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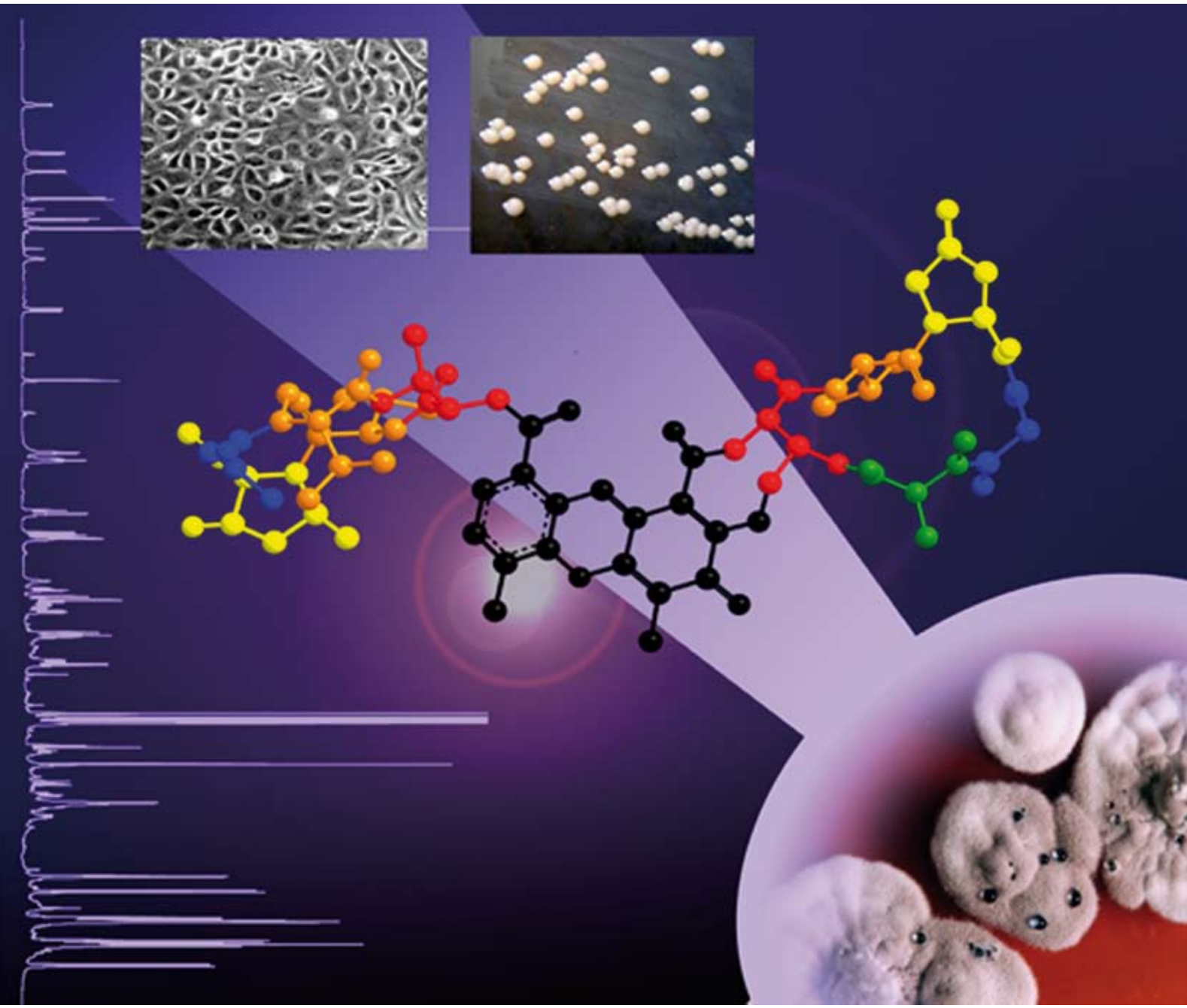

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FULL PAPER

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In this issue...

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First Y-type actinomycins from *Streptomyces* with divergent structure-activity relationships for antibacterial and cytotoxic properties†

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Streptomyces sp. strain Gö-GS12 was found to produce five novel actinomycins Y_1–Y_5 (**1–5**). Their amino acid pattern discloses them as members of a new family of this important class of antibiotics. Compounds **1–5** differ from Z-type actinomycins in their β-peptidolactone rings which here contain *trans*-4-hydroxyproline (Hyp) or 4-oxoproline (OPro) amino acids, and from the X-congeners by containing methylalanine (MeAla). Within the new Y-type actinomycins variations are not only in the rare chlorinated or hydroxylated threonine residue. Furthermore, the β-ring can undergo rearrangement by a two-fold acyl shift (compounds **3** and **4**) or show a unique additional ring closure with the chromophore (compound **5**), resulting in metabolites with yet unknown structural motifs, altered conformations and distinct bioactivities. The strongest bioactivity was found for the chlorine containing actinomycin Y_1 (**1**), the most surprising for Y_5 (**5**) with cytotoxic and antibacterial effects losing their coherence, which has been observed for the first time here.

