Synthesis and Properties of Some Peptide Analogues of Actinomycin D

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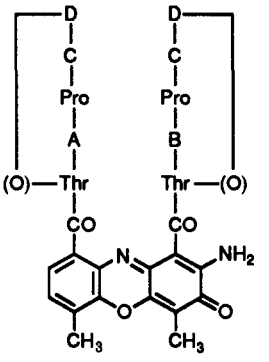
Analogues of actinomycin D (AMD) were synthesized in which amino acid replacements were made at various sites in the peptide moieties. These include (i) replacement of both *N*-methylvalines by *N*-methylleucine, (ii) replacement of both sarcosines by *N*-[2-(methoxycarbonyl)ethyl]glycine, and (iii) replacement of one or both D-valines by D-threonine. The purpose of replacements ii and iii was to ascertain the effect upon biological activity of introducing a new side chain which could be functionalized to allow the attachment of carrier molecules such as antibodies. NMR data indicated that none of the analogues had solution conformations significantly different from that of AMD. Difference spectra with DNA revealed that replacement i enhanced binding while the other analogues bound less strongly to DNA. All the analogues had lower antimicrobial activities than AMD. In contrast, 5,5′-(MeLeu)$_2$AMD displayed in vitro antitumor activity comparable with that of AMD at approximately 100-fold lower concentrations.

Analogues of the antitumor agent actinomycin D (AMD) have been produced by directed biosynthesis, partial synthesis, and total synthesis.[1-3] Relatively few synthetic analogues varying in the peptide moieties have been reported, because chromophoric analogues have been more readily prepared from AMD itself. The present study involves total synthesis of several new analogues varying at three different sites in the peptide units.

One aspect of structure–activity relationships in the actinomycin series involves the role of the C-terminal amino acid residues, which are *N*-methylvalines in AMD. In a previous study[4] it was shown that their replacement by *N*-methylalanine produces a substantial reduction in antimicrobial activity, and a loss of antitumor activity. This was unexpected in view of the potent biological activity[5] of the actinomycins of the Z complex, which contain C-terminal *N*-methylalanine in one of the two peptide units.[6] In the present study the effect of lengthening, instead of shortening, the side chains at this site is investigated by incorporating *N*-methylleucine.

Another aspect considered here is the effect of incorporating into an amino acid side chain a functional group which could be used to link a carrier molecule such as a tumor-specific antibody. Such a drug–antibody conjugate would be a candidate for targeting studies provided the analogue had antitumor potency comparable with that of AMD. Examination of models of the AMD–DNA complex[7] suggests that the side chains of D-valine present an area of the molecular periphery where modification would be expected to produce minimal disturbance of the associative contact within the DNA narrow groove. In the present study analogues of AMD having one or both D-valines replaced by D-threonine are described. The threonyl hydroxyl group could be used for ester linkage in various ways. Since the bulk and shape of the side chain are not markedly altered by this isosteric modification, the effect on the peptide conformation may be minimized. Synthetic analogues in which the D-valines were replaced by D-alanine or D-leucine were biologically inactive,[8] probably because of a change in conformation.

Chart I. Structures of Analogues (1–4) of Actinomycin D



	A	B	C	D
AMD	D-Val	D-Val	Sar	MeVal
1	D-Val	D-Val	Sar	MeLeu
2	D-Val	D-Val	Meg	MeVal
3	D-Thr	D-Thr	Sar	MeVal
4	D-Thr	D-Val	Sar	MeVal
	D-Val	D-Thr	Sar	MeVal

Another part of the molecular periphery of AMD which might be altered without drastic effect on the interaction with DNA is the *N*-methyl group of sarcosine. While its removal eliminates antimicrobial activity and the capacity to bind to DNA,[9] the effect of replacing it by a bulkier group has not been studied. Replacement of this *N*-CH_3 by *N*-CH_2CH_2COOH would provide an analogue which could be coupled to other molecules. As a test of the effect of this type of modification upon biological activity, an analogue is described here in which both sarcosyl *N*-methyls are replaced by *N*-$CH_2CH_2COOCH_3$. The amino acid replacing sarcosine, *N*-[2-(methoxycarbonyl)ethyl]-glycine, is given the abbreviation "Meg".

The structures of these AMD analogues are summarized in Chart I. They were compared with AMD with respect to (i) conformation (NMR), (ii) binding to DNA (difference

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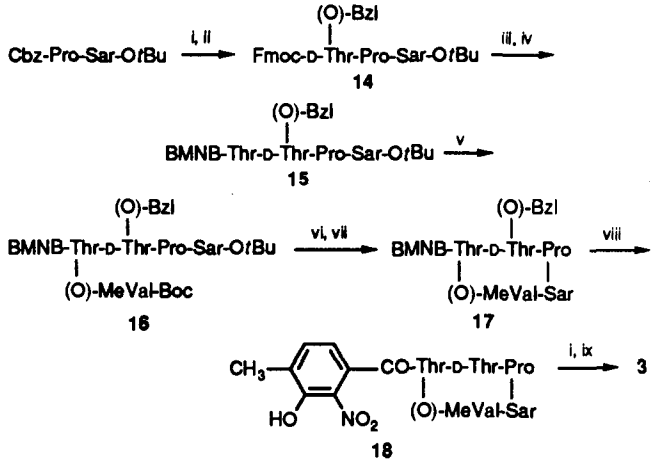
Scheme I.[a] Synthesis of AMD Analogue 3 Containing D-Thr in Place of D-Val



[a] BMNB = 3-(benzyloxy)-4-methyl-2-nitrobenzoyl. Reagents: (i) H_2/Pd/C, (ii) Fmoc-D-Thr(OBzl)-OH/DCC, (iii) $HNEt_2$, (iv) BMNB-Thr-OH/DCC/HONB, (v) Boc-MeVal-OH/DCC/DMAP, (vi) HCl/dioxane, (vii) BOP-Cl, (viii) TFMSA, (ix) $K_3FE(CN)_6$.

