^{13}C Nuclear Magnetic Resonance Study of Actinomycin D

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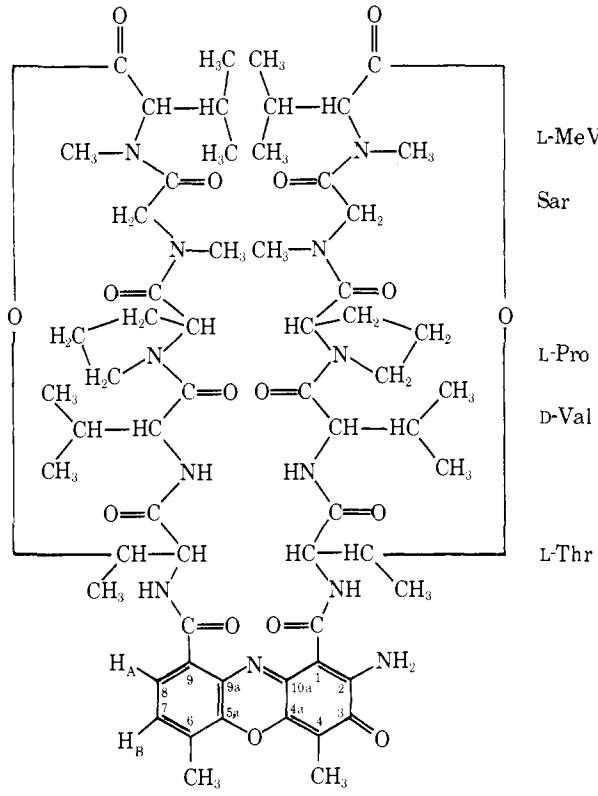
Abstract: Actinomycin D (**1**) has been investigated by ^{13}C spectroscopy. Signals were assigned by comparison with model substances 2-aminophenoxazone-3 (**2**), 2-amino-4,6-dimethyl-3-oxo-3*H*-phenoxazine-1,9-dicarboxylic acid (actinocin) (**3**), *p*-toluic acid (**4**), 2,2′-bisbenzoxazolinyl (**6**), 2,5-bis(dimethylamino)-1,4-benzoquinone (**7**), *p*-toluoylthreonine methyl ester (**5**), and other suitable amino acid derivatives. The signals fall into four groups: −180 to −166 ppm, chromophore and peptide carbonyls; −150 to −100 ppm, chromophore carbons; −75 to −17 ppm, amino acid C_α, C_β, C_γ, C_δ, and NCH_3; and −15 to −7 ppm, chromophore methyls. From relative shielding and deshielding effects several intramolecular interactions could be indicated. Because of the line width and intensities, the molecular framework is relatively rigid with free mobility of the methyls.

Actinomycin D (**1**) is an orange-red chromopeptide antibiotic from *Streptomyces antibioticus*.[1] The compound has



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aroused considerable interest over the last 30 years because of its highly effective antineoplastic action.[2] The exciting potentialities of actinomycin D have stimulated a great deal of research into its chemical and physical nature. One of the primary objectives of such studies has been the interpretation of the biological activity in terms of a plausible molecular structural model. Actinomycin D forms a complex with DNA whereby the planar chromophore unit intercalates between two successive base pairs and the two peptide rings interact with the adjacent nucleotides. The key to this interaction lies in the steric fit and thus in the conformation of the cyclic pentapeptides. Moreover, the effectiveness of the drug has been ascribed to a relatively small association and dissociation rate constant, in turn due to difficult conformational changes between the dissociated and the associated form of the cyclopentapeptides. The problem has been approached by studies involving proton nmr,[3] CD/ORD,[4] X-ray diffraction,[5] and energy minimizing calculations.[6] We report here a related study in which cmr has been used as a probing tool. The cmr spectrum of actinomycin D in chloroform was taken. Assignments could be made on the basis of broad-band and off-resonance decoupling, selective proton decoupling, and comparison of the chemical shifts with those of several model substances.