Introduction

The actinomycins are a family of chromopeptides with potent cytotoxic and antibiotic activities. They consist of an actinoyl chromophore (2-amino-4,6-dimethylphenoxazine-3-one-1,9-dicarboxylic acid) with two cyclic pentapeptidolactones connected to its carboxyl groups. Actinomycin D is the most prominent member of this family and has found clinical application as a chemotherapeutic agent, particularly in the treatment of infantile kidney tumors.[1]

The bacterial producers usually biosynthesize a mixture of four or more different actinomycins which are called actinomycin complexes.[2] About 25 naturally occurring single congeners have been described so far,[3,4] differing in the amino acid compositions of the two pentapeptidolactones and in their important cytotoxic properties. An updated overview on all natural congeners is given in the Supplementary Table S3. Several other actinomycins have been described without elucidation of their molecular structures.[3,5,6] The actinomycins act as intercalators with the DNA double helix. Their phenoxazinone chromophore fits between two guanine/cytosine base pairs, with the peptidolactone side chains lying inside the minor groove of the helix.[7] Thus, the binding strength is dependent on the amino acid composition.[8–10]

Clinically in use, actinomycin D may be regarded as a reference structure (Fig. 1).[2] Both peptidolactone units are identical (*iso*-actinomycin) although most other actinomycins feature two different rings (*aniso*-actinomycins).[11] Presently, the most variable position of the α- and β-peptidolactones turns out to be the third amino acid. Usually L-Pro, it can be replaced by Sar[12], 4-oxoproline (OPro), *trans*-4-hydroxyproline (Hyp), *cis*-5-methyl-4-oxoproline (MOPro), 3-hydroxy-*cis*-5-methylproline (HMPro) and *cis*-5-methylproline (MPro); all are known from G-,[4,13] X-,[14] and Z-type[15] actinomycins. Additionally some G- and Z-type analogues exhibit the rare amino acids 4-chlorothreonine (ClThr) or 4-hydroxythreonine (HThr) in position 1, and *N*-methyl-L-alanine (MeAla) in position 5 of the β-unit, while X-type actinomycins only obtain threonine (Thr) and *N*-methyl-L-valine (MeVal) in the respective positions.

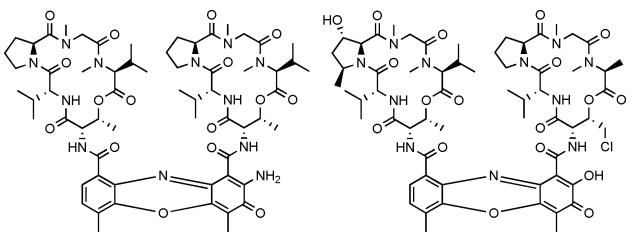

Fig. 1 Structural formulae of actinomycin D (left) and G_2 (right).

During the course of our chemical screening efforts with streptomycetes, strain Gö-GS12 attracted our attention by the production of orange pigments which were found in both mycelium and supernatant extracts. In the present work, we describe the production, isolation, and structure elucidation of five new actinomycins Y_1–Y_5 (**1–5**) with their distinct amino acid patterns in respect of the X- and Z-complex congeners. Their cytotoxic potency against different human tumor cell lines and their antimicrobial activity were determined in parallel. Structure-activity relationships (SAR) are discussed.

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† Electronic supplementary information (ESI) available: Overview of known actinomycins; general experimental procedures and those for amino acid analysis; detailed isolation scheme; additional biological data; assigned ^{1}H and ^{13}C NMR data for actinomycins Y_2–Y_5 (**2–5**); ^{1}H NMR spectra of **1–5**; ^{13}C NMR spectra of **1** and **3–5**. See DOI: 10.1039/b815689a

Results and discussion

Production, isolation and structure elucidation

Streptomyces sp. strain Gö-GS12 was found to produce several secondary metabolites, five of them orange-colored pigments. Their UV/Vis spectra (λ_{max} 238, 429, 441 nm) pointed to an aminophenoxazinone chromophore as a common structural feature. The presence of actinomycins was deduced with respect to the high molecular weights between 1269 and 1307 g/mol, though the exact values did not correspond with any known chromopeptide.

The individual components of the actinomycin complex were isolated to purity (see Experimental section for details), yielding one major component (12 mg/L, **1**) and four minor congeners (0.6–7.0 mg/L, **2–5**) (Fig. 2). In addition, an aromatic amide was isolated and chemically characterized. For structure elucidation, the compounds were subjected to high resolution ESI mass spectrometry (HR-ESIMS) and detailed 1D and 2D NMR analysis. It proved to be efficient to first unequivocally determine the molecular formulae by HR-ESIMS data, which was achieved by restricting the number of nitrogen atoms to 10–14 referring to the amino acid composition of the two peptidolactone rings (Table 1). The second step was the identification and NMR assignment of the chromophore and single amino acids by COSY, HSQC and HMBC correlations, supported by TOCSY experiments if necessary. Finally, as a third step, all residues were connected on the basis of HMBC and NOESY correlations, thus establishing the amino acid sequences and overall constitution.