Table I. Chemical Shifts (δ) and $J_{NH,C\alpha H}$ Values[a] (Hz) for Selected Protons in AMD and Analogues 1–4

proton[b]	AMD	1	2	3	4
NH	8.12 (5.7)	8.15 (5.0)	8.14 (5.5)	7.99 (6.5)	8.40, 8.20
	7.98 (5.9)	7.99 (5.8)	7.98 (5.9)	7.99 (6.5)	8.20, 7.94
	7.80 (6.6)	7.75 (6.2)	7.81 (6.6)	7.89 (6.3)	7.66, 7.62
	7.17 (6.8)	7.16 (6.4)	7.20 (6.9)	7.32 (6.5)	7.23, 7.18
8-H	7.65	7.64	7.68	7.67	7.72
7-H	7.38	7.37	7.38	7.39	7.37
Pro α-H	6.03	6.04	6.00	5.79	5.95
	5.98	5.97	5.92	5.79	5.85
N-CH_3	2.91	2.95	2.96	2.94	2.94
	2.90	2.93	2.93	2.88	2.91
	2.87	2.86	3.69[c]	2.86	2.87
	2.87	2.86	3.69[c]	2.86	2.85
6-CH_3	2.55	2.55	2.58	2.57	2.55
4-CH_3	2.24	2.24	2.27	2.24	2.24
Thr CH_3	1.26[d]	1.28[d]	obsc[e]	1.18–1.26[f]	1.10[d]

[a] In parentheses. [b] Numerals 4, 6, 7, 8 refer to positions on the phenoxazinone chromophore. [c] OCH_3 of Meg. [d] The two Thr CH_3 signals overlapped. [e] Obscured by Meg CH_2. [f] Four overlapping Thr CH_3 signals.

spectra), (iii) antimicrobial activity, and (iv) in the case of the most potent analogue based on the above criteria, 5,5′-(MeLeu)$_2$AMD (1), activity against various tumor cell lines in culture.

Synthesis

The AMD analogue in which MeLeu replaces MeVal (1) was synthesized by the same route as that described for AMD.[4] In the cyclization step (Pro-Sar) using the BOP-Cl reagent[10] a 57% yield was obtained. For the analogue with Meg replacing Sar (2) glycine *tert*-butyl ester was reacted with methyl acrylate and the resulting H-Meg-O*t*Bu used in place of H-Sar-O*t*Bu for an analogous synthesis. For the replacement of D-Val by D-Thr (3), the route was adapted as shown in Scheme I; the cyclization yield was 65%. The isomeric mixture of actinomycins having one or other of the two D-Val residues of AMD replaced by D-Thr (4) was obtained by a mixed oxidation of the two 3-hydroxy-4-methylanthraniloyl pentapeptide lactones. The resulting mixture was separated by silica gel column chromatography into three fractions: AMD, 4, and 3, but separation of 4 into its isomers was not achieved. The actinomycin analogues were characterized by Cf-252 plasma desorption mass spectrometry and proton NMR.

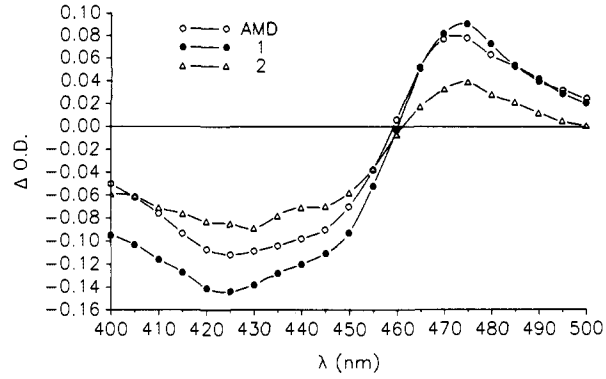


Figure 1. Difference spectra with calf thymus DNA: comparison of 1 and 2 with AMD.

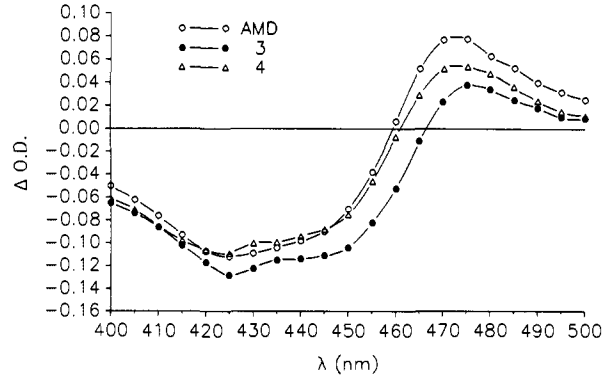


Figure 2. Difference spectra with calf thymus DNA: comparison of 3 and 4 with AMD.

