Actinomycins with Altered Threonine Units in the β-Peptidolactone

Jens Bitzer,[†] Victoria Gesheva,[‡] and Axel Zeeck*[,†]

Institute of Organic and Biomolecular Chemistry, University of Göttingen, Tammannstrasse 2, D-37077 Göttingen, Germany, and Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Strasse 26, BG-1113 Sofia, Bulgaria

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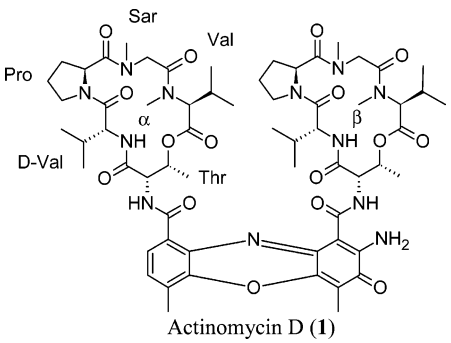
Five new members of the actinomycin family, actinomycins G_2−G_6 (**2**−**6**), are produced by *Streptomyces iakyrus* strain DSM 41873. Their structures were established by spectroscopic methods. Unlike actinomycin D (**1**), the α-ring of the novel compounds contains the unusual amino acid 3-hydroxy-5-methylproline, while the β-ring includes *N*-methylalanine and either a chlorinated or hydroxylated threonine moiety. The chlorine-containing actinomycin G_2 (**2**) is the main product; it exhibits strong cytotoxic and antibacterial activities. Actinomycin G_5 (**5**) is the first actinomycin with an additional ring closure between the β-peptidolactone and the actinoyl chromophore. Actinomycin G_6 (**6**) resulted from the 4-hydroxythreonine-containing actinomycin G_3 (**3**) by a 2-fold acyl shift of the β-unit, which has not been observed before for this class of chromopeptides. The structural modification of compounds **5** and **6** goes along with an evident reduction of the biological activity. The biosynthesis of *aniso*-actinomycins is discussed.

Actinomycins are a well-known class of chromopeptides produced by various species of streptomycetes. About 20 naturally occurring actinomycins are known, and more than 40 variants have been obtained by precursor-directed biosynthesis.[1] All actinomycins share the same actinoyl chromophore (2-amino-4,6-dimethylphenoxazine-3-one-1,9-dicarboxylic acid) but differ in the amino acid composition of the two pentapeptidolactone residues.[2]

These chromopeptides show strong antineoplastic and antibacterial activities. Actinomycin D has found clinical application as an anticancer drug, for example in the treatment of infantile kidney tumors (Wilm's tumor).[3] The mechanism of action is based on intercalation of the phenoxazinone chromophore between two guanine/cytosine base pairs of the DNA double helix.[4,5] The peptidolactone side chains bind in both directions with the minor groove of the DNA. Thus, the binding strength is dependent on the flanking sequence as well as the amino acid composition.[6]

Actinomycin D (**1**) is the most common compound of this group of antibiotics and may be regarded as a reference structure. It is an *iso*-actinomycin because it contains the same amino acids (L-Thr, D-Val, L-Pro, Sar,[7] MeVal) in each peptidolactone unit.[2] *Aniso*-actinomycins consist of two different rings.[8] The third amino acid in both peptidolactones, usually L-Pro, seems to be the most variable position. For example, L-Pro is replaced by Sar,[9] 4-oxoproline (OPro), or 3-hydroxy-5-methylproline (HMPro). The latter are known from X-[10] and Z-type[11] actinomycins.



Actinomycin D (**1**)

Only four natural actinomycins have been described so far with variations of the amino acid in position 1 of the β-ring. Two include 4-chlorothreonine (ClThr), and the others 4-hydroxythreonine (HThr). The chlorine-containing actinomycins Z_3 and Z_5 possess a particularly strong cytotoxicity[11] compared with the HThr-containing actinomycins Z_1 and G_1.[12,13] Additionally, the Z- and G-type actinomycins include *N*-methyl-L-alanine (MeAla) instead of MeVal in position 5 of the β-unit, and the proline moieties are selectively methylated and oxidized (Table 1).

Here we describe the isolation, structure elucidation, and biological activities of five new G-type actinomycins. Two of these contain the unusual amino acid 4-chlorothreonine (ClThr) and 4-hydroxythreonine (HThr), respectively. Actinomycin G_3 (**3**), with HThr, may be susceptible to cyclization or rearrangement reactions, which result in actinomycins with previously unknown structural features.

