Structures of Five Components of the Actinomycin Z Complex from *Streptomyces fradiae,* Two of Which Contain 4-Chlorothreonine

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Structure elucidation of five components of the actinomycin Z complex (Z_1−Z_5) isolated from *Streptomyces fradiae* is described. The components were separated by Si gel column chromatography and TLC/PLC and analyzed by ESIMS, FABMS, LC−MS of derivatized hydrolysates, and 2D NMR techniques. This permitted determination of the complete structures of actinomycins Z_1−Z_5. In Z_3 and Z_5, site 1 of the β-depsipeptide is occupied by the rare 4-chloro-L-threonine, an amino acid not previously found in an actinomycin. The structural variants of the actinomycin Z complex have the molecular architecture typical of other actinomycins but possess greater structural diversity resulting from the presence of several highly unusual amino acids. Actinomycins Z_3 and Z_5, but not Z_1, were more potent than actinomycin D in cytotoxicity assays against three tumor cell lines.

The actinomycins are a family of chromopeptide antitumor antibiotics isolated from various *Streptomyces* strains, of which more than 30 native and many synthetic variants are known. Actinomycin complexes termed A, B, C, D, I, X, Z, and so forth have been reviewed.[1] The natural actinomycins all share the same phenoxazinone chromophore, varying only in the amino acid content of their two depsipentapeptide moieties. Actinomycins C_3 and D have found clinical application as anticancer drugs, particularly in the therapy of Wilm's tumor[2] and soft tissue sarcoma[3] in children, and are still of interest in molecular biology, for example, in studying the binding to single-stranded DNA.[4] Recently, actinomycin D has been proposed as a therapeutic agent for AIDS, because it is a potent inhibitor of HIV-1 minus-strand transfer.[5]

The actinomycin Z complex was first isolated in 1958 from *Streptomyces fradiae* strain ETH-20675 and found to contain six components termed Z_0−Z_5.[6] In that report, it was stated that the amino acids identified in hydrolysates differed from those from all other known actinomycins in the absence of proline and the presence of *N*-methylalanine. Later, the absence of proline was explained when novel proline congeners were discovered in these actinomycins, namely *cis*-5-methylproline in actinomycin Z_5,[7,8] 4-keto-5-methylproline in actinomycins Z_1[9] and Z_5,[10] and *trans*-3-hydroxy-*cis*-5-methylproline in actinomycin Z_1.[11,12] Also, 4-hydroxythreonine, not found in other actinomycins, was identified in actinomycin Z_1.[13] The experimental evidence supported the concept that these compounds resembled other actinomycins in having two pentapeptide lactone moieties attached to the same phenoxazinone chromophore but differing at several amino acid sites. Sequence information was not obtained, nor were assignments of the various amino acids to the α- or β-peptide units established prior to the investigation reported here.

4-Chlorothreonine, which was identified in the present study as a component of actinomycins Z_3 and Z_5, has been found as the free amino acid in *Streptomyces* sp. OH-5093,[14] as well as in hydrolysates of the lipopeptides syringotoxin, syringostatin, and syringomycins A, E, and G.[15,16] Biosynthetic studies have established that L-threonine is the precursor of the 4-chlorothreonine in syringomycin.[17]

Results and Discussion

Actinomycin Z complex from three sources was used in this investigation: A, an old sample from cultures of the original *S. fradiae* strain ETH-20675;[6] B, a sample from a recent fermentation of the same organism; C, a sample from the collection of the National Cancer Institute. These three samples were found to be essentially identical by a variety of chromatographic and spectroscopic techniques. The main components Z_1−Z_5 are named in order of increasing R_f values on TLC and reversed-phase HPLC. A minor, more polar component designated Z_0 is a mixture of unknown variants[18] and was not studied.