Conformation

Chemical shifts and NH,CαH coupling constants obtained from proton NMR spectra of AMD analogues 1–4 are compared with those for AMD in Table I. Upon the basis of these data and the conformational sensitivity of N-methyl and prolyl α-proton chemical shifts in such cyclopeptides,[4,11] no conformational change resulting from the amino acid replacements represented by these analogues is apparent.

NMR spectra were also obtained from the various pentapeptide lactone synthetic intermediates. Previous studies on the AMD-related peptide lactone and its 5-MeAla analogue revealed solvent-dependent conformational duality.[4,11,12] In chloroform solution the 3-benzyloxy compounds exist as two conformers, designated A and C, while the corresponding 3-hydroxy compounds adopt only the C conformation in which all the peptide bonds are trans. In acetone solution both sets of compounds prefer the A conformation, which contains two cis peptide bonds (Val-Pro and Pro-Sar) and approximates that present in AMD. The analogous peptide lactones reported here produced similar NMR results apart from minor anomalies for 17 and 18 in which D-Val is replaced by D-Thr. Chemical shift data for the N-CH_3 and ArCH_3 singlets consistent with those reported for the corresponding AMD-related compounds,[4] are shown in Table II.

Binding to DNA

Difference spectra of the AMD analogues 1–4 with calf thymus DNA were compared with that for AMD in Figures

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Table II. Chemical Shifts (δ) of Methyl Singlets in Proton NMR Spectral Data of Peptide Lactone Intermediates

		NCH_3				$ArCH_3$	
		confmr C		confmr A			
compd	solvent	Sar	MeVal	Sar	MeVal	confmr C	confmr A
VI	$CDCl_3$	3.40	3.19	2.90	3.29	2.38	2.31
VII	$CDCl_3$	3.37	3.19			2.34	
XII	$CDCl_3$	3.66[a]	3.20	3.68[a]	3.29	2.39	2.30
	CD_3COCD_3			3.61[a]	3.32		2.42
XIII	$CDCl_3$	3.64[a]	3.17			2.34	
XVII	$CDCl_3$	3.41	3.19			2.34	
	CD_3COCD_3	3.40	3.16	2.85	3.29	2.42	2.44
XVIII	$CDCl_3$	3.35	3.19	2.88	3.13	2.30	2.32
	CD_3COCD_3			2.85	3.23		2.34

[a] OCH_3 of Meg.

Table III. Minimal Inhibitory Concentrations (μg/mL)

actinomycin	*S. aureus*	*B. subtilis*
AMD	0.25	0.09
1	0.5	0.2
2	2.5	2.0
3	2.5	2.0
4	0.6	0.4

1 and 2. Studies with other AMD analogues indicated that this measure of affinity to DNA correlates roughly with biological potency.[13,14] Upon the basis of the present results, 2–4 would be expected to be less potent than AMD whereas 1 might be more potent. Replacement of both D-Val residues by D-Thr was more deactivating than replacement of one of them, an observation which parallels those made with analogues where proline is replaced by pipecolic acid[13] and 4-methylproline.[14]

Antimicrobial Activities

Minimum inhibitory concentrations of 1–4 against *Staphylococcus aureus* and *Bacillus subtilis* are compared with those of AMD in Table III. These results correlate with the DNA binding proclivities except in the case of 1, which has about $^1/_2$ the antimicrobial activity of AMD.

Antitumor Activities

The most potent analogue, 1, was evaluated in the National Cancer Institute's in vitro disease-oriented primary antitumor screen, consisting of 60 tumor cell lines in culture.[15] Comparison with AMD showed that 1 is on average approximately 100-fold more potent. Response parameters GI_{50}, TGI, and LC_{50} are interpolated values representing the drug concentrations at which percentage cell growth is +50, 0, and −50% (respectively) of the control values. Mean values of these parameters were computed for the entire set of 60 tumor cell lines. For AMD these mean concentrations, expressed as log molar, were −8.67, −7.10, and −6.11, respectively. The corresponding concentrations for 1 were −11.28, −9.38, and −8.40, about 100-fold lower than for AMD. Subpanel specificities for the two compounds were not significantly dissimilar; the melanoma subpanel was particularly sensitive. Detailed data are available as supplementary material.

In an earlier phase of the study, 2 was tested against the B16 melanocarcinoma in mice. It had marginal activity, with T/C (median survival time) of 129 with three injections (ip) of 16 mg/kg, compared with a T/C of 165 at 0.256 mg/kg for AMD.[16] The other analogues were not tested in vivo.

Conclusions

Several peptide analogues of AMD have been synthesized and compared with AMD with respect to various parameters. Difference spectra with DNA suggested that, with the exception of 1, all the analogues would have lower biological potencies than AMD. This was confirmed by the observed antimicrobial activities. The replacement of one or both D-valines by D-threonine or the attachment of a bulkier group in place of the *N*-methyls of sarcosine was accomplished without observable change in peptide conformation, but biological activity was reduced nonetheless. Similar results were obtained with a series of analogues produced by directed biosynthesis,[17] in which one or both proline residues were replaced by other cyclic amino acids.[18]

The 5,5′-(MeLeu)$_2$ analogue 1 appeared to bind to DNA more strongly than AMD, possibly because of more effective hydrophobic interactions by the longer side chains of *N*-methylleucine. While it had only $^1/_2$ the antimicrobial activity of AMD, it displayed approximately 100 times the in vitro antitumor potency of AMD. This represents the only known example of a peptide analogue of actinomycin with more antitumor potency than AMD itself. The dramatic discrepancy between toxicity to bacteria and to tumor cells may reflect differences in uptake.

