CCH_3), 0.78–0.90 [6 H, d, D-Val 2′-C(CH_3)$_2$]. Anal. Calcd for $C_{25}H_{32}N_4O_7$: C, 60.0; H, 6.4; N, 11.2. Found: C, 59.6; H, 6.3; N, 11.0.

The intermediate was reduced and coupled to yield **1f** which recrystallized from ethanol–ether as red needles: 80%; mp 296–299 °C; IR (KBr) 3380–3290 (NH_2, NH, OH), 1670–1630 (amide C=O), 1570–1560 cm^{-1} (phenoxazone); UV λ_{max} (CH_3OH) 240 nm (log ϵ 4.569), 443 (4.383); ^1H NMR [(CD_3)$_2$SO] δ 10.38 (1 H, d, J = 5.7 Hz, D-Val NH), 8.79 (1 H, d, J = 6.0 Hz, D-Val NH),

8.18 (1 H, d, J = 6.3 Hz, Thr NH), 7.86 and 8.05 (3 H, m, D-Val NH_2, NH_2), 7.62 (1 H, d, J = 7.9 Hz, 8-CH), 7.40 (1 H, d, J = 7.9 Hz, 7-CH), 5.26–5.34 (2 H, m, Thr 1′-CH and OH, β chain), 4.60–4.66 (2 H, m, 2 × methylamide NH), 4.37–4.43 (2 H, m, Thr 1′-CH and OH, α chain), 3.95–4.18 (4 H, m, 2 × Thr 2′-CH, 2 × D-Val 1′-CH), 2.57, 2.58, and 2.59 (6 H, 3 s, 2 × NCH_3), 1.96–2.20 (2 H, m, 2 × D-Val 2′-CH), 208 (3 H, s, 4-CCH_3), 1.12 and 1.18 (6 H, dd, J = 5.9 Hz each, 2 × Thr 2′-CCH_3), 0.85 [12 H, d, J = 6.5 Hz, 2 × D-Val 2′-$C(CH_3)_2$]. Anal. Calcd for $C_{36}H_{50}N_8O_{10}$: C, 57.3; H, 6.7; N, 14.8. Found: C, 56.9; H, 6.5; N, 14.7.

Actinocylbis(threonyl-D-valine hydrazide) (1g). This was prepared in an identical manner to **1e**, using hydrazine hydrate in place of ethanolamine. The intermediate (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine hydrazide (**5**) was obtained from ethanol–ether as white microcrystals: 84%; mp 236–238 °C; IR (Nujol) 3375–3280 (NH_2, NH, OH), 1675–1640 (amide C=O), 1550–1540 (amide C=O and NO_2), 750 and 700 cm^{-1} (substituted benzene); UV λ_{max} (CH_3OH) 209 nm (log ϵ 4.876); ^1H NMR [$(CD_3)_2SO$] δ 9.15 (1 H, s, hydrazide NH), 8.35–8.50 (1 H, d, D-Val NH), 7.80–7.95 (1 H, d, Thr NH), 7.60 (2 H, s, 6-CH and 5-CH), 7.38 (5 H, s, C_6H_5), 4.95 (2 H, s, benzylic CH_2), 3.80–4.55 (4 H, m, Thr 1′-CH, 2′-CH, and OH, D-Val 1′-CH), 3.4–3.5 (2 H, br s, NH_2), 2.39 (3 H, s, 4-CCH_3), 2.25–2.38 (1 H, m, D-Val 2′-CH), 1.05–1.15 (3 H, d, Thr 2′-CCH_3), 0.80–0.90 [6 H, d, D-Val $C(CH_3)_2$]. Anal. Calcd for $C_{24}H_{31}N_5O_4$: C, 51.4; H, 6.2; N, 14.0. Found: C, 57.3; H, 6.4; N, 13.9.