Results and Discussion

Production and Isolation. A good production of actinomycins by *Streptomyces iakyrus* DSM 41873 was achieved by cultivation in a medium consisting of malt extract, yeast extract, and D-glucose. After harvesting and workup a green-colored and a more polar yellow-colored fraction were obtained by silica gel chromatography. The green fraction was purified using Sephadex LH-20 followed by preparative reversed-phase HPLC with acid-free solvents. The green color resulted from a mixture of four yellow pigments (**2**−**5**, 1−10 mg/L) and a blue one as minor component. Another yellow compound (**6**, 7 mg/L) was obtained from the polar fraction by repeated gel permeation chromatography.

Structure Elucidation. The typical UV/vis absorption spectra revealed the presence of an aminophenoxazinone chromophore in all of the isolated metabolites. Together with their molecular formulas, which were established by HRESIMS, they were recognized as members of the actinomycin family, especially by the presence of 12 nitrogen atoms (11 in the case of **5**). The amino acid compositions and complete structures were elucidated by 1D and 2D NMR spectroscopy. The assignment of the amino acids was done primarily by analysis of the HSQC and $^1H^1H$-COSY correlations and completed by an HMBC spectrum. The latter allowed the mapping of the quaternary carbonyl atoms and enabled some missing connections, particularly within the proline rings. The amino acid sequences in both peptidolactone units were unambiguously established by HMBC correlations. While the relative configuration of the residues was depicted from ^1H coupling constant analysis and NOESY correlations, the absolute configuration should be identical to that of actinomycin D (**1**). The exchange of an amino acid with its enantiomer would lead to a

* To whom correspondence should be addressed. Tel: +49 (0)551-393263. Fax: +49 (0)551-3912593. E-mail: azeeck@gwdg.de.
[†] University of Göttingen.
[‡] Bulgarian Academy of Sciences.

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Table 1. Amino Acid Sequences of G-[13] and Z-type[11] Actinomycins[a]

	α-ring	β-ring
actinomycin Z_1	Thr-D-Val-**HMPro**-Sar-MeVal	**HThr**-D-Val-**MOPro**-Sar-**MeAla**
actinomycin Z_2	Thr-D-Val-**HMPro**-Sar-MeVal	Thr-D-Val-**MOPro**-Sar-**MeAla**
actinomycin Z_3	Thr-D-Val-**HMPro**-Sar-MeVal	**ClThr**-D-Val-**MOPro**-Sar-**MeAla**
actinomycin Z_4	Thr-D-Val-**MPro**- Sar-MeVal	Thr-D-Val-**MOPro**-Sar-**MeAla**
actinomycin Z_5	Thr-D-Val-**MPro**- Sar-MeVal	**ClThr**-D-Val-**MOPro**-Sar-**MeAla**
actinomycin G_1	Thr-D-Val-Pro- Sar-MeVal	**HThr**-D-Val-**HMPro**-Sar-**MeAla**
actinomycin G_2 (**2**)	Thr-D-Val-**HMPro**-Sar-MeVal	**ClThr**-D-Val-Pro -Sar-**MeAla**
actinomycin G_3 (**3**)	Thr-D-Val-**HMPro**-Sar-MeVal	**HThr**-D-Val-Pro -Sar-**MeAla**
actinomycin G_4 (**4**)	Thr-D-Val-**HMPro**-Sar-MeVal	Thr-D-Val-Pro -Sar-**MeAla**
actinomycin G_5 (**5**)	Thr-D-Val-**HMPro**-Sar-MeVal	**HThr**[b]-D-Val-Pro −Sar-**MeAla**
actinomycin G_6 (**6**)	Thr-D-Val-**HMPro**-Sar-MeVal	**HThr**[c]-D-Val-Pro −Sar-**MeAla**

[a] Structural deviations from actinomycin D (**1**) are given in bold letters. These appear in position 1 (ClThr = 4-chlorothreonine, HThr = 4-hydroxythreonine) and position 5 (MeAla = *N*-methylalanine) of the β-ring, and partly in position 3 of the α- or β-ring or both (MPro = *cis*-5-methylproline, MOPro = *cis*-5-methyl-4-oxoproline, HMPro = *trans*-3-hydroxy-*cis*-5-methylproline). [b] HThr (β-ring) performs an additional ring closure with the chromophore. [c] Rearrangement of the HThr (β-ring) connectivities.