The five members of the actinomycin Z complex were separated and purified by column chromatography (CC) and PLC/TLC on Si gel; they could also be separated by reversed-phase HPLC. They had UV-visible spectra typical of all the actinomycins (visible λ_{max}. 443 nm). Their complete structures (Figure 1) were determined by ESIMS, FABMS, LC−MS of derivatized amino acids from hydrolysates, and 2D NMR techniques. MS data both from HPLC−ESIMS of the actinomycin Z complex and from FABMS of the separated actinomycins established molecular formulas in accord with the proposed structures (Table 1).

Isotopic patterns in the mass spectra of actinomycins Z_3 and Z_5 indicated that these two components each contain one chlorine atom, and this was confirmed by microanalysis.[19] The amino acids of each actinomycin were identified by LC−MS after hydrolysis and chiral derivatization by the Marfy[20] and GITC[21] techniques and were in accord with the structures in Figure 1. Because 4-keto-5-methylproline is destroyed during acid hydrolysis,[10] the actinomycins were also analyzed after reduction of the keto groups to hydroxyls with $NaBH_4$. Actinomycins Z_3 and Z_5 contained an amino acid with MW = 153 and 155 (minor Cl isotope),

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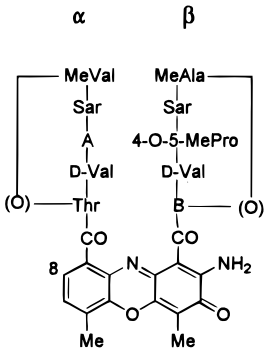

	A	B
Z_1	3-OH-5-MePro	4-OH-Thr
Z_2	3-OH-5-MePro	Thr
Z_3	3-OH-5-MePro	4-Cl-Thr
Z_4	5-MePro	Thr
Z_5	5-MePro	4-Cl-Thr

Figure 1. Structures of Z actinomycins. Nonstandard amino acid abbreviations: 4-O-5-MePro = 4-keto-*cis*-5-methyl-L-proline; 3-OH-5-MePro = *trans*-3-hydroxy-*cis*-5-methyl-L-proline; 4-OH−Thr = 4-hydroxy-L-threonine; 4-Cl-Thr = 4-chloro-L-threonine.

Table 1. HPLC−ESIMS[a] and FABMS of Actinomycin Z Complex

	ESIMS[b]		
	actinomycin	*m/z*	MW
Z_1	(10.73)	1301.2	1301.4
Z_2	(12.61)	1285.2	1285.4
Z_3	(13.14)	1319.2	1319.9
Z_4	(14.17)	1269.2	1269.4
Z_5	(14.90)	1303.2	1303.9

	FABMS		
	$(M + Cs)^+$ [c]	(mmu)[d]	mol formula
Z_1	1433.5034	+0.4	$C_{62}H_{84}N_{12}O_{19}$
Z_2	1417.5011	−7.0	$C_{62}H_{84}N_{12}O_{18}$
Z_3	1451.4639	−5.2	$C_{62}H_{83}ClN_{12}O_{18}$
Z_4	1401.5087	−4.5	$C_{62}H_{84}N_{12}O_{17}$
Z_5	1435.4725	−1.7	$C_{62}H_{83}ClN_{12}O_{17}$

[a] HPLC retention times (min.) of the actinomycins are in parentheses. [b] ^{12}C MH^+. [c] Measured lowest isotype. [d] Mass error.

and the presence of 4-chlorothreonine in these two actinomycins was confirmed by ^1H and ^{13}C NMR (Tables 2−4).

The amino acid sequences in each of the two peptide moieties in actinomycins Z_1−Z_5 were derived from connectivities in the 2D NMR studies (HMBC, ROESY, and TOCSY). For example, the connectivity diagram for actinomycin Z_3 is shown in Figure 2.[22] They were consistent with the results obtained in linked-scan FABMS of the daughter ions obtained from the protonated molecular ions resulting from the fragmentation of the depsipeptide rings.[23,24] In the latter experiments, ions were observed representing loss of the C-terminal tripeptides and tetrapeptides from one or both rings. These data confirmed that the MeAla and 4-keto-5-MePro residues occupy the same depsipeptide. An example[25] (for Z_5) is shown in Table 5.