Results and Discussion

The proton broad-band decoupled low-field (22.63 MHz) spectrum of actinomycin D is shown in Figure 1. Figures 2a,b and 3 represent the high-field (67.88 MHz) spectrum of the carbonyl region, the carbonyl and chromophore region, and the aliphatic region. There are 53 discernible signals in the entire high-field spectrum, 16 of which belong to the actinocin (2-amino-4,6-dimethyl-3-oxo-3*H*-phenoxazine-1,9-dicarboxylic acid) moiety and 37 to the peptide rings. Actinomycin D possesses 62 carbons of which 16 compose the actinocin chromophore and 23 compose each of the two identical cyclopentapeptides. The chemical shifts, together with their assignments, are listed in Table I. Several carbons, equivalent in the twin cyclopentapeptides, show different chemical shifts, particularly in the high-field spectrum, indicating their magnetic nonequivalence in the molecule. The assignments are based on peak multiplicities seen in the off-resonance spectrum (column 4), on selective proton decoupling, and by comparison with the models benzoic acid, toluene, *o*-aminophenol, 2-aminophenoxazone-3 (**2**), actinocin (**3**), *p*-toluic acid (**4**), 2,2′-bisbenzoxazolinyl (**6**), 2,5-bis(dimethylamino)-1,4-benzoquinone[7] (**7**), *p*-toluoylthreonine methyl ester (**5**), Boc-L-MeVal-OH, Boc-Sar-OH, Boc-L-Pro-OH, Boc-L-Val-OH, Boc-L-Thr-OH, Boc-L-Thr-Cys(Bzl)-Ile-OMe, Boc-L-Val-Val-OiPr, Ac-DL-Val-OH, H-Gly-Sar-OMe, and H-Gly-DL-Thr-OMe, taking into consideration the commonly known substituent effects. The chemical shifts of the amino acid models are listed in Table II. The chemical shifts of the models for the actinocin system are shown in the structures **2** through **7**. C_1 and C_4 in **1**, both carbons adjacent to exocyclic double bonds in the quinoidal ring, are compared with the equivalent carbon in **7**. The shifts in going from **7** *via* **2** and **3** to **1** are due to the effects of newly introduced groups. Model substances benzoic acid, toluene, *o*-aminophenol, **4, 5, 6,** and **7** are used, and the additivity of substituent effects is assumed. Similarly, the correlation of C_2 and C_3 follows from **7**. The bridgehead carbons C_{4a}, C_{5a}, C_{9a}, and C_{10a} are correlated to the corresponding carbons in **2** and **3,** taking into consideration substituent effects. C_7 and C_8 are com-