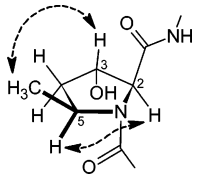
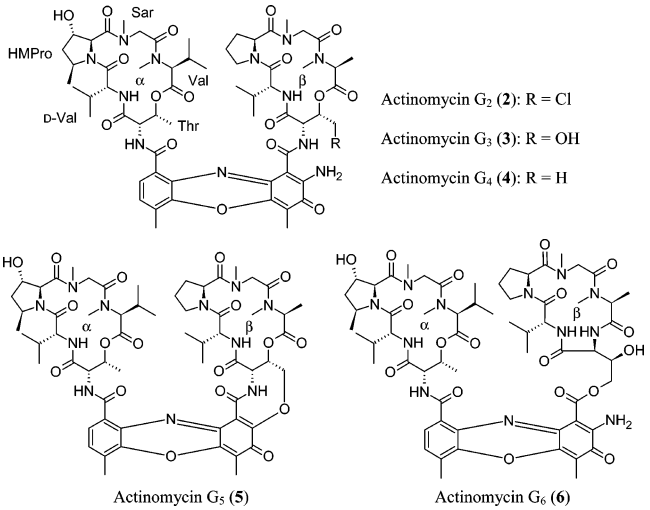
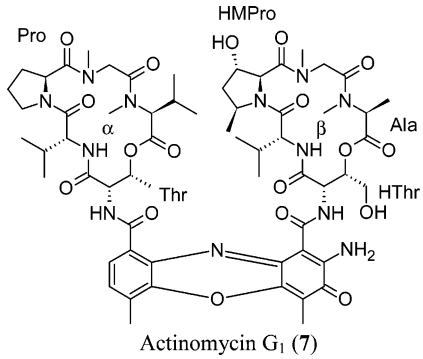


Figure 1. Relative configuration of the HMPro moiety, determined by NOESY correlations.

different conformation of the peptidolactone ring and thus to biologically inactive metabolites.[5] The replacement of all residues can be excluded due to the negative optical rotation values of the metabolites (except of **6**), which is also observed for **1**,[14] and by the fact that the enantiomer of actinomycin D is totally inactive.[15] Additional support for the identical absolute configuration of compounds **2**−**5** is given by their CD spectra, which all exhibit a negative Cotton effect near 210 nm.



The isotopic pattern found in the ESIMS of actinomycin G_2 (**2**) indicated the presence of one chlorine atom. HRMS confirmed this result and determined the molecular formula as $C_{61}H_{83}N_{12}O_{17}Cl$. Analysis of the NMR spectra revealed seven amino acids to be identical to those of actinomycin D (**1**) (MeVal, 2 × Sar, Pro, 2 × Val, and Thr) and three to be modified: *N*-methylalanine (MeAla), *trans*-3-hydroxy-*cis*-5-methylproline (HMPro), and 4-chlorothreonine (ClThr). MeAla was indicated by a C-methyl group (δ_H 1.32) resonating at lower frequency compared with Val. It showed a $^3J_{HH}$ coupling with a CH group at δ_H 3.33, which is the α-proton of MeAla. The α-proton of HMPro (δ_H 5.97) appears as a singlet; the dihedral angle H−C(2)−C(3)−H is thus supposed to be approximately 90°. Consequently, 3-OH is in a *trans* position to the amide group, while the *cis* configuration of the C-methyl group (δ_H 1.48) attached to C-5 is proven by the NOEs between 3-H and 5-CH_3 and between 2-H and 5-H (Figure 1). ClThr revealed an additional methylene group instead of a C-methyl. Its chemical shift (δ_H 3.94/3.99) is due to the C-4 chlorine atom. From the HMBC spectrum, the connectivities between the amino acids and between the threonine units and the chromophore were determined (see Supporting Information). The complete NMR data of actinomycin G_2 are given in Table 2. Its structure **2** is closely related to that of actinomycin G_1[1,13] (**7**), which differs from **2** by the HMPro location and by the presence of HThr instead of ClThr in the β-ring.



Compared with **2**, the molecular formula of actinomycin G_3 (**3**) ($C_{61}H_{84}N_{12}O_{18}$) suggested the replacement of the chlorine by an oxygen atom. This was confirmed by the downfield shift of C-4 (δ_C 59.4) of the Thr unit in the β-ring (Table 3). The amino acid sequence is the same as depicted for **2**. Actinomycin G_3 (**3**), thus, is an isomer of actinomycin G_1 (**7**), but bears the HMPro moiety in the opposite peptidolactone. The ^{1}H NMR spectrum of actinomycin G_4 (**4**) is almost identical with that of **2** and **3**, but the characteristic 3-H signal of a substituted Thr residue is missing. An additional methyl group at δ_H 1.30 indicates the presence of a second unmodified Thr. The structure of **4** is consistent with the formula $C_{61}H_{84}N_{12}O_{17}$, which followed from HRESIMS data.