Experimental Section

All reactions where the temperature is not specified were performed at room temperature. Elementary analyses were provided by Galbraith Laboratories, Inc., Knoxville, TN.

Medium pressure "flash" chromatography[19] used columns containing silica gel (40 μm average particle diameter) from J.T. Baker Chemical Co. Purified products gave a single spot on TLC in an appropriate solvent using E. Merck silica gel GF Uniplates from Analtech, Inc.

Chemical ionization mass spectrometry was performed on a Finnigan 1015 mass spectrometer with CH_4 or NH_3 as specified. Cf-252 plasma desorption mass spectra[20] were obtained on an instrument at the National Institutes of Health constructed by Dr. R. MacFarlane (Texas A & M University).

Proton NMR spectra were obtained on a Varian HR220 in the CW mode and chemical shifts were measured in ppm from internal Me_4Si.

UV and visible absorption spectra were obtained on a Beckman DB spectrophotometer. Difference spectra[13] with calf thymus DNA were obtained by subtracting spectra of the actinomycin

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(0.02 μmol/mL) in 0.01 M phosphate buffer (pH 7.0) from those obtained with the same actinomycin in a solution of DNA (38 μg/mL) in the same buffer.

Melting points were obtained on a Thomas-Hoover capillary apparatus and are uncorrected.

For antimicrobial activities of actinomycins, *S. aureus* or *B. subtilis* was inoculated into 50 mL of trypticase soy broth (TSB, BRL, Baltimore, MD) and incubated overnight at 37 °C. Each culture (0.1 mL) was then inoculated into fresh TSB medium (50 mL) and reincubated for 8 h at which time 0.1 mL of the rapidly growing culture was mixed with an additional 50 mL of TSB medium. The diluted cultures (0.1 mL) were employed for determination of the MIC's in 2 mL of TSB medium.

Antitumor activities were evaluated at the National Cancer Institute, Bethesda, MD. The in vitro primary drug screen[15] utilizes 60 human tumor cell lines, derived from seven cancer types (lung, colon, melanoma, renal, ovarian, brain, and leukemia). Cell growth is assayed by a spectrophotometric method and compared with controls at several concentrations of drug.

Abbreviations used are BOP-Cl, *N,N*-bis(2-oxo-3-oxazolidinyl)phosphorodiamidic chloride; CIMS, chemical ionization mass spectrometry; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; HONB, *N*-hydroxy-5-norbornene-2,3-dicarboximide; MeLeu, *N*-methylleucine; MeVal, *N*-methylvaline; PDMS, Cf-252 plasma desorption mass spectrometry; TFMSA, trifluoromethanesulfonic acid.

N-(3-BzlO-4-Me-2-nitrobenzoyl)-O-(Boc-MeLeu)-Thr-D-Val-Pro-Sar-OtBu (5). A solution of *N*-(3-BzlO-4-Me-2-nitrobenzoyl)-Thr-D-Val-Pro-Sar-O*t*Bu[4] (427 mg, 0.600 mmol) and Boc-MeLeu-OH (221 mg, 0.901 mmol) in DCM (5 mL) was stirred during addition of DCC (185 mg, 0.897 mmol) and DMAP (50 mg) in DCM (2 mL). After stirring at 0 °C for 16 h the mixture was filtered and evaporated. Flash chromatography with EtOAc/$CHCl_3$ (3:2) afforded **5** as a gum, yield 415 mg (74%). PDMS: ions at m/z 940.3 $(M + H)^+$ and 961.7 $(M + Na)^+$ indicate M = 939 ($C_{48}H_{70}N_6O_{13}$ requires M = 939.09). NMR ($CDCl_3$): δ 5.07 (s, $ArCH_2$), 3.20 (s, NCH_3), 2.79 (s, NCH_3), 1.53 (s, O*t*Bu), and 1.45 (s, O*t*Bu).

(3-BzlO-4-Me-2-nitrobenzoyl)-Thr-D-Val-Pro-Sar-MeLeu Lactone (6). A solution of **5** (717 mg, 0.764 mmol) in 4 N HCl/dioxane (12 mL) was kept for 2 h, then evaporated in vacuo. The residue was dissolved in H_2O (15 mL) and adjusted to pH 7.0 with 1 N NaOH. After evaporation the vacuum-dried residue was dissolved in DCM (180 mL) and DIPEA (0.13 mL) was added, followed by a solution of BOP-Cl (240 mg, 0.943 mmol) in DCM (40 mL). After 7 days the solution was filtered through Celite and evaporated. Flash chromatography with EtOAc/EtOH (12:1) afforded **6** as an amorphous solid, yield 331 mg (57%). CIMS (CH_4): ion at m/z 765 (M + 1, relative intensity 0.025) indicates M = 764 ($C_{39}H_{52}N_6O_{10}$ requires M = 764.88). NMR: see Table II. In addition ($CDCl_3$, C conformer): δ 9.69 (d, Thr NH), 7.39 (s, ArH), 6.53 (d, Val NH), 5.76 (q, Thr β-H), 5.25 (t, MeLeu α-H), 5.02 (AB, $ArCH_2$), 4.92, (d, Thr α-H), 4.85 (d, Pro α-H), 4.75 (d, Sar α-H), 4.41 (dd, Val α-H), 3.03 (d, Sar α-H), 1.27 (d, Thr CH_3), and 1.23 (d, Thr CH_3).