The regiospecificities of the amino acids (Figure 1) emerged from the 2D NMR experiments and particularly

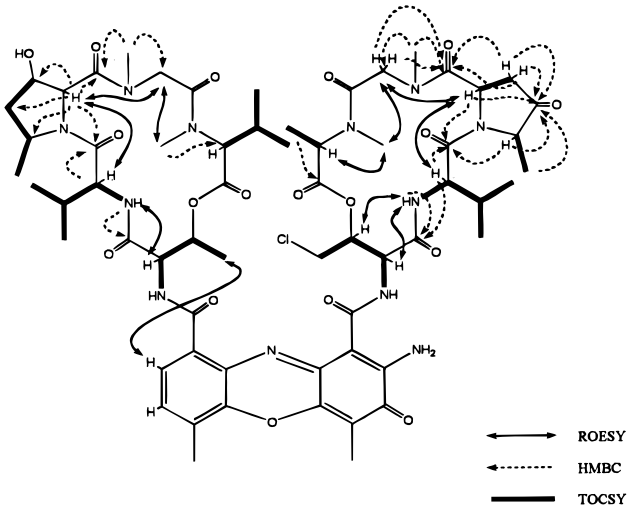

Figure 2. 2D NMR connectivities in actinomycin Z_3.

from the NOE observed between the 8-proton of the chromophore and the methyl group of threonine (Figure 2), which is invariably located in the α-peptide. The corresponding site in the *β*-peptide is occupied either by 4-hydroxythreonine (Z_1), threonine (Z_2, Z_4), or 4-chlorothreonine (Z_3, Z_5). In every case, the 4-keto-5-methylproline is located in the *β*-depsipeptide, while the corresponding site in the α-depsipeptide is always occupied by either 5-methylproline (Z_4, Z_5) or 3-hydroxy-5-methylproline (Z_1−Z_3). This is analogous to the earlier observation that the 4-ketoproline in actinomycin X_2 is located in the *β*-peptide.[26] In the Z actinomycins, *N*-methylalanine is invariably located in the *β*-peptide.

The 2D NMR experiments revealed that the Z actinomycins possess the same general architecture (pseudo-C_2 symmetrical) as the other actinomycins, including the presence of two cis peptide bonds (D-Val → *ψ*Pro and *ψ*Pro → Sar) in each peptide moiety,[27] but possess far greater structural diversity resulting from the presence of several highly unusual amino acids. In the case of actinomycin Z_3, the structure described here has been confirmed by X-ray crystallography,[28] and the conformation is similar to that found in solution by NMR.[29] The presence of 4-chlorothreonine in two of these actinomycins (Z_3 and Z_5) is reported for the first time in an actinomycin.

The cytotoxicities of actinomycins Z_1, Z_3, and Z_5 were compared with those of actinomycin D in three tumor cell lines representing stomach, liver, and breast cancer (Table 6). Actinomycin Z_3 was more active than actinomycin D in all three cell lines, and actinomycin Z_5 was more active in two of them. In earlier studies,[30] actinomycin D was more effective than actinomycins Z_1 and Z_5 in inhibiting RNA synthesis in *Bacillus subtilis* and in HeLa cells. Antimicrobial activities (MIC values) of actinomycins D, Z_1, Z_3, and Z_5 are also given in Table 6. The relative inactivity of actinomycin Z_1 in both the cytotoxicity and antimicrobial assays presumably results from the presence of a 4-hydroxythreonine residue, which is the only feature of the molecule absent from all of the other three actinomycins studied. This is possibly because its hydroxyl group is in close proximity to the chromophoric amino group,[28] which plays an important role in the intercalative complexation of actinomycins with DNA.[27]

The elucidation of the precise structures (Figure 1), general molecular architecture, and comparative biological activities of the Z actinomycins significantly extends our

Table 2. Proton NMR Data for Actinomycins Z_1, Z_3, and Z_5[a]