The UV spectrum of actinomycin G_5 (**5**) exhibited an additional maximum at 260 nm, indicating structural changes near the chromophore. The molecular formula $C_{61}H_{81}N_{11}O_{18}$ shows two peculiarities: **5** has one nitrogen atom less than compounds **2**−**4**, and the calculated number of double-bond equivalents is 27, instead of 26 for compounds **2**−**4**. Since the number of sp^2 carbon atoms was the same, an additional ring must exist. The NMR spectra revealed the same amino acid composition and sequence as deduced for actinomycin G_3 (**3**). However, the chemical shifts of the chromophore and of HThr show significant deviations from those of **3**. These are due to an additional ring closure between 4-OH of the HThr and C-2 of the chromophore, which is proven by a $^3J_{CH}$ HMBC correlation (Figure 2). This novel ether bond causes a downfield ^{13}C shift of both the methylene group at position 4 of HThr (δ_C 68.5) and C-2 of the chromophore (δ_C 168.0).

Table 2. NMR Data of Actinomycin G_2 (**2**) ($CDCl_3$, ^1H: 600 MHz, ^{13}C: 150.8 MHz)

Pentapeptidolactones									
α-ring	pos.	δ_C	δ_H	*J* [Hz]	β-ring	pos.	δ_C	δ_H	*J* [Hz]
Thr	1	168.49			ClThr	1	168.07		
	2	54.82	4.44	dd, 7.0, 2.9		2	53.54	5.27	dd, 7.6, 2.7
	3	74.72	5.28	qd, 6.3, 2.9		3	74.98	5.12	dt, 5.0, 2.5
	4	17.15	1.20	d, 6.3		4	43.21	3.94	dd, 11.4, 2.5
	NH		6.96	d, 7.1				3.99	dd, 11.4, 6.0
						NH		7.88	d, 7.3
d-Val	1	173.91			d-Val	1	173.23		
	2	59.38	3.37	dd, 10.0, 5.0		2	58.57	3.66	m
	3	31.77	2.10	m		3	31.78	2.08	m
	4	19.25	1.10	d, 6.7		4	19.28	0.90	d, 6.7
	5	19.04	0.86	d, 6.8		5	19.00	1.09	d, 6.7
	NH		8.20	d, 5.2		NH		8.12	d, 6.2
HMPro	1	170.95			Pro	1	173.17		
	2	68.22	5.97	s		2	56.82	5.90	d, 9.0
	3	75.44	4.06	d, 5.5		3	31.07	1.84	dd, 12.0, 6.5
	4	43.21	2.11	m				2.91	m
	5	53.78	2.19	m		4	22.67	2.07	m
			4.76	m				2.18	m
	6	18.73	1.48	d, 6.0		5	47.48	3.68	m
								3.80	m
Sar	1	166.12			Sar	1	166.52		
	2	51.51	3.64	d, 17.5		2	51.25	3.61	d, 17.5
			4.74	d, 17.5				4.69	d, 17.5
	NMe	34.97	2.86	s		NMe	34.84	2.83	s
MeVal	1	167.53			MeAla	1	168.85		
	2	71.27	2.65	d, 9.3		2	59.60	3.33	q, 7.0
	3	26.92	2.63	m		3	13.42	1.32	d, 7.0
	4	21.63	0.93	d, 6.4		NMe	36.71	2.91	s
	5	19.00	0.71	d, 6.8					
	NMe	39.40	2.91	s					

Chromophore

δ_H 2.13 (s, 3H, 12-H_3), 2.49 (s, 3H, 11-H_3), 7.31 (dq, $J = 7.7$, 0.9 Hz, 1H, 7-H), 7.53 (d, $J = 7.6$ Hz, 1H, 8-H).

δ_C 7.79 (CH_3, C-12), 14.97 (CH_3, C-11), 99.92 (C, C-1), 113.81 (C, C-4), 125.36 (CH, C-8), 127.57 (C, C-6), 128.86 (C, C-9a), 130.26 (CH, C-7), 132.65 (C, C-9), 140.47 (C, C-5a), 145.18 (C, C-4a), 146.04 (C, C-10a), 147.67 (C, C-2), 166.52 (C, C-13), 168.96 (C, C-14), 178.49 (C, C-3).