The mass spectrum of the main product, compound **1**, showed a quasi-molecular ion at $m/z = 1305$ $[M + H]^+$. The isotopic pattern indicated the presence of a chlorine atom, and high resolution data provided the molecular formula $C_{61}H_{81}N_{12}O_{18}Cl$. Detailed analysis of ^1H, ^{13}C, COSY, HSQC, and HMBC NMR spectra revealed ten amino acids for **1**, six of which being identical with those of actinomycin D (MeVal, Thr, 2 × Sar, 2 × Val). The four altered amino acids were *N*-methyl-L-alanine (MeAla), *trans*-3-hydroxy-*cis*-5-methylproline (HMPro), 4-oxoproline (OPro), and 4-chlorothreonine (ClThr). The amino acid composition is similar to those of G- and Z-type actinomycins, the latter additionally contain the *cis*-5-methyl-4-oxoproline (MOPro), but both types never show the very rare 4-oxoproline (OPro).[3,4] Hence, we named the unusual, newly found compounds as Y-type members.

OPro was easily identified by the ketone moiety (δ_C 208.6) and the altered chemical shifts and coupling patterns of the neighbouring methylene groups (Fig. 3A, Table 2). For MeAla, HMPro, and ClThr, we published a detailed spectroscopic characterization recently for the G-type actinomycins.[4] In short, the relative configuration of HMPro is based on interpretation of coupling patterns (2-H is a singlet, indicating a 90° dihedral angle to 3-H) and nuclear Overhauser effects (between 5-H and 2-H, and 5-CH_3 and 3-H). Therefore, 3-OH is *trans* and 5-Me is *cis* to the HMPro-carbonyl group. Having identified the individual building blocks, the connectivity of actinomycin Y_1 (**1**) was established successfully by HMBC correlations (Fig. 3B).

The α-peptidolactone ring bears the rare 'Z-type' HMPro in position 3 as the only modification compared to actinomycin D (*cf.* Fig. 1). In contrast, the β-peptidolactone bears MeAla instead of MeVal in position 5, while OPro and ClThr are located in position 3 and 1, respectively. The absolute configurations of the amino acids were supposed to be identical with actinomycin D, as indicated by the negative optical rotation values and the strong cotton effect at about 210 nm in the CD spectra. Both

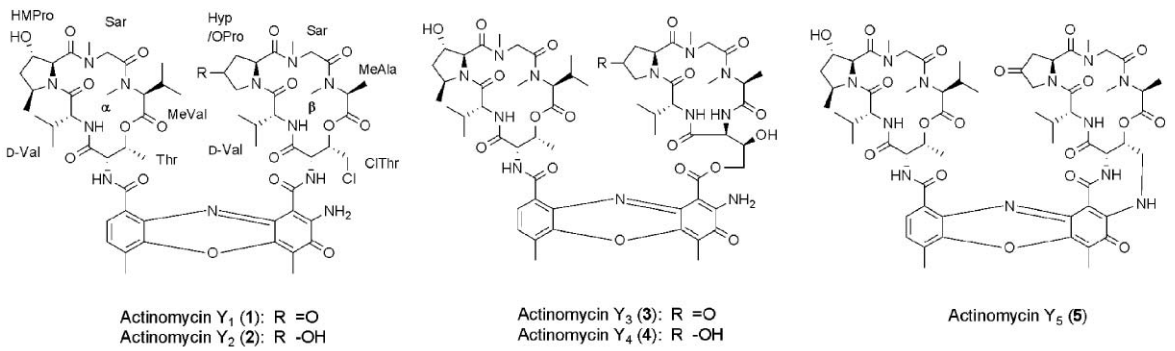

Fig. 2 The novel Y-type actinomycins.

Table 1 Analytical data of compounds **1–6**

					HR-ESIMS $[M + 2H]^{2+}$		
	R_f [a])	Molecular formula	M_r [g/mol]	$M_{monoisot.}$[g/mol]	measured m/z	calcd. m/z	Δ [ppm]
1	0.57	$C_{61}H_{81}N_{12}O_{18}Cl$	1305.82	1304.55	653.28125	653.28129	0.06
2	0.38	$C_{61}H_{83}N_{12}O_{18}Cl$	1307.84	1306.56	654.28886	654.28913	0.39
3	0.26	$C_{61}H_{82}N_{12}O_{19}$	1287.38	1286.58	644.29822	644.29824	0.03
4	0.23	$C_{61}H_{84}N_{12}O_{19}$	1289.39	1288.60	635.29295	635.29295	0.00
5	0.30	$C_{61}H_{80}N_{12}O_{18}$	1269.36	1268.57	645.30588	645.30606	0.28
6	0.12	$C_{10}H_{12}N_2O_3$	208.21	208.08	231.07393	231.07401[b]	0.37

[a] R_f values were determined on silica gel using chloroform/methanol (9:1) as mobile phase. [b] $[M + Na]^+$.

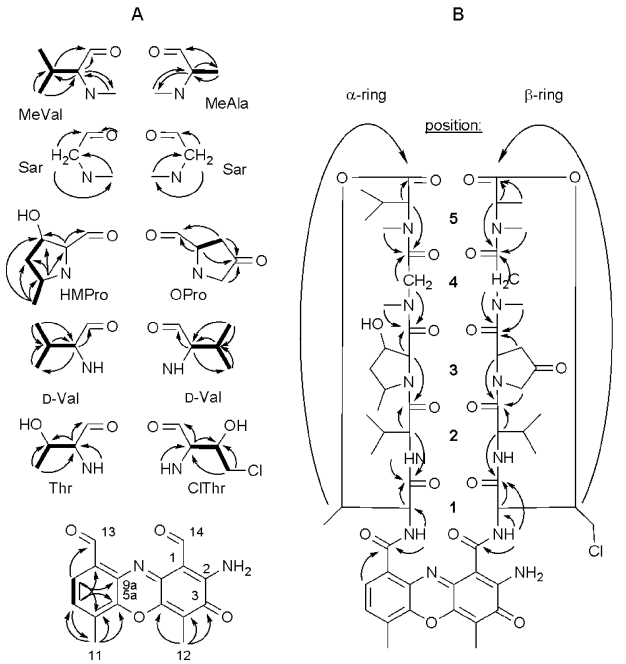

Fig. 3 **A** Intra-residual COSY and HMBC connectivities for actinomycin Y_1 (**1**); **B**. inter-residual couplings for **1**; bold lines: COSY correlations; arrows: HMBC correlations.