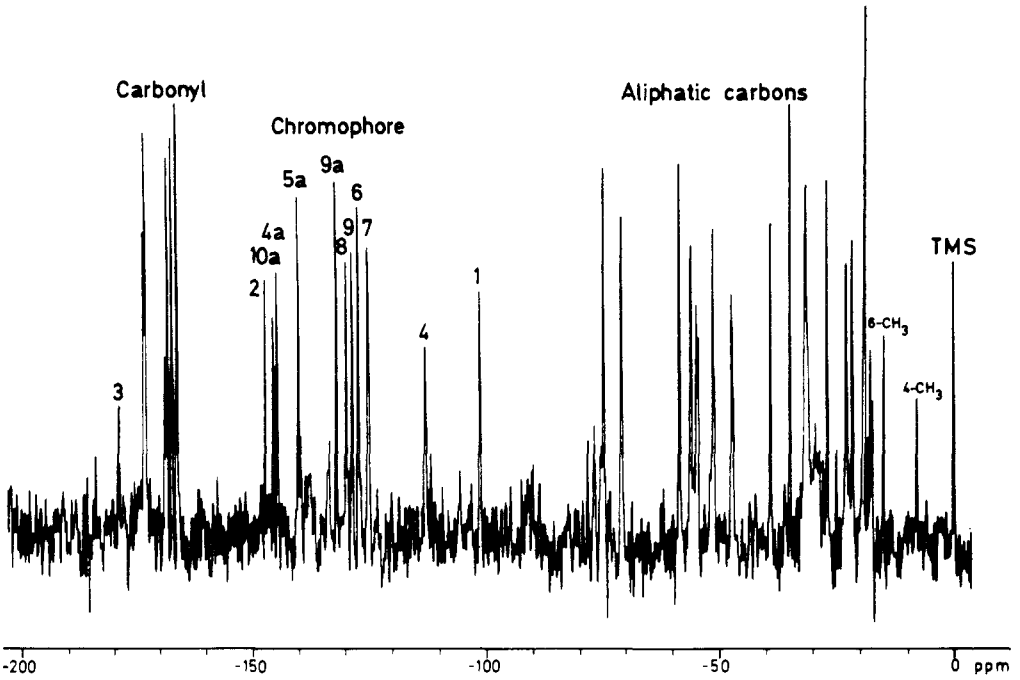
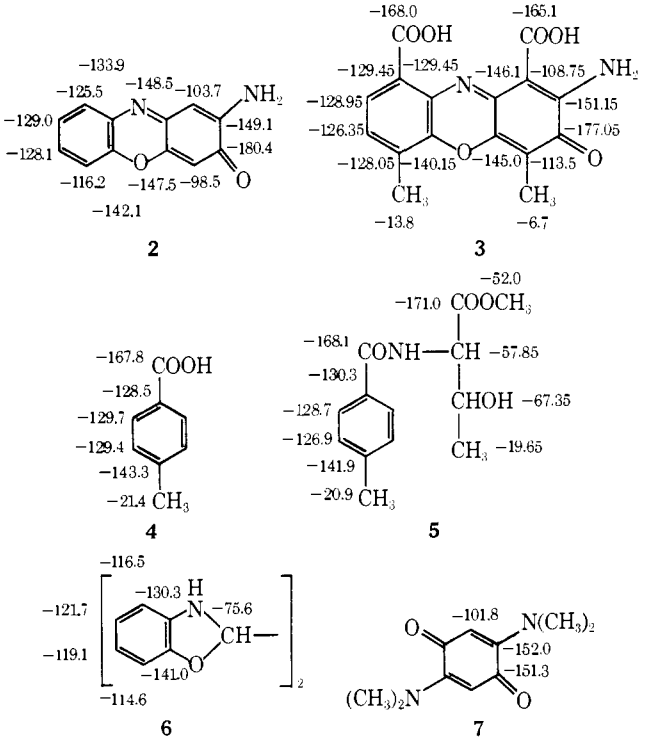


Figure 1. The 22.63-MHz ^{13}C [1H] spectrum of actinomycin D: 100 mg in 1 ml of $^{12}CDCl_3$ at 300°K, 16K scans; internal reference, TMS. Reduced pulse angle for better observation of slowly relaxing carbons.

pared with the corresponding carbons in **2, 3, 4, 5,** and **6.** Their assignments are further secured from the doublets in the off-resonance spectrum. The positions of C_9 and C_6 follow the pattern in **6,** taking into account the substituent effects of CH_3 and COOH. The carbonyls on C_1 and C_9 follow directly from the carboxyl absorption in **4** and **5,** C_9 CO being more closely related to the carboxyl signal of **4.** The methyls on C_4 and C_6 are deduced from **3** and **4** and confirmed by their multiplicity (quartet) in the off-resonance spectrum. The five intense carbonyl peaks are assigned to the carbonyls of the five amino acids by comparison with the model amino acid and peptide compounds (Table II). In the high-field cmr spectrum (67.88 MHz), three of these



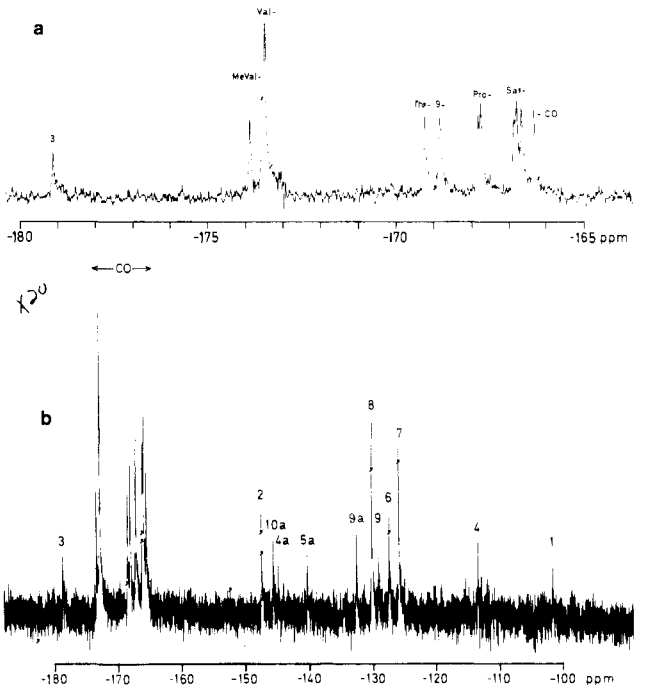


Figure 2. The 67.88-MHz ^{13}C [1H] spectrum of actinomycin D: about 100 mg in 1 ml of $CDCl_3$ at 313°K, 64K scans; internal reference, TMS. (a) Carbonyl region, expanded output; (b) carbonyl and chromophore region.