		Z_1		Z_3		Z_5	
site	H	α-ring	β-ring	α-ring	β-ring	α-ring	β-ring
1	2	4.62 dd, br	5.01 dd, br	4.49 dd (3.0, 7.0)	5.21 m	4.77 m	4.62 m
	3	5.24 dq (3.2, 10.1)	5.10 m	5.21 m	5.21 m	5.28 dq (3.0, 9.7)	5.20 m
	4	1.12 d (6.4)	3.69 m	1.11 d (6.1)	3.97 m	1.14 d (6.2)	4.09 m
			4.50 m		4.06 dd (5.5, 11.5)		4.13 m
	NH	7.18 s, br	8.13 d (6.2)	6.91 d (7.0)	7.90 d (6.2)	7.50 s, br	7.83 d (5.8)
	OH		3.22 s, br				
2	2	3.44 dd (6.9, 9.9)	3.83 dd (6.2, 9.9)	3.42 dd (5.0, 9.8)	3.81 dd (6.1, 9.8)	3.58 dd (5.9, 9.8)	3.74 dd (6.1, 10.1)
	3	2.11 m	2.18 m	2.14 m	2.14 m	2.12 m	2.23 m
	3-Me	0.90 d (6.2)	0.92 d (6.2)	0.90 d (6.8)	0.92 d (6.8)	0.93 d (6.9)	0.95 d (5.9)
		1.12 d (6.3)	1.15 d (6.6)	1.12 d (6.5)	1.13 d (6.7)	1.16 d (6.2)	1.16 d (6.2)
	NH	8.15 d (6.9)	8.30 s, br	7.92 d (5.0)	8.24 d (6.1)	8.02 d (5.9)	8.55 d (6.2)
3	2	5.86 s	6.42 dd (2.0, 11.0)	5.91 s	6.49 dd(2.0, 11.1)	6.08 d (8.9)	6.53 dd (2.0, 11.1)
	3	4.16 dd (5.2, 11.3)	2.32 d (17.6)	4.11 dd (5.0, 11.0)	2.33 d (17.6)	2.00 m	2.28 d (17.4)
			3.93 dd (11.0, 17.6)		3.97 m	2.23 m	3.75 dd (11.1, 17.4)
	4	2.11 m		2.14 m		1.82 m	
		2.18 m		2.23 m		2.68 m	
	5	4.71 m	4.55 q (7.1)	4.73 m	4.54 q (7.0)	4.30 m	4.62 q (6.7)
	5-Me	1.49 d (5.8)	1.59 d (7.1)	1.15 d (5.8)	1.59 d (7.0)	1.51 d (6.2)	1.62 d (6.7)
	OH	4.46 m		5.27 m			
4	2	3.61 d (17.5)	3.65 d (17.5)	3.65 d (17.5)	3.67 d (17.5)	3.61 d (17.5)	3.67 d (17.5)
		4.73 d (17.5)	4.56 d (17.5)	4.76 d (17.5)	4.58 d (17.5)	4.60 d (17.5)	4.6 d (17.5)
	N-Me	2.88 s	2.85 s	2.86 s	2.84 s	2.87 s	2.89 s
5	2	2.69 d (9.3)	3.22 q (6.9)	2.67 d (9.0)	3.38 q (7.0)	2.68 m	3.40 q (7.0)
	3	2.64 m	1.32 d (6.9)	2.63 m	1.38 d (7.0)	2.68 m	1.41 d (7.0)
	3-Me	0.73 d (6.7)		0.73 d (6.5)		0.73 d (6.2)	
		0.94 d (6.4)		0.94 d (6.1)		0.94 d (6.6)	
	N-Me	2.88 s	2.89 s	2.92 s	2.92 s	2.84 s	2.95 s
Chrom.	4-Me	1.93 s		2.15 s		1.80 s	
	6-Me	2.51 s		2.50 s		2.53 s	
	7	7.34 d (7.6)		7.31 d (7.8)		7.36 d (7.6)	
	8	7.48 d (7.6)		7.51 d (7.8)		7.55 d (7.6)	
	NH_2	7.20 + 8.10 br		6.72 + 7.97 br		7.20 + 8.45 br	

[a] Chemical shifts (δ) are in ppm relative to TMS. Spin coupling constants (J) are in parentheses (Hz).