values resemble well published data for other actinomycins.[2,4,13,15] Additionally, a small amount of **1** was hydrolyzed, and the free amino acids were analyzed by HPLC after chiral derivatization with Marfey's reagent.[16] Comparison with authentic standards revealed the presence of L-MeVal, L-Thr and D-Val, as expected. Although neither the altered proline and threonine moieties nor *N*-methylated alanine were available as references, they can be assumed to possess L-configurations due to the fact that the exchange of a single amino acid with its enantiomer leads to significant conformational changes of the respective peptidolactone ring, resulting in reduced biological activities as well as chemical shift deviations that have not been observed here.[17]

The molecular formula of actinomycin Y_2 (**2**), $C_{61}H_{83}N_{12}O_{18}Cl$, indicated two additional hydrogen atoms compared to **1**. This was confirmed by NMR analysis, which revealed a *trans*-4-hydroxyproline moiety instead of 4-oxoproline in the β-ring. For actinomycin Y_3 (**3**), HR-ESIMS gave the molecular formula $C_{61}H_{82}N_{12}O_{19}$ which indicated the lack of chlorine. In addition, the chemical shifts of all β-ring amino acids significantly differed from those obtained for **1** and **2**, indicating strong conformational changes in the β-unit of **3**. While NMR analysis proved that the α-unit is retained unchanged, the threonine residue in position 1 of the β-ring revealed an oxygen-substitution at C-4 which is linked to the carbonyl group of the chromophore by an ester bond. The amino group of **3**, instead, mediates ring closure to the fifth amino acid (MeAla) (see Supplementary Fig. S2). Hence, the β-ring is a true cyclopentapeptide and explains the observed conformational changes of actinomycin Y_3 (**3**) compared to Y_1 (**1**) and Y_2 (**2**). The altered connectivity of the β-ring has been observed and discussed before for actinomycin G_6.[4] It can be explained by a nucleophilic substitution of the chlorine atom by a hydroxy group. The resulting intermediate undergoes a two-fold acyl shift, which may be thermodynamically driven and catalyzed by neighbouring-group effects, though enzyme participation cannot be excluded without further investigations. The structure of actinomycin Y_4 (**4**) coincides with that of **3** except for the β-ring 4-oxoproline (OPro) residue, which again is Hyp as found for **2**.

The structural uniqueness of actinomycin Y_5 (**5**) was indicated by its surprising instability under aqueous acidic conditions, a decreased extinction of the UV/Vis maxima and its molecular formula $C_{61}H_{80}N_{12}O_{18}$ which was obtained from HR-ESIMS data and implied an additional double bond equivalent compared to **1** and **3**. NMR analysis revealed an unchanged α-peptidolactone ring, while again signals of the threonine residue showed deviations in position 1 of the β-ring. The chemical shift of C-4 (δ_C = 45.3) with regard to the molecular formula indicated the presence of a nitrogen atom in this threonine moiety of **5**. Additionally, the HMBC experiment showed a correlation between Thr-4-H_2 and the chromophore (Fig. 4), proving an unprecedented ring closure of the β-peptidolactone to the amino group of the chromophore creating an eight-membered heterocyclic lactam-enamine ring. Actinomycin Y_5 (**5**) probably emerges from **1** by nucleophilic attack of the amino group of the chromophore to C-4 of ClThr, followed by elimination of HCl. Notably, this unique structural feature has not been described before; neither for the G-type actinomycins[4,13] nor for the Z-complex with actinomycins Z_1–Z_5[15], although some of these compounds also bear a ClThr residue in the same position. Therefore **1–5**, exhibiting unusual structural features, cannot be classified within known families. The most similar structure presently known is actinomycin G_5,[4] which reveals a ring closure in the same structural position but with an oxygen ether bridge instead of the amino group.

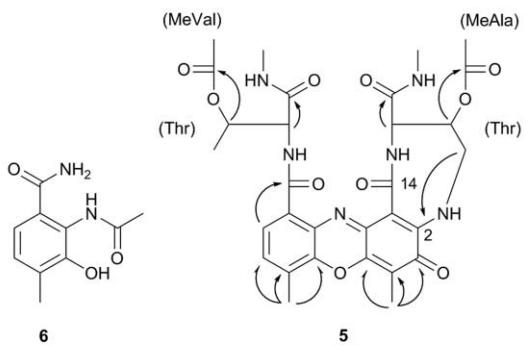

Fig. 4 Structural formula **6** and selected HMBC correlations indicating the structural deviations of actinomycin Y_5 (**5**).