carbonyl signals are split into two signals. They appear to be magnetically nonequivalent. Some of the assignments, as shown in Table I, should be considered as tentative, as long as no direct comparison with a synthetic tri-, tetra-, and pentapeptide is possible. The signals at about 173.5 ppm are distinctly shifted downfield relative to those of all other peptide carbonyls, appearing at about 167 ppm. This might reflect hydrogen bonding of the Val CO and the lactone linking of the MeVal CO.

The Thr C_β atoms follow directly from comparison. The downfield shift observed for Thr C_β (about 7 ppm with respect to the model compound) and the Thr C_α (about 14

Table I. Chemical Shift Values and Assignments for Actinomycin D[a]

Carbon class	δ, ppm— 22.63 MHz	67.88 MHz	Degree of protonation[b]	Assignment
Carbonyl	179.1	179.2	CO	C_3
	173.7	173.85	CO	1 MeVal CO
	173.4	173.45 intense	CO	2 Val CO
		173.25	CO	1 MeVal CO
	169.2	169.05	CO	2 Thr CO
	168.6	168.65	CO	C_9 CO
	167.7	167.65, 167.55	CO	2 Pro CO
	166.7	166.6, 166.45	CO	2 Sar CO
	166.4	166.1	CO	C_1 CO
Hetero-aromatic	147.7	147.85	C	C_2
	146.0	146.05	C	C_{10a}
	145.1	145.25	C	C_{4a}
	140.5	140.55	C	C_{5a}
	132.6	132.8	C	C_{9a}
	130.4	130.3	CH	C_8
	129.2	129.25	C	C_9
	127.8	127.55	C	C_6
	125.8	125.95	CH	C_7
	113.6	113.6	C	C_4
	101.9	101.9	C	C_1
		77.5, 77.05, 76.55		$CDCl_3$
Aliphatic	75.1	75.1 intense	CH	2 Thr C_β
	71.4	71.65, 71.5	CH	2 Thr C_α
	58.9	59.0, 58.85	CH	2 MeVal C_α
	56.6	56.5, 56.35	CH	2 Val C_α
	55.2	55.45	CH	2 Pro C_α
	54.8	55.11	CH	
	51.5	51.5 intense	CH_2	2 Sar C_α
	47.7	47.7, 47.4	CH_2	2 Pro C_δ
	39.4	39.3, 39.2	CH_3	2 Sar NCH_3
	35.1	34.9 intense	CH_3	2 MeVal NCH_3
	31.6	31.9, 31.6	CH	2 Val C_β
		31.4, 31.1	CH_2	2 Pro C_β
	29.7	29.7 weak		Impurity
	27.1	27.05 intense	CH	2 MeVal C_β
	23.1	23.1, 22.9	CH_2	2 Pro C_γ
	21.7	21.7, 21.6	CH_3	2 Thr C_γ
	19.1	19.3 intense	CH_3	4 MeVal C_γ
		19.1 very intense	CH_3	2 Val C_γ
	17.8	17.85	CH_3	1 Val C_γ
	17.4	17.4	CH_3	1 Val C_γ
	15.1	14.95	CH_3	C_6 CH_3
	7.9	7.8	CH_3	C_4 CH_3

[a] Solvent $^{13}CDCl_3$ (at 22.63 MHz) and $CDCl_3$ (at 67.88 MHz); internal standard TMS. [b] As following from proton off-resonance decoupling.

ppm with respect to the model compounds) can be explained only to a minor extent in terms of the acylation of the C_β alcohol group; *e.g.*, up to 5 ppm downfield shifts are observed by esterification of 2-propanol (*e.g.*, Boc-L-Val-Val-OiPr, δ_{CH} −68.6 ppm; 2-propanol, δ_{CH} −63.7 ppm). The remainder of the Thr C_α and C_β shifts in actinomycin D must be due to field effects from the heterocycle. It is reasonable that these field effects influence the Thr residue rather than the residues of all other four amino acids.