Table 3. NMR Data of the 4-Chlorothreonine and 4-Hydroxythreonine Moiety, Respectively, as Part of Actinomycins G_2 (**2**) and G_3 (**3**) ($CDCl_3$, ^1H: 600 MHz, ^{13}C: 150.8 MHz)

	ClThr in **2**		HThr in **3**	
atom	δ_C	δ_H	δ_C	δ_H
1	168.1		168.4	
2	53.5	5.27, dd (7.5, 2.5)	52.0	5.20, dd (7.0, 2.0)
3	75.0	5.12, ddd (5.0, 2.5, 2.5)	75.4	5.05, ddd (8.5, 5.0, 2.5)
4	43.2	3.94, dd (11.5, 2.5)	59.4	3.23, dd (12.0, 9.0)
		3.99, dd (11.5, 6.0)		3.76, dd (12.0, 4.5)
NH		7.88, d (7.5)		8.28, d (6.5)

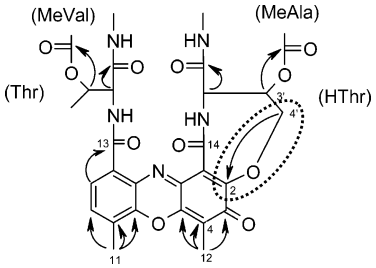


Figure 2. Important HMBC correlations for actinomycin G_5 (**5**).

The molecular mass and formula of actinomycin G_6 (**6**) coincide with those of **3**. The UV spectrum shows an intact phenoxazinone chromophore. From the NMR spectra followed that both compounds have the same amino acid composition, although the signals of the β-ring show remarkable deviations from their normal values. This indicates the loss of the so-called A-conformation[5,16] of the peptidolactone unit, which includes two *cis* peptide bonds (Sar→L-Pro, L-Pro→D-Val). The lower geminal coupling constant of the methylene group of sarcosin (14.5 instead of 18 Hz) refers to a *trans*-configurated peptide bond.[5] The α-protons of Pro (upfield shift from δ_H 6.1 to 4.9) and of MeAla (downfield shift from δ_H 3.5 to 5.4) provided additional evidence for strong configurational

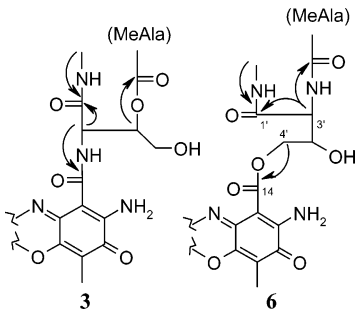


Figure 3. Comparison of important HMBC correlations for actinomycins G_3 (**3**) and G_6 (**6**).

Table 4. Antibacterial Activity of Actinomycins G_2−G_6 (**2**−**6**) Compared with Actinomycin D (**1**)[a]

test organism	*E. coli*	*S. aureus*	*B. subtilis*
2	15	27	45
3	0	21	33
5	0	10	20
6	0	0	0
actinomycin D (**1**)	20	30	55

[a] Diameter of inhibition zones in the plate diffusion assay in mm (50 μg of compound on 6 mm filter disk).

Table 5. Cytotoxic Activity of Actinomycins G_2−G_6 (**2**−**6**) against Different Tumor Cell Lines: HM02 (gastric adenocarcinoma), HepG2 (hepatocellular carcinoma), and MCF7 (breast adenocarcinoma)[a]

	HM02		HepG2		MCF7	
	GI_{50}	TGI	GI_{50}	TGI	GI_{50}	TGI
2	0.0013	0.0043	0.0029	0.018	0.0039	0.012
3	0.13	0.55	1.1	>7.9	0.46	1.6
4	0.065	0.52	0.64	1.4	0.40	1.5
5	2.1	4.4	2.7	7.8	1.4	2.5
6	1.0	2.0	1.6	>7.9	1.4	4.6
actinomycin D (**1**)	0.0016	0.0064	0.0012	0.052	0.0019	0.0088
2-aminophenoxazinone	4.5	10	6.6	26	0.61	2.0

[a] The GI_{50} and TGI values are given in μM. GI_{50} is the concentration at which half of the cells were inhibited in their growth; TGI is the concentration at which a total inhibition of cell growth was observed.

changes. This resulted from a change in the connectivities coming from HThr of the β-ring, which were established by HMBC correlations (Figure 3). HThr is connected with MeAla by an amide bond and with the β-carboxylic acid group of the chromophore by an ester bond originating from 4-OH. The 3-OH group of HThr remains free. Thus, the peptidolactone in β-position was changed to a cyclopentapeptide.