A minor component caught our attention during the careful chromatography sequence of the actinomycin complex and was classified as a rather small aromatic compound according to HPLC-MS-analysis. HPLC purification strategy allowed for the isolation of a white solid as a pure compound which was characterized as the new 2-acetylamino-3-hydroxy-4-methylbenzamide (**6**) using 2D NMR and HR-ESIMS. Key steps for the unambiguously assigned data included interpretation of ^1H,^{15}N-HSQC and -HMBC spectra. Particularly, the methyl group at δ_H 2.15 showed a $^3J_{NH}$ correlation with an amide nitrogen atom at δ_N 130, while the exchangeable signals at δ_H 7.50 and 7.92 were shown to

Table 2 NMR data of actinomycin Y_1 (**1**) in $CDCl_3$ (^1H: 600 MHz ^{13}C: 150.8 MHz). Data for actinomycins Y_2–Y_5 (**2–5**) are given in the supplementary information

Pentapeptidolactones									
α-ring	pos.	δ_C	δ_H	J [Hz]	β-ring	pos.	δ_C	δ_H	J [Hz]
Thr	1	168.7	—	—	ClThr	1	168.0	—	—
	2	54.5	4.49	m		2	53.8	5.16	m
	3	74.5	5.20	m		3	74.3	5.25	dt, 5.0, 2.5
	4	17.0	1.10	d, 6.0		4	43.6	3.96	m
	NH	—	6.97	d, 5.5				4.04	dd, 11.5, 5.5
						NH	—	7.91	d, 6.5
D-Val	1	174.2	—	—	D-Val	1	173.7	—	—
	2	59.1	3.42	dd, 10.0, 5.0		2	57.0	3.85	dd, 10.0, 6.0
	3	31.9	2.04	m		3	32.0	2.16	m
	4	19.2	1.10	d, 6.5		4	19.3	0.91	d, 6.5
	5	19.0	0.90	d, 6.5		5	18.8	1.14	d, 6.5
	NH	—	7.74	d, 5.0		NH	—	8.33	d, 5.5
HMPro	1	170.8	—	—	OPro	1	172.7	—	—
	2	68.5	5.89	s		2	54.6	6.52	d, 10.5
	3	75.6	4.12	br s		3	41.8	2.34	d, 17.5
	4	41.3	2.13	m				3.90	dd, 17.5, 11.0
			2.23	dd, 13.5, 7.0		4	208.6		
	5	54.1	4.71	m		5	52.8	3.97	d, 18.0
	6	18.8	1.50	d, 6.0				4.54	d, 18.0
Sar	1	166.1	—	—	Sar	1	166.0	—	—
	2	51.5	3.65	d, 17.5		2	51.2	3.66	d, 17.5
			4.73	d, 17.5				4.51	d, 17.5
	*N*Me	35.0	2.88	s		*N*Me	34.8	2.87	s
MeVal	1	167.5	—	—	MeAla	1	168.7	—	—
	2	71.2	2.66	d, 9.0		2	59.9	3.38	q, 7.0
	3	27.0	2.62	m		3	13.5	1.38	d, 7.0
	4	21.6	0.94	d, 6.5		*N*Me	37.1	2.95	s
	5	19.0	0.72	d, 6.5					
	*N*Me	39.4	2.92	s					

Chromophore

δ_H 2.16 (s, 3H, 12-H_3), 2.50 (s, 3H, 11-H_3), 7.32 (d, J = 7.5 Hz, 1H, 7-H), 7.50 (d, J = 7.5 Hz, 1H, 8-H)

δ_C 7.8 (CH_3, C-12), 15.0 (CH_3, C-11), 100.0 (C, C-1), 113.9 (C, C-4), 125.8 (CH, C-8), 127.7 (C, C-6), 128.9 (C, C-9a), 130.3 (CH, C-7), 132.6 (C, C-9), 140.4 (C, C-5a), 145.2 (C, C-4a), 146.1 (C, C-10a), 147.6 (C, C-2), 166.1 (C, C-13), 168.7 (C, C-14), 178.6 (C, C-3)

belong to a second amide at δ_N 110. Positioning of the two amide moieties, a hydroxyl group and another methyl group was achieved by further 2D NMR and chemical shift interpretation. Compound **6** constitutes the *N*-acetylated monomeric unit of the actinoyl chromophore, hence being a putative precursor that could be loaded on the PCP unit of the non-ribosomal peptide synthetase (NRPS) responsible for actinomycin biosynthesis. The *N*-acetylation of arylamines by arylamine *N*-acetyltransferases (NATs) has been shown to be widely distributed both in eukaryotes and prokaryotes, including streptomycetes.[18,19]

Biological activity and structure-activity relationships

Actinomycins Y_1–Y_5 (**1–5**) were evaluated for their *in vitro* antimicrobial and cytotoxic activity and revealed surprising and novel insights into SAR which are discussed as follows. i) The main metabolite **1**, actinomycin Y_1, was the most potent compound in both assays. Surprisingly, its cytotoxic activity was an order of magnitude lower than that of actinomycin D as clinical reference, though still in the nanomolar range (Table 3). This was interesting since actinomycin Z_3[15] and G_2[4] which differ from **1** only in the 4-oxoproline residue of the β-ring (Z_3: MOPro, G_2: Pro) showed equal or slightly better activity than actinomycin D.