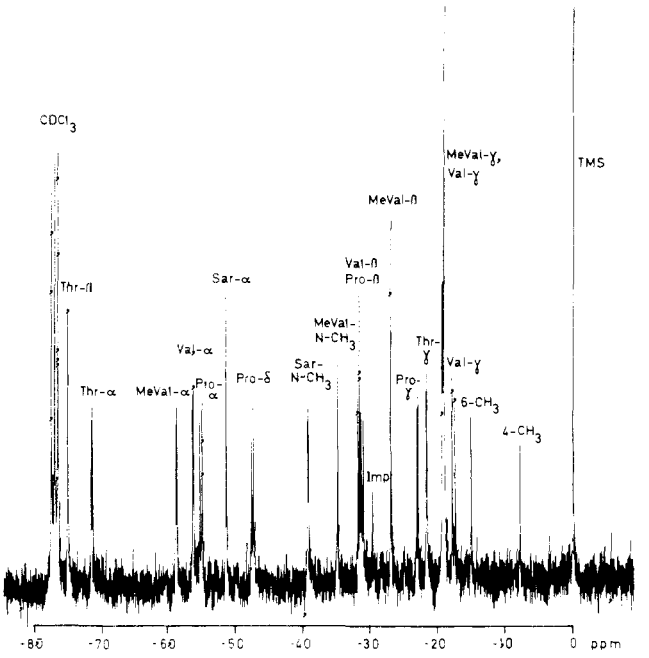


Figure 3. The 67.88-MHz ^{13}C [1H] spectrum of actinomycin D, aliphatic region (see caption to Figure 2).

Correlation with the models (Table II) and selective proton decoupling, using the proton Larmor frequencies as assigned in previous work,[3] leads to the assignment of MeVal C_α, Val C_α, Pro C_α, and Sar C_α. In the low-field cmr spectrum (22.63 MHz), no magnetic nonequivalence of the α carbons, except for those of Pro, is found. The high-field cmr spectrum (67.88 MHz), however, shows two signals for the C_α carbons, except for those of Sar. The assignment of the Pro C_β, C_γ, and C_δ atoms follows from ref. 8 and 9. The Val and MeVal C_β and C_γ atoms follow from the model compounds (Table II). In the high-field cmr spectrum two signals are observed only for Val C_β and Pro C_β. The γ carbons of MeVal and Val give rise to two strong unresolved signals between 19.1 and 19.3 ppm which cannot be resolved, even in high-field cmr spectrum. The commonly observed magnetic nonequivalence of the Val and MeVal C_γ atoms in the range of 0.5 to 1.5 ppm is also found in actinomycin D. The assignment of Thr C_γ (21.7, 21.6 ppm) arises directly from comparison with isopropyl acetate (20.4 ppm) or model compound Boc-L-Val-Val-OiPr (21.8 ppm). The *N*-methyl carbon signals of MeVal and Sar, as assigned in Table I, are remarkably shifted to lower field, relative to the model compounds in Table II. The ^{13}C shift values assigned to Pro C_β and Pro C_γ (Table I) correspond better to a cis configuration of the peptide bond than to the trans configuration,[8] supporting X-ray[5] and calculation[6] results.

The carbonyl at C_9 in **1**, as assigned by comparison with **4**, is slightly more deshielded than in **3**, possibly because of the change from carboxyl to carboxamide and because of lack of coplanarity with the ring system. The C_1 CO in **3** is slightly shielded relative to **4**, but the effect is less in **1**. An intramolecular hydrogen bond between C_2 NH_2 and C_3=O in **1** may also be present in **3** because of the inverse shielding direction pattern for C_2 and C_3 in going from **2** *via* **3** to **1**. In **3**, however, hydrogen bonding between C_1 COOH and C_2 NH_2 is also possible.