Biological Activity. The actinomycins **2**, **3**, **5**, and **6** were tested against different bacteria in a plate diffusion assay (Table 4). Actinomycin G_2 (**2**) exhibited the highest antibacterial activity, although it was still lower than that of actinomycin D as reference. The HThr-containing actinomycin G_3 (**3**) showed significantly lower antibacterial effects. The cyclized actinomycin G_5 (**5**) follows next, while the cyclopeptide ester **6** is inactive.

The cytotoxic activity of **2** against three human tumor cell lines (Table 5) is comparable with that of actinomycin D. The other actinomycins exhibit significantly lower inhibition values in the descending order **4** > **3** > **5** > **6**. Noteworthy, the actinomycins **5** and **6**, with modified core structures, are still weakly cytotoxic.

Conclusion. *S. iakyrus* DSM 41873 was found to produce five new actinomycins, which differ around the threonine moiety of the β-ring. Actinomycin G_4 (**4**), with an unmodified Thr, was found to be a minor compound (1 mg/L), while actinomycin G_2 (**2**), with ClThr, is the main product of the strain (10 mg/L). The chloro substituent at C-4 of Thr does not change the cytotoxicity (Table 5), as it was already described for actinomycins Z_3 and Z_5.[11] HThr in the β-ring lowers the biological activity significantly. Although actinomycin Z_1 revealed HThr in the same position as actinomycin G_3 (**3**), no derivatives have been described with a subsequent ring closure or rearrangement, as it has been observed for actinomycins G_5 (**5**) and G_6 (**6**), respectively. The 2-fold acyl rearrangement leading to a completely different actinomycin core structure seems to be a privileged ability of strain DSM 41873.

Presumed Biosynthesis of *Aniso*-Actinomycins. The biosynthesis of the actinomycins proceeds in three steps: (i) The precursor 4-methyl-3-hydroxyanthanilic acid (4-MHA) is built from L-tryptophan,[17] (ii) three multifunctional enzymes catalyze the formation of the pentapeptidolactone using 4-MHA as starter unit, and (iii) an oxidative coupling of two 4-MHA-pentapeptidolactone units occurs to complete the chromopeptide, resulting in an *iso*-actinomycin.[18,19] The enzyme catalyzing the dimerization has not been identified yet; a formerly assumed participation of a common phenoxazinone synthase was not confirmed.[18,20]

Astonishingly, no investigation has been done to establish the biosynthesis of *aniso*-actinomycins with two different peptidolactone units. In the case of C-[8,21] and F-type[9,22] actinomycins it is likely that a low substrate specificity of the second and third adenylation domain of the NRPS leads to the incorporation of different amino acids, which therefore may appear in one or both peptidolactone units. In contrast, the new G-type and the Z-type[11] actinomycins are known to bear a MeVal moiety only in the α-ring and a MeAla moiety only in the β-ring (Table 1). Because no mixture within the isolated metabolites was observed, the dimerizing enzyme should be responsible for the observed selectivity. From this assumption follows (i) an exclusion of molecular oxygen in order to prevent an uncontrolled dimerization[20] and (ii) different strains of streptomycetes having different dimerizing enzymes to guarantee the biosynthesis of strain-specific actinomycins. To rule out enzyme specificity at this level, a biosynthetic interconversion of the amino acids MeVal and MeAla must be assumed, which seems unlikely. On the other hand, the regioselective methylation and oxidation of the proline residues in G-, X-, and Z-type actinomycins could be done by *post*-NRPS enzymes, accepting either the monomeric units or the dimers. Thus a comparative analysis of the biosynthetic genes of different actinomycin producers seems to be forward-looking for understanding the molecular background of the diversity within this important class of antibiotics.

Putative Biosynthetic Relationship of Actinomycins G_2−G_6 (2−6). Actinomycin G_4 (**4**) is considered to be the precursor for further modifications in the β-ring. Chlorination or hydroxylation of Thr leads to actinomycins G_2 (**2**) and G_3 (**3**), respectively. A nonheme Fe^{II} α-ketoglutarate- and O_2-dependent halogenase that chlorinates threonine during the syringomycin E biosynthesis has been characterized recently.[23] As similar enzymes are known to catalyze hydroxylations,[24] **2** and **3** could be produced by the same enzyme that lacks specificity. Alternatively, the ClThr of **2** could be hydrolyzed to give **3**.