Introduction of Hyp into the β-ring, accomplished with the new structure **2**, led to a further 100-fold decrease of cytotoxicity (HM02 or HepG2 cells) compared to **1**. That is in total a 1000-fold lower activity regarding actinomycin Z_3 and G_2 (**2**) only caused by a MOPro-Hyp exchange. This clearly indicates a yet unknown negative impact of the OPro and Hyp residue on the cytotoxic potential (while Pro and MOPro are tolerated). Additionally, the cytotoxicity test panel confirmed that replacement of ClThr with cyclized HThr (cHThr), obtained from a two-fold acyl shift, resulted in a 100-fold decrease of activity observed by comparison of **1** and **3**, even 1000-fold was reported for actinomycin G_2 and G_6 (Table 3).[4] No cytotoxic effect at all was observed for the unusually cyclized compound **5**.

ii) Cell cycle investigations with HM02 cells proved that both actinomycin D and **1** reduced the ratio of cells in the S-phase (Suppl. Table S1), as expected for intercalating DNA and RNA polymerase inhibitors.[20]

iii) The antibacterial effect of actinomycin Y_1 (**1**) was in a similar range compared to actinomycin D, however, somewhat

Table 3 Cytotoxic and antibacterial activities of actinomycins Y_1–Y_5 (**1–5**) in comparison with that of actinomycin D and G_2, G_3, and G_5[4]

Cytotoxicity assays[a]								Antibacterial assays[b]		
HM02		HepG2		MCF7			SAR-significant			
GI_{50}	TGI	GI_{50}	TGI	GI_{50}	TGI	Compound	alterations in β-ring	*E. coli*	*S. aureus*	*B. subtilis*
0.015	0.036	0.02	0.3	0.025	0.16	Act. Y_1 (**1**)	1-ClThr, 3-OPro	18	30	39
1.7	3.3	0.33	9.6	1.4	2.7	Act. Y_2 (**2**)	1-ClThr, 3-Hyp	8	18	30
1.0	5.2	1.6	5.4	0.81	1.7	Act. Y_3 (**3**)	1-rHThr, 3-OPro	0	8	13
>10	>10	3.5	9.8	9.5	>10	Act. Y_4 (**4**)	1-rHThr, 3-Hyp	0	0	0
>10	>10	>10	>10	>10	>10	Act. Y_5 (**5**)	1-cThr, 3-OPro	13	22	25
0.0016	0.0064	0.0012	0.052	0.0019	0.0088	Act. D	1-Thr, 3-Pro	20	30	55
0.0013	0.0043	0.0029	0.018	0.0039	0.012	Act. G_2	1-ClThr, 3-Pro	15	27	45
0.13	0.55	1.1	>7.9	0.46	1.6	Act. G_3	1-HThr, 3-Pro	0	27	45
2.1	4.4	2.7	7.8	1.4	2.5	Act. G_5	1-rHThr, 3-Pro	0	10	20
>10	>10	>10	>10	>10	>10	Compound **6**	—	11	9	14

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.[a] Cytotoxicity was determined against human tumor cell lines HM02 (gastric adenocarcinoma), HepG2 (hepatocellular carcinoma), and MCF7 (breast adenocarcinoma); GI_{50} and TGI values are given in μg/mL. [b] Antibacterial activities are given as diameter in mm of inhibition zones in plate diffusion assays (50 μg compound on 6 mm filter disc).

lower (Table 3), while none of the compounds inhibited growth of the yeast *Candida albicans*. Here, unexpectedly, the effects of the replacements of β-ring Pro (actinomycin D or G_2) by OPro (for **1**) were marginal. And towards β-Hyp (for **2**) only one order of magnitude lower activity was observed. That is, the diameters of the inhibition zones of the plate diffusion assays were approximately cut in half, corresponding to a 10-fold decrease in activity.

iv) The strong antiparasitic effects observed for **1** are probably due to its cytotoxicity, which is deduced from a total loss of activity for compound **3** with low cytotoxicity (Supplementary Table S2).

In summary, the actinomycins Y_3 (**3**) and Y_4 (**4**) exhibited only low cytotoxic and antibacterial properties as expected. This is in accordance with previous results for the analogously assembled actinomycin G_6.[4] We state that the exchange of OPro to Hyp in the β-rings of **3** and **4**, respectively, provoked the decrease in both, cytotoxic and antibacterial activity as also found for compounds **1** and **2**. However, unusual results were obtained for the cyclized peptidolactone congener actinomycin Y_5 (**5**). Although being the only compound without any detectable cytotoxic activity (up to 10 μg/mL), it was the second most powerful antibiotic among the novel Y- and known G-type[4] actinomycins, with potency against both Gram-positive and Gram-negative germs.

Conclusion and outlook

In summary, five novel *aniso*-actinomycins Y_1–Y_5 (**1–5**) with a common phenoxazinone chromophore (actinocin), but with novel asymmetrically attached peptidolactone and peptide rings were isolated from *Streptomyces* sp. strain Gö-GS12. The Y-type actinomycins share the rare OPro and Hyp residue only with the X-type actinomycins, but instead of MeVal they reveal MeAla in the β-ring and HMPro in the α-ring which are only present in Z- and recently found G-actinomycins. Consequently, the novel actinomycins were classified as the new Y-type family. Chromopeptides **3** and **4** result from a two-fold acyl shift, while the additional ring closure of **5** is probably formed by a nucleophilic substitution reaction.