There is no substantial difference in the line widths and intensities between the C_α and most of the C_β atoms. This indicates that the intramolecular mobility is less than the freedom of overall molecular tumbling. However, some methyl signals (C_4 CH_3, C_6 CH_3, Val C_γ, MeVal C_γ) show

Table II. ^{13}C Chemical Shift Values (δ in −parts per million Relative to Internal TMS) and Assignments for Various Amino Acid Model Compounds (Solvent $CDCl_3$; Temperature 27°)

Model	C_α	C_β	C_γ	C_δ	NCH_3	COO	CONH	Boc/Ac	Others
Boc-*Sar*-OH	50.1[a] 50.8[a]	—	—	—	35.6	173.6	—	Boc CO	156.0[a] 156.6[a]
								Boc C	80.7
								Boc CH_3	28.25
H-Gly-*Sar*-OCH_3	51.5	—	—	—	34.85	165.4	163.35	OCH_3	54.15
								Gly C_α	44.55
Boc-L-*Val*-OH	58.8[a] 60.5[a]	31.4	17.8[h] 18.3[h]	—	—	176.6	—	Boc CO	156.35[a] 157.85[a]
								Boc C	80.25[a] 80.0[a]
								Boc CH_3	28.6
Ac-DL-*Val*-OH	57.7	30.2	18.3[h] 19.5[h]	—	—	173.6	—	Ac CO	170.35
								Ac CH_3	22.65
Boc-L-*Val*-*Val*-OiPr	57.3[d] 60.0[e]	28.5[d] 31.0[e]	17.9[e] 19.3[e] } h 18.4[d] 19.0[d] } h	—	—	172.3[g]	171.3[f]	Boc CO Boc C	156.0 79.2
								Boc CH_3	28.4
								i-Pr OCH	68.6
								i-Pr $(CH_3)_2$	21.8
Boc-L-*MeVal*-OH	64.2[c] 64.95[a]	27.5	18.9[h] 19.75[h]	—	31.05[a] 31.3[a]	175.75	—	Boc CO	155.7[a] 156.55[a]
								Boc C	80.6
								Boc CH_3	28.15
Boc-L-*Pro*-OH	58.5[b] 58.7[c]			46.1[c] 46.4[b]	—	176.3[b] 177.05[c]	—	Boc CO	153.85[c] 154.85[b]
		29.4[c] 30.5[b]	23.3[b] 24.0[c]					Boc C	80.15
								Boc CH_3	27.95
Boc-L-*Thr*-OH	58.9	68.1	19.4	—	—	174.7	—	Boc CO	156.75
								Boc C	80.25
								Boc CH_3	28.4
H-Gly-L-*Thr*-OCH_3	58.05	67.85	19.95	—	—	171.65	159.55	OCH_3	52.55
								Gly C_α	43.9
p-Tol-L-*Thr*-OCH_3	57.85	67.35	19.65	—	—	171.0	168.1	OCH_3	52.0
								p-Tol CH_3	20.95
								p-Tol C-1	130.25
								p-Tol C-2,6	128.7
								p-Tol C-3,5	126.9
								p-Tol C-4	141.9
Boc-L-*Thr*-Cys-(Bzl)-Ile-OCH_3	59.0	67.5	18.45	—	—	—	170.35	Boc CO	156.0
								Boc C	79.95
								Boc CH_3	28.25
								Cys C_α	56.85
								Cys C_β	28.25
								Cys CONH	171.0
								Ile C_α	52.65
								Ile C_β	37.55
								Ile C_γ (CH_3)	15.45
								Ile C_γ (CH_2)	25.15
								Ile C_δ	11.45
								Ile COO	171.85
								OCH_3	52.0
								Bzl CH_2	56.85
								Bzl C-1	137.9
								Bzl C-2,6	128.5
								Bzl C-3,5	128.95
								Bzl C-4	127.1

[a] Cis–trans isomer. [b] Cis isomer. [c] Trans isomer. [d] N terminal. [e] C terminal. [f] –CONH. [g] –COOR. [h] Diastereotopic.

reduced intensities and line widths in the 22.63-MHz spectrum, reflecting a rotational freedom at the amino acid side chains and at the chromophore.