Actinomycin G_5 (**5**) is build from **3** by nucleophilic attack of the 4-OH of HThr on C-2 of the chromophore. Finally, a 2-fold acyl displacement of **3** with hexa- and pentacyclic intermediates results in actinomycin G_6 (**6**). The participation of an enzyme on these transformations remains unclear. Although all components were detected in the crude extract by HPLC, **5** and **6** could be formed nonenzymatically during workup of the culture broth. However, no analogues are known from the Z-type actinomycins, which were obtained using a similar procedure for extract preparation,[11] and actinomycin G_3 (**3**) is stable in acidic solution.

Structure−Activity Relationships. Actinomycin G_2 (**2**) exhibits the highest cytotoxic and antibacterial activities. This agrees with previous results reported for the Z-type actinomycins; the chlorinated Z_3 and Z_5 showed the highest cytotoxicity.[11] By the change to a hydroxy group in the same position, the biological activity is significantly reduced. This is probably due to hydrogen bonding of 4-OH of HThr and the 2-amino group of the chromophore, diminishing the required participation of the amino group in DNA intercalation.[25] The substitution of the 2-amino group in **5** and the loss of the actinomycin core structure in the β-ring of **6** lead to metabolites with very low cytotoxicity.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Varian Inova 600 spectrometer at 298 K. Chemical shifts were determined relative to the solvent as internal standard ($CDCl_3$: δ_H 7.25, δ_C 77.00; CD_3OD: δ_H 3.30, δ_C 49.00). Optical rotations were obtained in MeOH with a Perkin-Elmer 241 polarimeter. UV and CD spectra were obtained in methanol on a Varian Cary 3E and a Jasco J-500 spectrometer, respectively. Infrared spectra were recorded on a Perkin-Elmer FTIR 1600 spectrometer as KBr pellets. ESIMS spectra were

obtained on a Finnigan LC-Q and high-resolution ESIMS spectra on a Bruker Apex-Q III (field strength 7 T). TLC was carried out on Si gel 60 F_{254} plates (Merck, 0.2 mm).

Biological Material. The producing microorganism *S. iakyrus* DSM 41873 was isolated from the rhizosphere of an orange tree (*Citrus sinensis*), growing in Zur Moshe, Israel.[26] The taxonomical identification was done using both chemotaxonomic and genetic methods (16S rDNA sequencing). The strain is on deposit in the German Collection of Microorganisms and Cell Cultures (DSMZ). Fermentations were carried out in 300 mL Erlenmeyer flasks with three baffles. Each flask was filled with 100 mL of medium consisting of malt extract 1%, yeast extract 0.4%, and D-glucose 0.4% in deionized H_2O (pH 7.0). The flasks were inoculated with a 1 cm^2 piece of a well-grown agar plate and cultivated for 4 days at 28 °C on a rotary shaker (180 rpm). At harvest time the cultures had become greenish; the pH value was 7.1.

Sample Workup and Isolation Procedure. A 4 L amount of whole fermentation broth was centrifuged (4500 rpm, 10 min). The supernatant was discarded, and the mycelium was extracted with acetone (3 × 1.2 L). The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography. Elution with CH_2Cl_2/MeOH (gradient from 0% to 20% MeOH, column 50 × 5 cm) yielded two fractions, A and B. Fraction A was separated further by gel chromatography (Sephadex LH-20, acetone, column 80 × 2.5 cm) into fractions A1 and A2. Both portions were applied to preparative HPLC using a Nucleodur 100 C18 column (5 μm, 250 × 16 mm, endcapped, Macherey-Nagel) with a flow rate of 17 mL/min and UV detection at 254 nm. Isocratic elution of fraction A1 with 54% CH_3CN in H_2O led to **2** (40 mg) and **4** (3.5 mg), and isocratic elution of fraction A1 with 51% CH_3CN in H_2O led to **3** (12 mg) and **5** (8 mg). Fraction B was purified by repeated gel chromatography (Sephadex LH-20, MeOH and acetone, columns 100 × 2.5 cm and 80 × 2.5 cm, respectively) to yield pure **6** (28 mg).

Biological Tests. For plate diffusion assays, 50 μg of the desired compound was solved in acetone or MeOH and dropped on paper disks (ϕ 6 mm, thickness 0.5 mm). These were dried under sterile conditions and put on agar plates inoculated with the test organism (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*). The plates were cultivated at 37 °C (bacteria) or 25 °C (yeast) for 24 h. The cytotoxic activity was determined according to the NCI guidelines[27] with the tumor cell lines HM02 (gastric adenocarcinoma), HepG2 (hepatocellular carcinoma), and MCF7 (breast adenocarcinoma).