Interestingly, the biosynthesis of the clinically relevant actinomycins with different α- and β-units has not been thoroughly investigated yet. The crystal structure of the phenoxazinone synthase from *S. antibioticus* has been published recently, revealing a novel type 2 copper center as catalytic core.[21] However, the structure gives no hint how unsymmetrical actinomycins evolve, implying the future need to analyze the whole gene cluster responsible for actinomycin biosynthesis.

The new set of actinomycins Y_1–Y_5 (**1–5**) with unprecedented structural features allowed for detailed SAR studies. The oxidation level of the β-ring proline residue influenced both cytotoxic and antibacterial activity in the same tendency (Pro > OPro > Hyp) but in different orders of magnitude. The co-linearity of both activities is lost for actinomycin Y_5 (**5**) and therefore is not mandatory in the structural class of actinomycins. The new Y-type actinomycin family described here also significantly deepened our insight into the late stage modifications of actinomycin biosynthesis and clearly invites for future detailed studies.

Experimental section

General experimental procedures

For general experimental procedures see Supplementary Information.†

Biological material and fermentation

The producing microorganism strain Gö-GS12 was isolated from a soil sample collected in Alice Springs/Australia and characterized as streptomycetes by morphological and physiological data. Strain Gö-GS12 was maintained on agar plates containing malt extract 10 g L^{-1}, D-glucose 4 g L^{-1}, and yeast extract 4 g L^{-1}. Precultures were grown in 300 mL Erlenmeyer flasks, equipped with three intrusions/flow spoilers and filled with 100 mL medium (oatmeal 20 g L^{-1} and trace element solution 2.5 mL L^{-1} in deionized water; pH 7.0 prior to sterilization). Trace element solution: $CaCl_2 \times 2 H_2O$ 3 g L^{-1}, Fe(III)-citrate 1 g L^{-1}, $MnSO_4$ 0.2 g L^{-1}, $ZnCl_2$ 0.1 g L^{-1}, $CuSO_4 \times 5 H_2O$ 25 mg L^{-1}, $Na_2B_4O_7 \times 10 H_2O$ 20 mg L^{-1},

$CoCl_2$ 4 mg L^{-1}, $Na_2MoO_4 \times 2\ H_2O$ 10 mg L^{-1}. After inoculation with 1 cm^2 of an agar plate, the flasks were incubated on a rotary shaker (180 rpm, 28 °C, 48 h). 400 mL of preculture were used to inoculate a 4 L batch cultivation in a stirred vessel bioreactor (Braun-Diessel Biostat B, 500 rpm, 2.5 vvm, 28 °C, 72 h). Growth medium was the same as for the preculture, and the pH value at harvest time was 5.8.

Sample workup and isolation

The culture broth was centrifuged (3000 rpm, 10 min). The residue was extracted three times with 500 mL acetone each, using an ultrasonic bath (10 min). Drying of the combined organic phases led to 2.47 g mycelium extract. The supernatant was adsorbed on XAD-2 (500 mg, Pharmacia). The resin was washed with water (2 L) and eluted with methanol (1 L). Removal of the solvent under reduced pressure yielded 1.8 g of supernatant extract. Actinomycins were found in both, mycelium and supernatant extracts, and their isolation was achieved by almost identical procedures for each using subsequently silica gel chromatography (silica gel 60, 25 × 4 cm, CH_2Cl_2/MeOH 4:1), gel chromatography (Sephadex LH-20, 100 × 2.5 cm, acetone), and semi-preparative HPLC (Nucleosil C-18 ec, 5 µm, 250 × 16 mm, H_2O/MeCN with 0.1% formic acid). The first separation step on silica gel led to five fractions. Fractions 1 and 2 from the mycelium additionally were degreased by partitioning between petrol ether and water. Gel chromatography of fraction 1 led to compound **1** (48 mg in total). Compounds **2** (2.5 mg) and **3** (28 mg) were isolated from fractions 2 and 3, respectively, by applying gel chromatography and then HPLC (H_2O/MeCN 52:48). Purification of compounds **4** (11 mg) and **5** (21 mg) was done by HPLC (H_2O/MeCN 58:42). **5** proved to be instable under acidic conditions, implying the need to carefully and rapidly remove formic acid from the fractions derived by preparative HPLC. This was achieved by repeated addition of methanol followed by concentration *in vacuo* to 30% of volume. In a final step, the solvent was removed completely. A detailed isolation scheme is given in the Supplementary Information (Figure S1).

Amino acid analysis

For amino acid analysis see Supplementary Information.†

Bioassays

For plate diffusion assays, 50 µg of the desired compound were dissolved in acetone and dropped on paper disks (ø 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 in compliance with the NCI guidelines[22] with the human tumor cell lines HM02 (gastric adenocarcinoma), HepG2 (hepatocellular carcinoma) and MCF7 (breast adenocarcinoma). Cell cycle investigations were carried out with HM02 cells as described previously.[23] *In vitro* determination of antiparasitic activity against *Plasmodium falciparum*, *Trypanosoma b. rhodesiens*, *Trypanosoma cruzi*, and *Leishmania donovani* was determined following published protocols.[24] For detailed results see Supplementary Information, Table S2.