Table 3. Chromophoric ^{13}C NMR Assignments for Actinomycins Z_1, Z_3, and Z_5

carbon	Z_1	Z_3	Z_5
C-1	98.1	99.8	100.9
C-2	148.5	147.8	148.3
C-3	113.3	114.0	113.0
C-4a	145.7	145.3	145.0
C-5a	140.2	140.6	140.2
C-6	127.3	127.8	127.3
C-7	130.4	130.4	130.1
C-8	126.0	125.6	126.1
C-9	133.1	132.4	132.9
C-9a	128.7	128.9	129.0
C-10a	145.1	146.2	145.7
4-Me	7.2	7.8	7.1
6-Me	15.0	15.0	15.1
1-CO	168.9	168.9	168.7
3-CO	178.4	178.6	179.0
9-CO	166.3	166.4	166.2

knowledge of this important class of naturally occurring antitumor agents.

Experimental Section

General Experimental Procedures. Melting points are uncorrected, Kofler block. UV: Kontron Uvicon 860. NMR: Varian Unity 300, Inova 500; ^1H at 500 MHz; ^{13}C at 125 MHz. MS: Finnigan MAT 95 and LCQ (ESIMS). TLC plates: 10 × 20 cm, Si gel 60 F_{254}, 0.25 mm with concentrating zone (Merck); solvent system for R_f values: chloroform−acetone (3:1). PLC plates: 20 × 20 cm, Si gel 60 F_{254}, 0.5 mm (Merck, Darmstadt). CC: Si gel 60, 0.025−0.04 mm (Macherey, Nagel & Co., Düren). Circular paper chromatography (CPC): *n*-butyl acetate−*n*-dibutyl ether−10% aqueous sodium *m*-cresotinate, saturated with cresotic acid (3:1:4); paper type 2043b mgl, 30 × 30 cm (Schleicher & Schüll, Dassel). HPLC: column, SMT HYP300 OD-5-300, C_{18}, 250 × 4.6 mm. Solvent system: 40% A (20 min) and 90% B (A = MeCN, B = 0.05% TFA−H_2O). FABMS: JEOL SX102 (Peabody, MA). ESIMS: HPL1100 LCMS (San Jose, CA).

Origin of Actinomycin Z Samples. The *S. fradiae* strain ETH-20675, isolated from a soil sample found in Horsham, England, produced the Z complex for the original investigation by Bossi et al.[6] About 35 years ago Prof. W. Keller-Schierlein at ETH in Zurich provided the strain to the Institute of Organic Chemistry in Göttingen (Prof. H. Brockmann), from which sample A was isolated. Recently, the original strain, stored in a special soil in Göttingen, was cultivated again for comparison purposes (generating sample B). An additional sample of the actinomycin Z mixture was obtained from the repository of the National Cancer Institute, originally supplied by Prof. W. Loeffler, Universität Tübingen, Germany (sample C).

The three samples, A, B, and C, were compared by HPLC, TLC, CPC, and MS and NMR spectroscopy of their separated components, and proved to be identical except for slight differences in the relative amounts of the individual actinomycins.

Cultivation and Extraction of *S. fradiae*. Sample A, (strain ETH-20675) was cultivated in a 120-L fermenter for 3 days at 28 °C (medium: 2% ungreased soy flour, 2% mannite, pH 7.5; see also Bossi et al.[6]). The actinomycin mixture was extracted with EtOAc and the evaporated residue filtered over a column (40 × 5 cm) of neutral alumina III−IV with toluene, EtOAc, acetone, and MeOH, and the EtOAc fraction (6.2 g) was isolated. Sample B was obtained using the same culture medium in shaken Erlenmeyer flasks (3 days at 28 °C).