(3-OH-4-Me-2-nitrobenzoyl)-Thr-D-Val-Pro-Sar-MeLeu Lactone (7). To a solution of **6** (325 mg, 0.425 mmol) in DCM (11 mL) was added TFMSA (0.55 mL, 6.2 mmol) and the solution was stirred for 10 min with exclusion of moisture. After dilution with EtOAc (35 mL) the solution was washed with aqueous NaCl, dried (Na_2SO_4), and evaporated. Flash chromatography with $CHCl_3$/MeOH (11:1) afforded **7** as a yellow solid, yield 258 mg (90%), which crystallized from EtOAc as large colorless prisms which collapsed in air or vacuum and had no definite mp. CIMS (CH_4): ions at m/z 675 (M + 1, relative intensity 1.00) and 703 (M + 29, relative intensity 0.055) indicated M = 674 ($C_{32}H_{46}N_6O_{10}$ requires M = 674.76). NMR: see Table II. In addition ($CDCl_3$, C conformer): δ 9.55 (d, Thr NH), 7.48 (d, ArH), 6.92 (d, ArH), 6.82 (d, Val NH), 5.79 (q, Thr β-H), 5.17 (t, MeLeu α-H), 4.95 (d, Thr α-H), 4.86 (d, Pro α-H), 4.70 (d, Sar α-H), 4.41 (dd, Val α-H), 3.00 (d, Sar α-H), and 1.33 (d, Thr CH_3).

5,5'-(MeLeu)$_2$-actinomycin D (1). A solution of **7** (170 mg, 0.252 mmol) in MeOH (15 mL) was hydrogenated over 10% Pd/C for 2 h, then filtered through Celite and diluted to 35 mL with MeOH. This solution was added to a stirred solution of $K_3Fe(CN)_6$ (215 mg) in 0.067 M phosphate buffer (pH 7.12, 35 mL). After 10 min the solution was diluted with aqueous NaCl and extracted three times with EtOAc. The extracts were washed with aqueous NaCl, dried (Na_2SO_4), and evaporated. Flash chromatography with EtOAc/EtOH (30:1) afforded **1**, which crystallized from EtOH as red prisms. Mp: 242–244 °C dec. Yield: 130 mg (80%). PDMS: ions at m/z 1284.2 $(M + H)^+$ and 1306.7 $(M + Na)^+$ indicated M = 1283 ($C_{64}H_{90}N_{12}O_{16}$ requires M = 1283.45). NMR ($CDCl_3$): see Table I.

1-[[(*tert*-Butoxycarbonyl)methyl]amino]-2-(methoxycarbonyl)ethane (H-Meg-OtBu) (8). A mixture of glycine *tert*-butyl ester (2.40 g, 18.3 mmol) and methyl acrylate (0.92 g, 11 mmol) in MeOH (20 mL) was kept for 48 h, then evaporated. Distillation of the residue at 0.10 Torr gave **8** as a colorless oil: Bp 83–85 °C. Yield, 2.18 g (91%). NMR ($CDCl_3$): δ 3.68 (s, OCH_3), 3.29 (s, NCH_2CO), 2.88 (t, J = 6.6 Hz, NCH_2), 2.50 (t, J = 6.6 Hz, CH_2CO), and 1.46 (s, O*t*Bu). Picrolonate, yellow needles from EtOH. Mp: 179–180 °C. Anal. ($C_{20}H_{27}N_5O_9$) C, H, N.

Cbz-D-Val-Pro-Meg-OtBu (9). A solution of Cbz-D-Val-Pro-OH[21] (3.50 g, 10.0 mmol) and **8** (1.74 g, 8.00 mmol) in DCM (30 mL) was stirred at 0 °C during addition of DCC (3.50 g, 17.0 mmol). The solution was kept at 22 °C for 16 h, then filtered and evaporated. Flash chromatography with $CHCl_3$/EtOAc (1:1) afforded **9** as a gum, yield 4.02 g (92%). CIMS (NH_3): ions at m/z 548 (M + 1, relative intensity 1.00) and 565 (M + 18, relative intensity 0.026) indicated M = 547 ($C_{28}H_{41}N_3O_8$ requires M = 547.63). NMR ($CDCl_3$): δ 7.32 (s, ArH), 3.68 (s, OCH_3), 1.47 (s, O*t*Bu), 0.98 (d, Val CH_3), and 0.90 (d, Val CH_3).