Experimental Section

All cmr spectra were taken in $CDCl_3$ ($^{12}CDCl_3$ in Figure 1) or $(CD_3)_2SO$, with tetramethylsilane as internal reference. The low-field cmr spectra were measured at 27° on a Bruker HFX-90 instrument, operating at 22.63 MHz for ^{13}C and 90 MHz for ^1H. The high-field spectra were taken at 40° on a Bruker WH-270 spectrometer, operating at 67.88 MHz for ^{13}C and at 270 MHz for ^1H. The pulse Fourier transform technique was applied. The accumulated interferograms were Fourier transformed by a Digital PDP-8-I computer (8K, 9 bit for HFX-90) and a Nicolet-BNC-12 computer (32K, 20 bit for WH-270), respectively, to the phase corrected real parts of the cmr spectra. The ^2D signal of the deuterated solvent served for field/frequency stabilization at 13 (HFX-90) and 39 MHz (WH-270), respectively. The spectra of **2, 3, 4,** and **6** were taken in $(CD_3)_2SO$; the low-field spectrum of **1** was taken in $^{12}CDCl_3$, and all other spectra were taken in $CDCl_3$. The proton Larmor frequencies to be irradiated during selective ^1H decoupling were determined by taking a 90-MHz ^1H nmr spectrum of the sample before taking its ^{13}C [^1H] spectra.

2,5-Bis(dimethylamino)-1,4-benzoquinone (7). The cmr data (solvent $CDCl_3$) were taken from the literature.[7]

2-Aminophenoxazone-3 (2). This compound was obtained by oxidation of *o*-aminophenol with *p*-benzoquinone.[10]

Actinocin (2-Amino-4,6-dimethyl-3-oxo-3*H*-phenoxazine-1,9-dicarboxylic Acid) (3). This compound was synthesized according to the literature.[11] It had λ_{max} (MeOH) 447 nm (lit.[11] 446 nm; lit[12] 448 nm).

2,2′-Bisbenzoxazolinyl (6). This compound was synthesized according to the literature.[13]

p-**Toluoylthreonine Methyl Ester (5).** L-Threonine methyl ester hydrochloride was dissolved in an excess of cold 10% sodium carbonate. After three extractions with chloroform, drying over sodium sulfate, and evaporation of the solvent, 2.3 g (0.0173 mol) of a colorless oil was obtained. H-L-Thr-OMe was dissolved in 10 ml of methylene chloride. To the solution was added 2.35 g (0.0173 mol) of *p*-toluic acid (Fluka, puriss) in methylene chloride. The salt precipitated immediately. Sufficient methylene chloride was added so that the suspension could be stirred. At 0°, 3.56 g of dicyclohexylcarbodiimide in methylene chloride was added dropwise whereupon the suspension warmed up spontaneously. It was stirred for 16 hr at 25°. Methylene chloride was evaporated, and the residue was suspended in ethyl acetate and filtered. After addition of petroleum ether (30:50) to the filtrate, 3.1 g (72%) of white platelets were obtained, mp 106°. The material was pure by tlc and had R_f 0.62 in BuOH–AcOH–H_2O, 2:1:1, R_f 0.61 in BuOH–Pyr–AcOH–H_2O, 30:20:6:24, and R_f 0.66 in EtOH–H_2O–benzene–AcOH, 40:20:10:5, as detected by uv and Cl-tolidine. The compound was identified by proton nmr, ^{13}C nmr, and mass spectrum.

Anal. Calcd for $C_{13}H_{17}NO_4$: C, 62.15; H, 6.77; N, 5.58. Found: C, 62.12; H, 6.94; N, 5.46.