Actinomycin Y_1 (1). **1** was obtained as a red solid; $[\alpha]_D^{20}$ –162 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 238 (4.43), 429 (4.23), 441 (4.24) nm; CD (MeOH) λ_{max} ([Θ]) 212 (–115700), 241 (21800), 266 (–43600) nm; IR (KBr) $\tilde{\nu}_{max}$ 3426, 2965, 1747, 1660, 1581, 1515, 1479, 1307, 1191, 1103 cm^{-1}; ESIMS (pos. ions) *m/z* 653 $[M + 2H]^{2+}$, 1305 $[M + H]^+$, 1327 $[M + Na]^+$; ESIMS (neg. ions) *m/z* 1303 $[M - H]^-$. For R_f value and HR-ESIMS data see Table 1; for NMR data in $CDCl_3$ see Table 2.

Actinomycin Y_2 (2). **2** was obtained as a red solid; $[\alpha]_D^{20}$ –112 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 236 (4.40), 426 (4.19), 440 (4.20) nm; CD (MeOH) λ_{max} ([Θ]) 210 (–110000), 243 (12000), 270 (–19600) nm; IR (KBr) $\tilde{\nu}_{max}$ 3421, 2962, 1735, 1644, 1503, 1467, 1360, 1192, 1135 cm^{-1}; ESIMS (pos. ions) *m/z* 644 $[M + 2H]^{2+}$, 1307 $[M + H]^+$; ESIMS (neg. ions) *m/z* 1305 $[M - H]^-$. For R_f value and HR-ESIMS data see Table 1; for NMR data in CD_3OD see supplementary material.

Actinomycin Y_3 (3). **3** was obtained as a red solid; $[\alpha]_D^{20}$ –85 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 236 (4.50), 434 (4.22) nm; CD (MeOH) λ_{max} ([Θ]) 214 (–74300), 240 (30200), 274 (22700) nm; IR (KBr) $\tilde{\nu}_{max}$ 3430, 2958, 1737, 1648, 1515, 1464, 1379, 1181, 1123 cm^{-1}; ESIMS (pos. ions) *m/z* 654 $[M + 2H]^{2+}$, 1287 $[M + H]^+$, 1309 $[M + Na]^+$; ESIMS (neg. ions) *m/z* 1285 $[M - H]^-$. For R_f value and HR-ESIMS data see Table 1; for NMR data in CD_3OD see supplementary material.

Actinomycin Y_4 (4). **4** was obtained as a red solid; $[\alpha]_D^{20}$ ±0 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 237 (4.45), 435 (4.21) nm; CD (MeOH) λ_{max} ([Θ])208 (–121100), 238 (17000), 273 (21100) nm; IR (KBr) $\tilde{\nu}_{max}$ 3422, 2924, 1636, 1570, 1458, 1190, 1062 cm^{-1}; ESIMS (pos. ions) *m/z* 645 $[M + 2H]^{2+}$, 1269 $[M + H]^+$, 1291 $[M + Na]^+$; ESIMS (neg. ions) *m/z* 1267 $[M - H]^-$. For R_f value and HR-ESIMS data see Table 1; for NMR data in CD_3OD see supplementary material.

Actinomycin Y_5 (5). **5** was obtained as a red solid; $[\alpha]_D^{20}$ –58 (c 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 238 (4.33), 436 (4.02) nm; CD (MeOH) λ_{max} ([Θ]) 213 (–81600), 252 (6300), 284 (8000) nm; IR (KBr) $\tilde{\nu}_{max}$ 3420, 2963, 1750, 1635, 1560, 1458, 1363, 1192, 1097 cm^{-1}; ESIMS (pos. ions) *m/z* 645 $[M + 2H]^{2+}$, 1289 $[M + H]^+$, 1311 $[M + Na]^+$. For R_f value and HR-ESIMS data see Table 1; for NMR data in CD_3OD see supplementary material.

2-(Acetylamino)-3-hydroxy-4-methylbenzamide (6). **6** was obtained as a white solid; UV (MeOH) λ_{max} (log ε) 220 (4.22), 245 sh, 300 (3.18) nm; IR (KBr) $\tilde{\nu}_{max}$ 3402, 2935, 1657, 1534, 1450, 1373, 1292, 1041, 730, 643 cm^{-1}; ESIMS (pos. ions) *m/z* 231 $[M + Na]^+$, ESIMS (neg. ions) *m/z* 207 $[M - H]^-$; ^1H NMR (DMSO-d_6, 600 MHz) δ_H 2.15 (s, 3H, COMe), 2.19 (s, 3H, 4-Me), 7.05 (d, J = 7.5 Hz, 1H, 5-H), 7.11 (d, J = 7.5 Hz, 1H, 6-H), 7.50, 7.92 (br s, 1H each, NH_2), 9.58 (br s, 1H, NH), 11.05 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 150.8 MHz) δ_C 16.8 (q, 4-Me), 23.7 (q, COMe), 119.6 (d, C-6), 124.9 (s, C-2), 126.3 (s, C-1), 127.7 (d, C-5), 130.7 (s, C-4), 149.1 (s, C-3), 170.7 (1-CO), 171.6 (s, 4-CO). For R_f value and HR-ESIMS data see Table 1.

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