(3-BzlO-4-Me-2-nitrobenzoyl)-Thr-D-Val-Pro-Meg-OtBu (10). To a solution of **9** (2.51 g, 4.58 mmol) in EtOH (40 mL) was added 1,4-cyclohexadiene (5 mL) and 10% Pd/C and the mixture was stirred under N_2 for 18 h, then filtered through Celite and evaporated in vacuo. The residue in DMF (10 mL) was mixed with a solution of (3-BzlO-4-Me-2-nitrobenzoyl)-Thr-OH[21] (2.00 g, 5.15 mmol) in DMF (10 mL), and HONB (1.50 g) was added. The solution was stirred at 0 °C during addition of DCC (1.50 g, 7.28 mmol) and kept at 4 °C for 48 h. After filtration and evaporation in vacuo, flash chromatography with EtOAc/EtOH (40:1) afforded **10** as an amorphous solid, yield 2.75 g (76%). CIMS (NH_3): ions at m/z 784 (M + 1, relative intensity 1.00) and 801 (M + 18, relative intensity 0.47) indicated M = 783 ($C_{39}H_{53}N_5O_{12}$ requires M = 783.85). NMR ($CDCl_3$): δ 7.36 (s, ArH), 5.00 (AB q, $ArCH_2$), 3.67 (s, OCH_3), 2.38 (s, $ArCH_3$), 1.42 (s, O*t*Bu), 1.26 (d, Thr CH_3), 1.02 (d, Val CH_3), and 0.94 (d, Val CH_3).

N-(3-BzlO-4-Me-2-nitrobenzoyl)-O-(Boc-MeVal)-Thr-D-Val-Pro-Meg-OtBu (11). A solution of **10** (520 mg, 0.663 mmol) and Boc-MeVal-OH (230 mg, 1.00 mmol) in DCM (3 mL) was stirred during addition of DMAP (50 mg) followed by DCC (200 mg, 0.971 mmol) in DCM (2 mL). After stirring for 16 h the mixture was filtered and evaporated. Flash chromatography with $CHCl_3$/EtOAc (2:3) afforded **11** as a gum, yield 620 mg (94%). PDMS: ion at 1020.0 $(M + Na)^+$ indicated M = 997 ($C_{50}H_{72}N_6O_{15}$ requires M = 997.15). NMR ($CDCl_3$): δ 7.36 (s, ArH), 4.97 (AB q, CH_2), 2.81 (s, NCH_3), 2.36 (s, $ArCH_3$), 1.41 (s, O*t*Bu), and 0.93 (Val and MeVal C-CH_3).

(3-BzlO-4-Me-2-nitrobenzoyl)-Thr-D-Val-Pro-Meg-MeVal Lactone (12). A solution of **11** (577 mg, 0.579 mmol) in 4 N HCl/dioxane (9 mL) was kept for 2 h, then evaporated in vacuo. The residue was dissolved in H_2O (10 mL) and adjusted to pH 7.0 with 1 N NaOH. After evaporation the vacuum-dried residue was dissolved in DCM (200 mL) and DIPEA (0.1 mL) was added followed by a solution of BOP-Cl (177 mg, 0.695 mmol) in DCM (50 mL). After 3 days further DIPEA (50 μL) and BOP-Cl (90 mg) were added. After 3 more days the solution was filtered through Celite and evaporated. Flash chromatography with EtOAc/EtOH (20:1) afforded **12** as an amorphous solid, yield 185 mg (39%). NMR: see Table II. In addition: (C conformer, $CDCl_3$) δ 9.52 (d, Thr NH), 7.40 (s, ArH), 6.49 (d, Val NH), 5.80 (q, Thr β-H), 5.01 (AB, $ArCH_2$), and 1.28 (d, Thr CH_3); (A conformer, CD_3COCD_3) 8.15 (d, Val NH), 6.40 (d, Pro α-H), 5.29 (q,

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Thr β-H), 4.99 (s, $ArCH_2$), 3.61 (s, OCH_3), 3.32 (s, NCH_3), 2.42 (s, $ArCH_3$), and 1.18 (d, Thr CH_3).

(3-OH-4-Me-2-nitrobenzoyl)-Thr-D-Val-Pro-Meg-MeVal Lactone (13). To a solution of **12** (146 mg, 0.177 mmol) in DCM (3 mL) was added TFMSA (0.3 mL, 3.4 mmol) and the mixture was stirred for 10 min with exclusion of moisture. After dilution with EtOAc (25 mL) and washing with aqueous NaCl the product was extracted into aqueous $NaHCO_3$. The aqueous extract was washed with EtOAc, acidifed with 2 N HCl, and extracted three times with EtOAC. These extracts were washed with H_2O, dried (Na_2SO_4), and evaporated to afford **13** as a yellow amorphous solid, yield 80 mg (62%). CIMS (CH_4): ions at m/z 733 (M + 1, relative intensity 0.087) indicated M = 732 ($C_{34}H_{48}N_6O_{12}$ requires M = 732.79). NMR: see Table II. in addition ($CDCl_3$, C conformer): δ 9.27 (d, Thr NH), 7.44 (d, ArH), 6.89 (d, ArH), 6.81 (d, Val NH), 5.82 (q, Thr β-H), and 1.33 (d, Thr CH_3).