Preparative Separation of Actinomycins. The crude Z complex (500 mg of sample A) was separated by flash chromatography on a column (3 × 38 cm) of Si gel with $CHCl_3$−acetone (3:1). Fractions containing the components of interest were purified by a second CC procedure or by repeated PLC/TLC ($CHCl_3$−acetone 3:1 or 4:1, developed twice). Crystalliza-

Table 4. Peptidic ^{13}C NMR Assignments for Actinomycins Z_1, Z_3, and Z_5

		Z_1		Z_3		Z_5	
site	C	α-ring	β-ring	α-ring	β-ring	α-ring	β-ring
1	1	168.4/169.2[b]		168.8[a]/168.2		167.7/169.3[b]	
	2	54.7	52.3	54.5	54.5	54.6	54.5
	3	74.9	77.8	74.6	74.5	74.8	74.0
	4	16.9	59.1	17.6	43.6	17.0	44.1
2	1	174.0/174.5[b]		174.1/174.0[b]		173.9/174.0[b]	
	2	57.5/58.7[b]		59.2	57.3	59.0	57.5
	3	32.1/32.1		31.6	32.3	32.0	32.0
	3-Me	18.8[a]/19.0[a,b]		18.9[a]/19.1[a,b]		18.8[a]/19.0[a,b]	
		19.0[a]/19.3[a,b]		19.1[a]/19.1[a,b]		19.2[a]/19.2[a,b]	
3	1	170.5/172.7[b]		170.9	172.6	172.8/172.9[b]	
	2	67.8	53.1	68.5	54.0[a]	58.4	53.8
	3	75.0	41.2	75.6	41.2[a]	29.6	41.2
	4	41.2	212.3	41.3[a]	211.8	26.9	212.3
	5	53.9	59.2	54.0[a]	58.4	55.4	58.7
	5-Me	18.9	15.0	19.2[a]	15.2	18.3	15.0
4	1	166.0/166.0[b]		166.2/166.3[b]		166.1/166.2[b]	
	2	51.4/51.4[b]		51.4/51.6[b]		51.4/51.6[b]	
	N-Me	34.9/35.1[b]		34.9/35.1[b]		34.8/35.0[b]	
5	1	167.7/167.9[b]		167.7	168.6	167.1/167.6[b]	
	2	71.1	60.2	71.3	59.9	71.2	60.1
	3	27.0	13.5	27.1	13.5	26.9	13.6
	3-Me	19.5[a]/21.5[b]		19.3[a]/21.6[b]		9.3[a]/21.6[b]	
	N-Me	37.2/39.0[b]		38.4	37.1	37.3	39.0

[a] Can be interchanged with signals of very similar δ values. [b] Signals separated by "/" are not assigned to the α or β position.

Table 5. Daughter Ion Spectra in Linked-Scan FABMS of Actinomycin Z_5 (Parent Ion *m/z* 1303.6)

m/z	fragment lost (except those in parentheses)
1004	H-4-O-5-MePro-Sar-MeAla-OH
991	H-5-MePro-Sar-MeVal-OH
968	H-4-O-5-MePro-Sar-MeAla-OH + HCl
905	H−D-Val-4-O-5-MePro-Sar-MeAla-OH
891	H−D-Val-5-MePro-Sar-MeVal-OH
691	H-4-O-5-MePro-Sar-MeAla-OH + H-5-MePro-Sar-MeVal-OH
655	H-4-O-5-MePro-Sar-MeAla-OH + H-5-MePro-Sar-MeVal-OH + HCl
592	H−D-Val-4-O-5-MePro-Sar-MeAla-OH + H-5-MePro-Sar-MeVal-OH *or* H−D-Val-5-MePro-Sar-MeVal-OH + H-4-O-5-MePro-Sar-MeAla-OH
556	H−D-Val-4-O-5-MePro-Sar-MeAla-OH + H-5-MePro-Sar-MeVal-OH + HCl *or* H−D-Val-5-MePro-Sar-MeVal-OH + H-4-O-5-MePro-Sar-MeAla-OH + HCl
493	H−D-Val-4-O-5-MePro-Sar-MeAla-OH + H−D-Val-5-MePro-Sar-MeVal-OH
413	(H−D-Val-5-MePro-Sar-MeVal-OH + H)$^+$
314	(H-5-MePro-Sar-MeVal-OH + H)$^+$