Boc-Amino Acid Derivatives. The *tert*-butyloxycarbonyl derivatives of Sar, L-Pro, L-Val, and L-Thr were prepared according to the literature.[14] L-*N*-Methylvaline was prepared according to the literature.[12] It had mp 325–327° (sealed capillary) (lit.[12] 297–300° dec) and $[\alpha]^{21}D$ +31.35° (*c* 2.62, 5 *N* HCl) (lit.[12] +32.4 ± 0.8°, *c* 3.0, 5 *N* HCl). The compound was pure by electrophoresis and was further identified by proton nmr, ^{13}C nmr, and mass spectra (trimethylsilyl derivative). Blocking of its amino group by *tert*-butyloxycarbonyl according to ref. 14 gave only 8% if carried out at room temperature, and 16% at 40°. By methylating Boc-L-Val-OH (32.5 g, 0.15 mol) with methyl iodide (75 ml, 1.2 mol) and sodium hydride (19.8 g 55–60% dispersion, Fluka, 0.45 mol) during 28 hr according to the literature,[15] a light yellow oil was obtained which crystallized slowly upon standing at 5°. The crystals were very soluble in ether–petroleum ether or in pure petroleum ether, 40:60. Purification of the large crystals could be achieved by repeated washing with absolute petroleum ether (40:60) at 0°: yield 27 g (71%), mp 53–55° (lit.[14] Boc-DL-MeVal-OH, 86°). The material was pure by tlc (BuOH–AcOH–H_2O, 2:1:1) and by electrophoresis. It was identified by proton and ^{13}C nmr and by mass spectrometry. A sample was decomposed in 6 *N* HCl to MeVal which had $[\alpha]^{20}D$ +33.4° (*c* 1.5, 6 *N* HCl) (lit.[12] +32.4 ± 0.8, *c* 3.0, 5 *N* HCl). A sample of Boc-L-MeVal-OH was converted to its dicyclohexylammonium salt by mixing equimolar amounts of Boc-L-MeVal-OH and dicyclohexylamine in a little absolute ether. Evaporation of the ether gave crystals which were recrystallized from absolute petroleum ether (60:80), mp 105–106°.

Anal. Calcd for $C_{23}H_{44}N_2O_4$: C, 67.05; H, 10.72; N, 6.80. Found: C, 66.93; H, 10.84; N, 6.94.

tert-**Butyloxycarbonyl-L-threonyl-*S*-benzylcysteinylisoleucine Methyl Ester.** This compound was synthesized according to the literature.[16]

tert-**Butyloxycarbonyl-L-valylvaline Isopropyl Ester.** L-Valine (11.7 g, 0.1 mol) was suspended in 300 ml of 2-propanol. Anhydrous hydrochloric acid was introduced under refluxing for 5 hr. After evaporation, the residue was dissolved in chloroform and kept for 12 hr at 5°. Some unreacted valine was filtered off, and the filtrate was evaporated. Yield was 10.5 g (54%) of colorless needles of L-valine isopropyl ester hydrochloride, mp 120°. H-L-Val-OiPr · HCl (10.15 g, 0.052 mol) was dissolved in 100 ml of ethyl acetate, and 5.6 g (0.055 mol) of triethylamine in 30 ml of ethyl acetate was added in the cold. The precipitated hydrochloride was removed, and 12.2 g (0.056 mol) of Boc-L-Val and 11.55 g (0.056 mol) of dicyclohexylcarbodiimide were added. The mixture was stirred for 5 hr. Precipitated dicyclohexylurea was removed, and the filtrate was washed with 5% sodium bicarbonate, water, 3% hydrochloric acid, and water. After drying over sodium sulfate, the evaporation residue (11.2 g, 60%, mp 127–129°) was recrystallized from cyclohexane, *n*-hexane, or ether–petroleum ether (30:50), yielding colorless needles with mp 130.5°.

Anal. Calcd for $C_{18}H_{34}N_2O_5$: C, 60.31; H, 9.56; N, 7.82; O, 22.32. Found: C, 59.91; H, 10.00; N, 7.83; O, 22.11.

N-**Acetyl-DL-valine.** This material was purchased from Schuchardt.

Glycylsarcosine Methyl Ester. Glycylsarcosine (Fluka, 100 mg) was suspended in 3 ml of 95% aqueous methanol. At 0°, an excess ethereal solution of diazomethane was added. The suspension was stirred for several hours at 0° and filtered. The residue was subjected to the same procedure five times. The combined filtrates yielded 51 mg of glycylsarcosine methyl ester as colorless crystals.

Glycyl-DL-threonine Methyl Ester. Glycyl-DL-threonine (EGA-Chemie, 100 mg) was methylated as above yielding 60 mg of glycyl-DL-threonine methyl ester as colorless crystals.

Acknowledgment. We are indebted to Dr. B. Bradley of Bruker Physik, Karlsruhe, Germany, for measuring the high-field ^{13}C [1H] spectrum. A generous gift of actinomycin D by Merck Sharp and Dohme, New Jersey, and of $^{12}CDCl_3$ by Merck, Darmstadt, Germany, is gratefully acknowledged.

References and Notes

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