4,4′-Meg$_2$-Actinomycin D (2). A solution of **13** (71 mg, 0.10 mmol) in MeOH (10 mL) was hydrogenated over 10% Pd/C for 4 h, then filtered through Celite and diluted to 13 mL with MeOH. This solution was added to a stirred solution of $K_3Fe(CN)_6$ (103 mg) in 0.067 M phosphate buffer (pH 7.12, 15 mL). After 10 min the solution was diluted with aqueous NaCl and extracted three times with EtOAc. The extracts were washed with aqueous NaCl, dried (Na_2SO_4), and evaporated. Chromatography on a column of acid-washed alumina[22] with $CHCl_3$ afforded **2** as a red amorphous solid, yield 43 mg (63%). PDMS: ions at 1400.6 $(M + H)^+$ and 1422.3 $(M + Na)^+$ indicated M = 1399 ($C_{68}H_{94}N_{12}O_{20}$ requires M = 1399.54). NMR: see Table I.

Fmoc-D-Thr(OBzl)-Pro-Sar-OtBu (14). Cbz-Pro-Sar-OtBu[23] (1.50 g, 3.98 mmol) in EtOH (25 mL) was hydrogenated over Pd/C for 16 h. After filtration and evaporation the residue was dissolved in a solution of Fmoc-D-Thr(OBzl)-OH (1.30 g, 3.01 mmol) in DCM (20 mL) and stirred at 0 °C during addition of DCC (1.50 g) in DCM (5 mL). After 18 h at 22 °C the solution was filtered and evaporated. Flash chromatography with $CHCl_3$/EtOAc (3:1) afforded **14** as an amorphous solid, yield 1.58 g (80%). CIMS (NH_3): ion at m/z 656 (M + 1, relative intensity 0.047) indicated M = 655 ($C_{38}H_{45}N_3O_7$ requires M = 655.76). NMR ($CDCl_3$): δ 7.27 (s, Bzl ArH), 2.90 (s, NCH_3), 1.44 (s, OtBu), and 1.19 (d, Thr CH_3).

(3-BzlO-4-Me-2-nitrobenzoyl)-Thr-D-Thr(OBzl)-Pro-Sar-OtBu (15). A solution of **14** (1.15 g, 1.76 mmol) in DCM (30 mL) was mixed with $HNEt_2$ (2.5 mL) and stirred for 5 h, then evaporated in vacuo. The residue was dissolved in a solution of (3-BzlO-4-Me-2-nitrobenzoyl)-Thr-OH[24] (1.50 g, 3.86 mmol) and HONB (750 mg) in DMF (25 mL) and the solution was stirred at 0 °C during addition of DCC (1.50 g). After 18 h at 22 °C the solution was filtered and evaporated. Flash chromatography with EtOAc/EtOH (40:1) afforded **15** as an amorphous solid, yield 975 mg (69%). PDMS: ion at m/z 827.0 $(M + Na)^+$ indicated M = 804 ($C_{42}H_{53}N_5O_{11}$ requires M = 803.88). NMR ($CDCl_3$): δ 4.92 (s, $ArCH_2$), 2.96 (s, NCH_3), 2.34 (s, $ArCH_3$), 1.43 (s, OtBu), 1.23 (d, Thr CH_3), and 1.18 (d, Thr CH_3).

***N*-(3-BzlO-4-Me-2-nitrobenzoyl)-*O*-(Boc-MeVal)Thr-D-Thr-(OBzl)-Pro-Sar-OtBu (16).** A solution of **15** (975 mg, 1.21 mmol) and Boc-MeVal-OH (500 mg, 2.16 mmol) in DCM (25 mL) was stirred at 0 °C during addition of DMAP (100 mg) followed by DCC (500 mg) in DCM. After stirring at 22 °C for 18 h the solution was filtered and evaporated. Flash chromatography with EtOAc/$CHCl_3$ (3:2) afforded **16** as an amorphous solid, yield 870 mg (71%). PDMS: ion at m/z 1040.0 $(M + Na)^+$ indicates M = 1017 ($C_{53}H_{72}N_6O_{14}$ requires M = 1017.16). NMR ($CDCl_3$): δ 3.13 (s, NCH_3), 2.79 (s, NCH_3), 2.37 (s, $ArCH_3$), 1.44 (s, OtBu), 1.42 (s, OtBu), 1.32 (d, Thr CH_3), and 1.18 (d, Thr CH_3).

(3-BzlO-4-Me-2-nitrobenzoyl)-Thr-D-Thr(OBzl)-Pro-Sar-MeVal Lactone (17). A solution of **16** (870 mg, 0.855 mmol) in 4 N HCl/dioxane (30 mL) was kept for 2 h, then evaporated in vacuo. The residue was dissolved in H_2O (20 mL) and adjusted to pH 7.0 with 1 N NaOH, then evaporated in vacuo. The residue was dissolved in DCM (500 mL), and DIPEA (0.4 mL) and BOP-Cl (500 mg, 1.96 mmol) were added with stirring. After 2 days, additional DIPEA (0.2 mL) and BOP-Cl (250 mg) were added. After 2 more days the solution was evaporated and flash chromatography with EtOAc/EtOH (12:1) afforded **17** as an amorphous solid, yield 465 mg (65%). PDMS: ions at m/z 844.1 $(M + H)^+$ and 865.6 $(M + Na)^+$ indicated M = 843 ($C_{44}H_{54}N_6O_{11}$ requires M = 842.94). NMR: see Table II. In addition ($CDCl_3$, C conformer): δ 9.64 (d, J = 9.1 Hz, Thr NH), 7.50 (d, ArH), 7.37 (d, ArH), 7.25 (s, Bzl ArH), 6.88 (d, J = 8.0 Hz, D-Thr NH), 5.83 (q, Thr β-H), 4.96 (s, Bzl CH_2), 1.27 (d, Thr CH_3), 1.23 (d, Thr CH_3), 0.91 (d, MeVal C-CH_3), and 0.77 (d, MeVal C-CH_3).