This intermediate **5** was then reduced and coupled to yield **1g** which recrystallized from ethanol–ether as red microcrystals: 68% from **5**; mp 187–190 °C; IR (KBr) 3400–3270 (NH_2, NH, OH), 1675–1640 (amide C=O), 1580–1540 cm^{-1} (phenoxazone); UV λ_{max} (CH_3OH) 240 nm (log ϵ 4.519), 443 (4.284); ^1H NMR [$(CD_3)_2SO$] δ 9.15 (2 H, br s, 2 × hydrazide NH), 8.60–8.80 (2 H, m, 2 × D-Val NH), 7.90–8.10 (2 H, m, 2 × Thr NH), 7.65–7.75 (1 H, d, J = 7.8 Hz, 8-CH), 7.40–7.50 (1 H, d, J = 7.8 Hz, 7-CH), 5.15 (2 H, br s, NH_2), 3.80–4.75 (8 H, m, 2 × Thr 1′-CH, 2′-CH, and OH, 2 × D-Val 1′-CH), 3.40 (4 H, br s, 2 × NNH_2), 2.65 (3 H, s, 6-CCH_3), 2.15 (3 H, s, 4-CCH_3), 1.80–2.11 (2 H, m, 2 × D-Val 2′-CH), 1.20 (6 H, d, J = 6.0 Hz, 2 × Thr 2′-CCH_3), 0.88 [12 H, d, J = 6.2 Hz, 2 × D-Val 2′-$C(CH_3)_2$]. Anal. Calcd for $C_{34}H_{48}N_{10}O_{10}$: C, 54.0; H, 6.4; N, 18.5. Found: C, 54.0; H, 6.5; N, 18.3.

Actinocylbis(threonyl-D-valylproline methyl ester) (1h). (2-Nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine hydrazide (**5**) (0.3 g, 0.6 mmol) was suspended in glacial acetic acid (0.8 mL) at 0 °C. Hydrochloric acid (5 N, 0.2 mL), water (3 mL), and sodium nitrite (65 mg, 0.94 mmol) were added, respectively, to the stirred suspension and the stirring was continued for 20 min with the exclusion of light. The (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine azide generated in the reaction was extracted into precooled ether (3 × 30 mL), washed with precooled sodium bicarbonate solution (3 × 30 mL), and dried over magnesium sulfate. Proline methyl ester hydrochloride (0.138 g, 0.84 mmol) and triethylamine (91 mg, 0.9 mmol) in chloroform (25 mL) were added to the ether solution. Ether was removed from the reaction mixture in vacuo at 0 °C and the resulting chloroform solution was stirred for 1 h at 0 °C with the exclusion of light and stored at 0 °C overnight. The reaction mixture was diluted with ethyl acetate (60 mL) and washed with 1 N hydrochloric acid (3 × 30 mL), 1 M sodium bicarbonate (3 × 30 mL), and saturated sodium chloride solutions (3 × 30 mL), respectively. The organic phase was dried over magnesium sulfate and evaporated to dryness in vacuo to yield a gum which gave a major spot [R_f 0.65, TLC (silica gel) ethyl acetate] and a minor spot (R_f 0.90, same system as above). The product was chromatographed on a column of silica gel (2 × 50 cm) with ethyl acetate as solvent. The second fraction yielded (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valylproline methyl ester as white microcrystals which were recrystallized from ethyl acetate–petroleum ether (bp 40–60 °C): 0.12 g (34%); mp 78–83 °C; IR (KBr) 3430–3300 (NH, OH), 1745 (ester C=O), 1660–1630 (amide C=O), 1550–1540 (amide C=O, NO_2), 800 and 760 cm^{-1} (substituted benzene); UV λ_{max} (CH_3OH) 216 nm (log ϵ 4.773); ^1H NMR ($CDCl_3$) δ 7.20–7.60 (2 H, m, 2-NH), 7.40 (7 H, 2 s superimposed, 6-CH, 5-CH, C_6H_5), 4.95 (2 H, s, CH_2), 3.70–4.75 (7 H, m, Thr 1′-CH, 2′-CH, and OH, D-Val 1′-CH, and Pro 2′-CH and 5′-CH_2), 3.68 (3 H, s, OCH_3), 2.35 (3 H, s, 4-CCH_3), 2.30 (4 H, s, Pro 3′-CH_2 and 4′-CH_2), 2.11–2.27 (1 H, m, D-Val 2′-CH), 1.20–1.30 (3 H, d, Thr 2′-CCH_3), 0.95–1.05 [6 H, d, D-Val 2′-$C(CH_3)_2$]. Anal. Calcd for $C_{30}H_{38}N_4O_9$: C, 62.2; H, 6.4; N, 9.4. Found: C, 61.9; H, 6.3; N, 9.2.