Table 6. Cytotoxicities (μmolar) in Three Human Tumor Cell Lines and MIC Values (μg/mL) *in B. subtilis* of Actinomycins D, Z_1, Z_3, and Z_5

actinomycin	level	HMO2 (stomach)	HEP G2 (liver)	MCF 7 (breast)	MIC
D	GI_{50}	0.3	1.0	0.5	0.78
	TGI	0.8	4.0	2.2	
	LC_{50}	>50	>50	>50	
Z_1	GI_{50}	0.75	0.95	<0.5	12.5
	TGI	5.8	5.5	>50	
	LC_{50}	>50	>50	>50	
Z_3	GI_{50}	<0.1	<0.1	<0.1	0.20
	TGI	<0.1	1.4	<0.1	
	LC_{50}	0.28	>50	0.5	
Z_5	GI_{50}	<0.1	1.5	<0.1	0.78
	TGI	<0.1	10.0	0.12	
	LC_{50}	0.50	>50	0.5	

tion afforded pure actinomycins Z_1, Z_3, and Z_5. The smaller amounts of Z_2 and Z_4 could not be crystallized, and these two compounds were not as thoroughly characterized as the other three. R_f values on Si gel TLC ($CHCl_3$−acetone 3:1) were Z_1, 0.12; Z_2, 0.35; Z_3, 0.39; Z_4, 0.50; Z_5, 0.56.

Actinomycin Z_1: orange needles (benzene); mp 257−262 °C (dec); $[\alpha]^{20}_D$ −367° (*c* 0.20, MeOH), −310° (*c* 0.18, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 242 (4.50), 425 (4.30), 442 (4.32) nm; ^1H and ^{13}C NMR ($CDCl_3$), Tables 2−4; FABMS *m/z* (%) neg. 1301 (46), 1300 (100); ESIMS *m/z* (%) pos. 1325 (33), 1324 (73), 1323 (100) $[M + Na]^+$, 1301 (95) $[M + H]^+$; neg. 1299 (100) $[M - H]^-$; *anal.* C 56.62%, H 6.51%, N 12.61% (calcd for $C_{62}H_{84}N_{12}O_{19}$, 1301.37; C 57.20%, H 6.51%, N 12.91%).

Actinomycin Z_2: orange powder ($CHCl_3$−cyclohexane); FABMS *m/z* (%) pos. 1285 (100) $[M + H]^+$, neg. 1284 (100) $[M]^-$; calcd for $C_{62}H_{84}N_{12}O_{18}$, 1285.42; ^{13}C and ^1H NMR data ($CDCl_3$,), almost identical with those of Z_3, except for the β-Thr residue, which replaces Thr: δ_C (δ_H) 17.51 (1.28, 3H, d, J = 6.5 Hz, 4-H_3), 54.75 (4.92, 1H, dd, J = 6.7, 2.8 Hz. 2-H), 75.30 (5.20, 1H, m, 3-H), (7.72, 1H, d, J = 6.7 Hz, NH).

Actinomycin Z_3: orange crystals (benzene−cyclohexane); mp 238−242 °C (dec); $[\alpha]^{20}_D$ −244° (*c* 0.22, MeOH), −305° (*c* 0.16, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 240 (4.53), 424 (4.36), 442 (4.38) nm; ^1H and ^{13}C NMR ($CDCl_3$), Tables 2−4; FABMS *m/z* (%) pos. 1323 (37), 1322 (70), 1321 (84), 1320 (100), 1319 (24) $[M + H]^+$; neg. 1322 (28), 1321 (58), 1320 (75), 1319 (100), 1318 (18) $[M]^-$; ESIMS *m/z* (%) pos. 1344 (26); 1343 (54), 1342 (61), 1341 (89) $[M + Na]^+$, 1284 (74), 1283 (100) $[M - Cl]^+$; neg. 1355 (55), 1353 (68), 1317 (100) $[M - H]^-$, 1282 (38) $[M - HCl]^-$; *anal.* C 55.60%, H 6.44%, Cl 2.95%, N 12.36% (calcd for $C_{62}H_{83}ClN_{12}O_{18}$, 1319.84; C 56.42%, H 6.34%, Cl 2.69%, N 12.73%).