(3-OH-4-Me-2-nitrobenzoyl)-Thr-D-Thr-Pro-Sar-MeVal Lactone (18). To a stirred solution of **17** (421 mg, 0.500 mmol) in DCM (15 mL) was added TFMSA (0.7 mL). After 20 min, EtOAc (50 mL) was added and the solution was washed with aqueous NaCl, dried (Na_2SO_4), and evaporated. Flash chromatography with $CHCl_3$/MeOH (12:1) afforded **18** as a yellow amorphous solid, yield 265 mg (80%). CIMS (NH_3): ion at m/z 663 (M + 1, relative intensity 0.47) indicated M = 662 ($C_{30}H_{42}N_6O_{11}$ requires M = 662.69). NMR: see Table II. In addition ($CDCl_3$): δ 7.42 (d, ArH), 6.95 (d, ArH), 5.83 (q, Thr β-H), 1.35 (d, Thr CH_3), 1.29 (d, Thr CH_3), 0.90 (d, MeVal C-CH_3), and 0.77 (d, MeVal C-CH_3).

2,2′-D-Thr$_2$-actinomycin D (3). A solution of **18** (43 mg, 0.065 mmol) in MeOH (4 mL) was hydrogenated over Pd/C for 1 h. After filtration MeOH was added to 9 mL and this solution was added to a stirred solution of $K_3Fe(CN)_6$ (62 mg) in 0.067 M phosphate buffer (pH 7.12, 9 mL). After 15 min aqueous NaCl was added and the solution extracted (3×) with EtOAc. The extracts were washed with aqueous NaCl, dried (Na_2SO_4), and evaporated. Flash chromatography with $CHCl_3$/MeOH (15:1) afforded **3** as a red amorphous solid, yield 28 mg (68%). PDMS: ions at m/z 1260.9 $(M + H)^+$ and 1282.6 $(M + Na)^+$ indicated M = 1260 ($C_{60}H_{82}N_{12}O_{17}$ requires M = 1259.34). NMR: see Table I.

2-D-Thr-2′-D-Thr-actinomycin D (Isomeric Mixture) (4). A mixture of **18** (163 mg, 0.247 mmol) and (3-OH-4-Me-2-nitrobenzoyl)-Thr-D-Val-Pro-Sar-MeVal lactone[4] (163 mg, 0.247 mmol) in MeOH (30 mL) was hydrogenated over Pd/C for 1 h. After filtration MeOH was added to 60 mL and the solution was stirred during addition of a solution of $K_3Fe(CN)_6$ (475 mg) in 0.067M phosphate buffer (pH 7.12, 60 mL). After 15 min aqueous NaCl was added and the solution was extracted (3×) with EtOAc. The extracts were washed with aqueous NaCl, dried (Na_2SO_4), and evaporated. TLC on silica gel with $CHCl_3$/MeOH (12:1) gave three red spots and subsequent flash chromatography with $CHCl_3$/MeOH (19:1) gave three fractions: AMD (R_f 0.48, 74 mg), **4** (R_f 0.37, 96 mg), and **3** (R_f 0.30, 60 mg). PDMS of **4**: ions at m/z 1258.5 $(M + H)^+$ and 1280.1 $(M + Na)^+$ indicated M = 1257 ($C_{61}H_{84}N_{12}O_{17}$ requires M = 1257.37). NMR: see Table I.

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Registry No. **1**, 131545-48-7; **2**, 131545-49-8; **3**, 131545-50-1; **4** (isomer 1), 131566-44-4; **4** (isomer 2), 131545-51-2; **5**, 131545-64-7; **6**, 131545-65-8; **7**, 131566-45-5; **8**, 131545-52-3; **9**, 131545-53-4; **10**, 131545-54-5; **11**, 131545-55-6; **12**, 131545-56-7; **13**, 131545-57-8; **14**, 131545-58-9; **15**, 131545-59-0; **16**, 131545-60-3; **17**, 131545-61-4; **18**, 131545-62-5; BMNB-Thr-D-Val-Pro-Sar-OBu-*t*, 6041-34-5; Boc-MeLeu-OH, 53363-89-6; H-Gly-OBu-*t*, 6456-74-2; H_2C=CHCOOMe, 96-33-3; Cbz-D-Val-Pro-OH, 47450-18-0; BMNB-Thr-OH, 2441-62-5; BOC-MeVal-OH, 45170-31-8; Cbz-Pro-Sar-OBu-*t*, 5616-82-0; Cbz-Pro-Sar-OBu-*t*, 5616-82-0; Fmoc-D-Thr-(OBzl)-OH, 131545-63-6; (3-OH-4-Me-2-NO_2)C_6H_2CO-Thr-D-Val-Pro-Sar-MeVal lactone, 21148-64-1.

Supplementary Material Available: Dose–response curves and mean graphs for **1** and AMD from the in vitro antitumor screen performed by the National Cancer Institute (5 pages). Ordering information is given on any current masthead page.

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