This intermediate was reduced and coupled by the usual method to yield **1h** which recrystallized from ethyl acetate–petroleum ether (bp 40–60 °C) to give red microcrystals: 73%; mp 140–145 °C; IR (KBr) 3420–3320 (NH_2, NH, OH), 1745–1740 (ester C=O), 1660–1635 (amide C=O), and 1570 cm^{-1} (phenoxazone); UV λ_{max} (CH_3OH) 240 nm (log ϵ 4.540), 443 (4.357); ^1H NMR ($CDCl_3$) δ 8.00–9.40 (2 H, m, 2 × D-Val NH), 7.50–8.00 (2 H, m, 2 × Thr NH), 7.25–7.49 (2 H, dd, 8-CH and 7-CH), 5.00–5.20 (2 H, br s, NH_2), 4.00–5.00 (14 H, m, 2 × Thr 1′-CH, 2′-CH, and OH, 2 × D-Val 1′-CH, and 2 × Pro 2′-CH and 5′-CH_2), 3.55–3.65 (6 H, 2 s superimposed, 2 × OCH_3), 2.35 (3 H, s, 6-CCH_3), 1.90–2.30 (13 H, m, 4-CCH_3, 2 × Pro 3′-CH_2, and 2 × D-Val CH), 1.20–1.40 (6 H, m, 2 × Thr 2′-CCH_3), 1.09–1.10 [12 H, m, 2 × D-Val 2′-$C(CH_3)_2$]. Anal. Calcd for $C_{46}H_{62}N_8O_{14}$: C, 58.1; H, 6.5; N, 11.8. Found: C, 57.6; H, 6.4; N, 11.5.

Actinocylbis(threonyl-D-valyl-4′-oxoproline methyl ester) (1i). (a) Synthesis of 4-(Ethylenedioxy)proline Methyl Ester. N-Benzyloxycarbonyl-4-hydroxyproline[18] (6.3 g, 25.7 mmol) was dissolved in 2.5% methanolic hydrogen chloride (50 mL) and stood in a stoppered flask for 72 h. The methanol was evaporated in vacuo at room temperature. The resulting oil was dissolved in ethyl acetate (50 mL) which was then washed with 1 N hydrochloric acid (3 × 40 mL), 1 M sodium bicarbonate (3 × 40 mL), and saturated sodium chloride solutions (3 × 40 mL), respectively. The organic phase was dried over magnesium sulfate and evaporated in vacuo to yield N-benzyloxycarbonyl-4-hydroxyproline methyl ester (**7**), 5.37 g (75%). This was then oxidized as follows.

Chromic acid solution (8 N, 9 mL) was added over a period of 10 min to a stirred solution of N-benzyloxycarbonyl-4-hydroxyproline methyl ester (**7**) (2.4 g, 8.6 mmol) in acetone (130 mL) at 0 °C. The reaction mixture was stirred for a further 30 min. Excess chromic acid was destroyed by the addition of methanol (10 mL). The solution was shaken with Celite and filtered to remove precipitated chromium salts. The filtrate was concentrated in vacuo and the residue taken up in ethyl acetate (50 mL). This was washed with 1 N hydrochloric acid (3 × 50 mL), 1 M sodium bicarbonate (3 × 50 mL), and saturated sodium chloride solutions (3 × 50 mL), respectively, dried over magnesium sulfate, and evaporated to dryness in vacuo to yield a two-component mixture, a major spot [R_f 0.69, TLC (silica gel) benzene–ethyl acetate, 16:10] and a minor spot (R_f 0.17, same system as above). The two components were separated by column chromatography on silica gel (3 × 50 cm) with with same solvent. The major fraction gave N-benzyloxycarbonyl-4-oxoproline methyl ester (**8**) as an oil [R_f 0.69, TLC (silica gel) benzene–ethyl acetate, 16:10]: 2.02 g (85%); IR (film) 1770–1710 (cyclic C=O, ester C=O, carbamate C=O), 780 and 710 cm^{-1} (substituted benzene); ^1H NMR δ 7.30 (5 H, s, C_6H_5), 5.15 (2 H, s, benzylic CH_2), 4.70–5.05 (1 H, m, 2-CH), 3.90 (2 H, s, 5CH_2), 3.65 (3 H, s, OCH_3), 2.39–2.95 (2 H, m, 3CH_2); M^+ (high resolution) 277.0946 ($C_{14}H_{15}NO_5$ requires 277.0950, 1 ppm error). Anal. Calcd for $C_{14}H_{15}NO_5$: C, 60.6; H, 5.4; N, 5.1. Found: C, 60.3; H, 5.4; N, 5.0.