Actinomycin Z_4: orange powder ($CHCl_3$−cyclohexane); ESIMS *m/z* (%) pos. 1291 (100) $[M + Na]^+$, neg. 1267 (100) $[M - H]^-$, (calcd for $C_{62}H_{84}N_{12}O_{17}$, 1269.42); ^1H NMR data ($CDCl_3$), almost identical to those of Z_5, except for the β-Thr residue, which replaces ClThr: δ_H (β-Thr) 1.25 (d, J = 6.2 Hz, 4-H_3), 5.14 (m, 3-H), 4.58 (m, 2-H), 7.64 (d, J = 6.2 Hz, NH).

Actinomycin Z_5: orange needles (EtOAc−cyclohexane); mp 269−273 °C (dec); $[\alpha]^{20}_D$ −406° (*c* 0.17, MeOH, −260° (*c* 0.16,

$CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 239 (4.52), 426 (4.34), 443 (4.37) nm; ^1H and ^{13}C NMR, Tables 2−4; FABMS *m/z* (%) pos. 1307 (37), 1306 (66), 1305 (84), 1304 (100), 1303 (18) $[M + H]^+$, neg. 1305 (31), 1304 (57), 1303 (89), 1302 (100) $[M]^-$; ESIMS *m/z* (%) pos. 1328 (29); 1327 (57), 1326 (69), 1325 (100) $[M + Na]^+$, 1303 (50) $[M + H]^+$, 1267 (54) $[M - Cl]^+$; neg. 1306 (48); 1305 (80), 1304 (97), 1303 (100), 1302 (54) $[M]^-$, 1267 (39), 1266 (24) $[M - HCl]^-$; *anal.* C 55.83%, H 6.66%, Cl 2.81%, N 12.44% (calcd for $C_{62}H_{83}ClN_{12}O_{17,}$ 1303.84; C 57.11%, H 6.42%, Cl 2.72%, N 12.89%).

Biological Assays. The MICs were determined by 3-fold dilution tests performed in Kolmer broth (1% peptone, 0.5% NaCl, 0.25% glucose, 1% meat extract in H_2O, pH 7.4−7.6). The actinomycins (0.5 mg in 10 mL) were dissolved in acetone or methanol (0.3 mL) and diluted with sterile water (0.3 mL). This solution (1 mL) was added to the broth (1 mL), and this 1:1 dilution continued stepwise. The tubes were inoculated with *B. subtilis* (strain ATCC 6051, Department of Medicinal Microbiology, Universität Göttingen) for 18 h at 37 °C. The last clear sample of the series determined the MIC. The cytotoxicities were determined according to the method of the National Cancer Institute (NCI).[31] The human tumor cell lines [HMO2 (stomach), HEPG2 (liver), and MCF7 (breast), obtained from NCI] were cultivated on 96-well microtiter plates in the medium RPMI[32] 1640 with 10% fetal calf serum; 24 h after cell seeding, the actinomycin (1, 5, 10, and 25 μg/mL in MeOH or DMSO) was added and the cultivation continued for 48 h. The number of viable cells was then determined by protein assay with sulforhodamine. The maximum solvent concentration on the test plates was 1%.

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Supporting Information Available: 2D NMR connectivity diagrams (TOCSY and NOESY) for actinomycins Z_1 and Z_5 and linked-scan FABMS data (analogous to Table 5) for actinomycins Z_1−Z_4. This information is available on the World Wide Web at http://pubs.acs.org.

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