Actinomycin G_2 (2): orange solid; R_f 0.53 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D^{20}$ −214 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 240 (4.44), 426 (4.24), 440 (4.25) nm; CD (MeOH) λ_{max} ($[\theta]$) 212 (−178 300), 242 (33 700), 270 (−73 900), 378 (−24 600) nm; IR (KBr) ν_{max} 3421, 3055, 2963, 2931, 2862, 1749, 1668, 1635, 1580, 1507, 1475, 1360, 1306, 1192, 1097, 753 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Table 2; ^1H and ^{13}C NMR (CD_3OD), see Table S1 (Supporting Information); ESIMS *m*/*z* pos. 1291 $[M + H]^+$, 1313 $[M + Na]^+$; neg. 1289 $[M - H]^-$; HRESIMS *m*/*z* 646.291570 (calcd for $C_{61}H_{85}N_{12}O_{17}{}^{35}Cl$ $[M + 2H]^{2+}$, 646.291660).

Actinomycin G_3 (3): orange solid; R_f 0.51 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D^{20}$ −83 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 236 (4.39), 426 (4.12), 444 (4.13) nm; CD (MeOH) λ_{max} ($[\theta]$) 210 (−128 500), 240 (7700), 269 (−26 300) nm; IR (KBr) ν_{max} 3430, 2941, 1735, 1644, 1503, 1467, 1312, 1266, 1196, 1115, 1035, 804, 667 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Table S2 (Supporting Information); ^1H and ^{13}C NMR (CD_3OD), see Table S3 (Supporting Information); ESIMS *m*/*z* pos. 1273 $[M + H]^+$, 1295 $[M + Na]^+$; neg. 1271 $[M - H]^-$; HRESIMS *m*/*z* 637.308708 (calcd for $C_{61}H_{86}N_{12}O_{18}$ $[M + 2H]^{2+}$, 637.308603).

Actinomycin G_4 (4): orange solid; R_f 0.31 ($CHCl_3$/MeOH, 9:1); UV-(MeOH) λ_{max} (log ϵ) 238 (4.45), 426 (4.26), 439 (4.27) nm; CD (MeOH) λ_{max} ($[\theta]$) 210 (−111 300), 240 (5000), 277 (−18 700) nm; ^1H and ^{13}C NMR (CD_3OD), see Table S4 (Supporting Information); ESIMS *m*/*z* pos. 1257.5 $[M + H]^+$, 1279.6 $[M + Na]^+$; neg. 1255.6 $[M - H]^-$; HRESIMS *m*/*z* 629.311171 (calcd for $C_{61}H_{86}N_{12}O_{17}$ $[M + 2H]^{2+}$, 629.311146).

Actinomycin G_5 (5): orange solid; R_f 0.36 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D^{20}$ −38 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 234 (4.18), 260 (4.04), 418 (3.69), 443 (3.64) nm; CD (MeOH) λ_{max} ($[\theta]$) 212 (−57 900), 261 (2900), 304 (−9500) nm; IR (KBr) ν_{max} 3434, 2931, 2858, 1745, 1654, 1460, 1376, 1192, 1135, 800, 668 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD), see Table S5 (Supporting Information); ESIMS *m*/*z* pos. 1255.5 $[M + H]^+$, 1278.6 $[M + Na]^+$; neg. 1254.7 $[M - H]^-$; HRESIMS *m*/*z* 1256.583064 (calcd for $C_{61}H_{82}N_{11}O_{18}$ $[M + H]^+$, 1256.583381).

Actinomycin G_6 (6): orange solid; R_f 0.27 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D^{20}$ +16 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 237 (4.45), 435 (4.27) nm; CD (MeOH) λ_{max} ($[\theta]$) 238 (22 000), 252 (7600), 277 (25 600) nm; IR (KBr) ν_{max} 3435, 3313, 2966 (sh), 1732, 1644, 1582, 1525, 1447, 1318, 1475, 1194, 1101, 1001 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD), see Table S6 (Supporting Information); ESIMS *m*/*z* pos. 1273.6 $[M + H]^+$, 1295.6 $[M + Na]^+$; neg. 1271.6 $[M - H]^-$; HRESIMS *m*/*z* 637.30854 (calcd for $C_{61}H_{86}N_{12}O_{18}$ $[M + 2H]^{2+}$, 637.30860).

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Supporting Information Available: ^1H and ^{13}C NMR data of actinomycins G2 to G6 (Tables S1−S6) and different spectra of actinomycins G2 and G6 (Figures S1−S9) are available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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