The ethylene ketal of **8** (**9**) was then prepared. A solution of N-benzyloxycarbonyl-4-oxoproline methyl ester (**8**) (1.2 g, 4.3 mmol), ethanediol (8.4 mL, 150 mM), and a catalytical amount of p-toluenesulfonic acid (0.1 g) was heated under reflux with the exclusion of moisture in dry benzene (72 mL) with stirring for 72 h. The reaction mixture was cooled and washed with 1 N hydrochloric acid (3 × 30 mL), 1 M sodium bicarbonate (1 × 30 mL), and saturated sodium chloride solutions (3 × 30 mL), dried over magnesium sulfate, and evaporated to dryness in vacuo to yield an oil which was a two-component mixture, the major spot [R_f 0.65, TLC (silica gel) benzene–ethyl acetate, 2:1] and the minor spot (R_f 0.94, same system as above). Column chromatography of the mixture on silica gel (3 × 50 cm) with this system yielded compound **9** in the second fraction as an oil (R_f 0.65, same system as above) which was crystallized from ether–petroleum ether (bp 40–60 °C): 1.11 g (80%). This N-(benzyloxycarbonyl)-4-(ethylenedioxy)proline methyl ester (**9**) (0.41 g, 1.2 mmol) was next deprotected by hydrogenation over 5% palladium on charcoal (0.1 g) in methanol (10 mL) at room temperature and pressure for 3 h to yield the required 4-(ethylenedioxy)proline methyl ester as an oil which gave a positive reaction with ninhydrin [R_f 0.26,

TLC (silica gel) ethyl acetate]: 0.23 g (95%); IR (film) 3350 (NH), 1745 cm^{-1} (ester C=O); ^1H NMR ($CDCl_3$) δ 3.95–4.30 (1 H, s, 2-CH), 3.84 (4 H, s, OCH_2CH_2O), 3.70 (3 H, s, OCH_3), 2.75–3.20 (2 H, m, $5CH_2$), 2.45 (1 H, br s, NH), 2.07–2.55 (2 H, m, 3-CH_2); M^+ (high resolution) 187.08429 ($C_8H_{13}NO_4$ requires 187.08445, 1 ppm error).

(b) Synthesis of (2-Nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine (10). (2-Nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine *p*-nitrobenzyl ester (**4**) (0.63 g, 1 mmol) was dissolved in acetone (5 mL), 1 N sodium hydroxide (1.1 mL) added, and the mixture stirred for 1 h at 40 °C. The reaction mixture was diluted with water (50 mL) and washed with ethyl acetate (2 × 30 mL). The aqueous phase was acidified with 5 N hydrochloric acid and the resulting oil extracted with ethyl acetate (3 × 30 mL). The combined ethyl acetate extracts were washed with water (3 × 30 mL), dried over magnesium sulfate, and evaporated to dryness in vacuo to yield (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine (**10**) as an oil which was crystallized from nitromethane: 0.42 g (85%); mp 133–135 °C; IR (Nujol) 3290–3250 (OH, NH), 1725 (acid C=O), 1660–1635 (amide C=O), 1545–1530 (amide C=O, NO_2), 750 and 700 cm^{-1} (substituted benzene); UV λ_{max} (CH_3OH) 215 nm (log ϵ 4.345).

(c) Synthesis of the Actinocylbis(tripeptide) 1i. A solution of (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valine (**10**) (0.49 g, 1 mmol), 4-(ethylenedioxy)proline methyl ester (0.21 g, 13 mmol), triethylamine (0.2 mL), and *N,N'*-dicyclohexylcarbodiimide (0.22 g, 1.07 mmol) in dichloromethane (5 mL) was stirred for 4 h at 0 °C and 72 h at room temperature with the exclusion of moisture and light. The mixture was filtered to remove the precipitate of dicyclohexylurea. Acetone (0.5 mL) was added to the filtrate which was cooled and refiltered. The filtrate was taken up into ethyl acetate (30 mL) and washed with 5% citric acid (3 × 30 mL), 1 M sodium bicarbonate (3 × 30 mL), and saturated sodium chloride (3 × 30 mL) solutions. The organic phase was dried with magnesium sulfate and evaporated to dryness in vacuo to yield an oil which contained three components [R_f 0.77, 0.64, and 0.47, respectively, TLC (silica gel) acetone–chloroform, 3:7]. The major spot (R_f 0.47, same system as above) was isolated by preparative thin-layer chromatography (silica gel) with the same solvent to yield (2-nitro-3-benzyloxy-4-methylbenzoyl)threonyl-D-valyl-4-(ethylenedioxy)proline methyl ester, which was crystallized from ethyl acetate–petroleum ether (bp 40–60 °C): 0.28 g (42%); mp 69–72 °C; IR (Nujol) 3420–3290 (NH, OH), 1750 (ester C=O), 1660–1630 (amide C=O), 1550–1540 (amide C=O, NO_2), 760 cm^{-1} (substituted benzene); UV λ_{max} (CH_3OH) 209 nm (log ϵ 4.759); ^1H NMR ($CDCl_3$) δ 6.88–7.48 (9 H, m, C_6H_5, 6-CH, 5-CH, Thr NH, and D-Val NH), 4.98 (2 H, s, benzylic CH_2), 4.10–4.75 (5 H, m, Thr 1'-CH, 2'-CH, and OH, D-Val 1'-CH, and Pro 2'-CH), 3.92 (4 H, s, $-OCH_2CH_2O$), 3.65–3.70 (4 H, s, OCH_3, Pro 5'-CH_2), 2.32 (3 H, s, 4'-CCH_3), 1.85–2.23 (3 H, m, Pro 3'-CH_2, D-Val 2'-CH), 0.88–1.25 [9 H, m, Thr 2'-CCH_3, D-Val $C(CH_3)_2$]. Anal. Calcd for $C_{32}H_{40}N_4O_{11}$: C, 58.5; H, 6.1; N, 8.5. Found: C, 58.5; H, 6.1; N, 8.4.

This "monomer" intermediate was reduced and oxidatively coupled as usual and the product deprotected by dissolving in methanol (10 mL) and 8% v/v H_2SO_4 (4 mL). After stirring for 2 h at 40 °C, water was added to the solution and the product extracted with ethyl acetate (3 × 30 mL). The combined ethyl acetate extract was washed with 1 N hydrochloric acid (2 × 20 mL), 1 M sodium bicarbonate, and saturated sodium chloride solutions (1 × 20 mL), respectively, dried over magnesium sulfate, and evaporated to dryness in vacuo to give compound **1i** [R_f 0.50, TLC (silica gel) acetone–chloroform, 3:7] which was separated from a minor component (R_f 0.40, same system as above) by preparative TLC with the same solvent. Compound **1i** was crystallized from ethyl acetate–petroleum ether (bp 40–60 °C): 18 mg (66%); IR (KBr) 3420–3310 (NH_2, NH, OH), 1750–1740 (ester C=O, keto C=O), 1670–1630 (amide C=O), 1565 cm^{-1} (phenoxazone); UV λ_{max} (CH_3OH) 240 nm (log ϵ 4.578), 443 (4.410). Anal. Calcd for $C_{46}H_{58}N_8O_{16}$: C, 56.4; H, 6.0; N, 11.5. Found: C, 57.2; H, 6.0; N, 11.1.

Antibacterial Assays. Antibacterial activity was assessed by the cup–plate diffusion assay (6-mm stainless steel cylinders) against *B. subtilis* (ATCC 6633) on nutrient agar at 37 °C.

DNA Binding. All solutions were prepared in pH 7.00 1 M NaCl–0.008 M Tris buffer. A stock solution of calf thymus DNA (Sigma type 1) was prepared (1.2 mg/mL) and assayed using the figure $\epsilon\,(P)_{260} = 6600$. Actinomycin D solutions were assayed using the figure $\epsilon_{440} = 24\,800$. All the analogues obeyed Beer–Lambert's law at the concentrations used.

(a) Determination of Spectral Shifts. Six solutions of each drug (about 7.0×10^{-6} M) were prepared to contain 0, 1.875, 2.75, 5.5, 11.0, and 22.0×10^{-5} M DNA, respectively. The spectral shifts were determined after allowing 30 min for equilibration.

(b) Ultracentrifugation. Six solutions of each drug (about 5×10^{-6} M) were prepared to contain 0, 1.94, 2.27, 3.35, and 9.45×10^{-5} M DNA, respectively. After equilibration for 30 min each solution was centrifuged at 150 000g for 6 h at 25 °C. The concentrations of unbound drug were determined from the supernatants. The concentrations of bound drug were obtained by dissolving the DNA pellet in 50% DMF and reading absorbance at 443 nm compared to the drug in 50% DMF. This was repeated varying the drug/DNA ratio.

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