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TETRACENOMYCINS B_3 AND D_3, KEY INTERMEDIATES OF THE ELLORAMYCIN AND TETRACENOMYCIN C BIOSYNTHESIS

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Tetracenomycins B_3 and D_3, besides tetracenomycin D (D_1), were produced by a blocked mutant of the elloramycin producer *Streptomyces olivaceus* TÜ 2353. The compounds were isolated as red powders, and their structures were elucidated by comparing their physicochemical data with those of the known tetracenomycins A_2, B_1, B_2, D and E. Tetracenomycin B_3 (**2**), the main compound, and tetracenomycin D (**3**) were antibiotically inactive against Gram-positive and Gram-negative bacteria, whereas tetracenomycin D_3 (**1**) showed a moderate activity against *Bacillus subtilis* and *Arthrobacter aurescens*. Tetracenomycin B_3 (**2**) is the key intermediate where the biosynthesis of the elloramycins branches off from the line leading to tetracenomycin C (**5**) as the final product of the tetracenomycin biosynthesis branch.

Streptomyces olivaceus TÜ 2353 produces elloramycins A~F, a family of anthracycline-like antibiotics[2,3], which are active against Gram-positive bacteria, especially streptomycetes, and against murine L1210 leukemia cells. Surprisingly, elloramycin A (**6**††=elloramycin) showed a weaker cytotoxic activity than its sugarless relative, tetracenomycin C (**5**)[4]. One possible reason for this could be the high degree of methylation of elloramycin A, which disables its interaction with DNA. This hypothesis is confirmed by the better antibiotic activity of its less methylated congener elloramycin B (**7**)[3]. Thus we screened for a mutant producing late precursors of elloramycin, *i.e.* less methylated elloramycins, in a higher amount than the wild type TÜ 2353. In the course of this screening, a mutant was isolated which attracted attention by a dark red discoloration of the cells and the growing medium. We could not detect any compounds with the tetracenomycin C chromophore (=elloramycin chromophore), but an investigation of the product produced by this mutant, subsequently named TÜ 2353-R, seemed worthwhile, because red colored naphthacene quinones were suggested to be precursors of the yellow colored tetracenomycin C and elloramycin chromophore[5,6]. Indeed, three new naphthacene quinones, tetracenomycin D_3 (**1**), B_3 (**2**) and D (**3**, also called D_1[6]) were found. While **3** is already known as a metabolite of the tetracenomycin C producer *Streptomyces glaucescens* TÜ 49, **1** and the main compound of TÜ 2353-R, tetracenomycin B_3 (**2**), had not been found before, but were proposed as key intermediates of the tetracenomycin C biosynthesis[6].

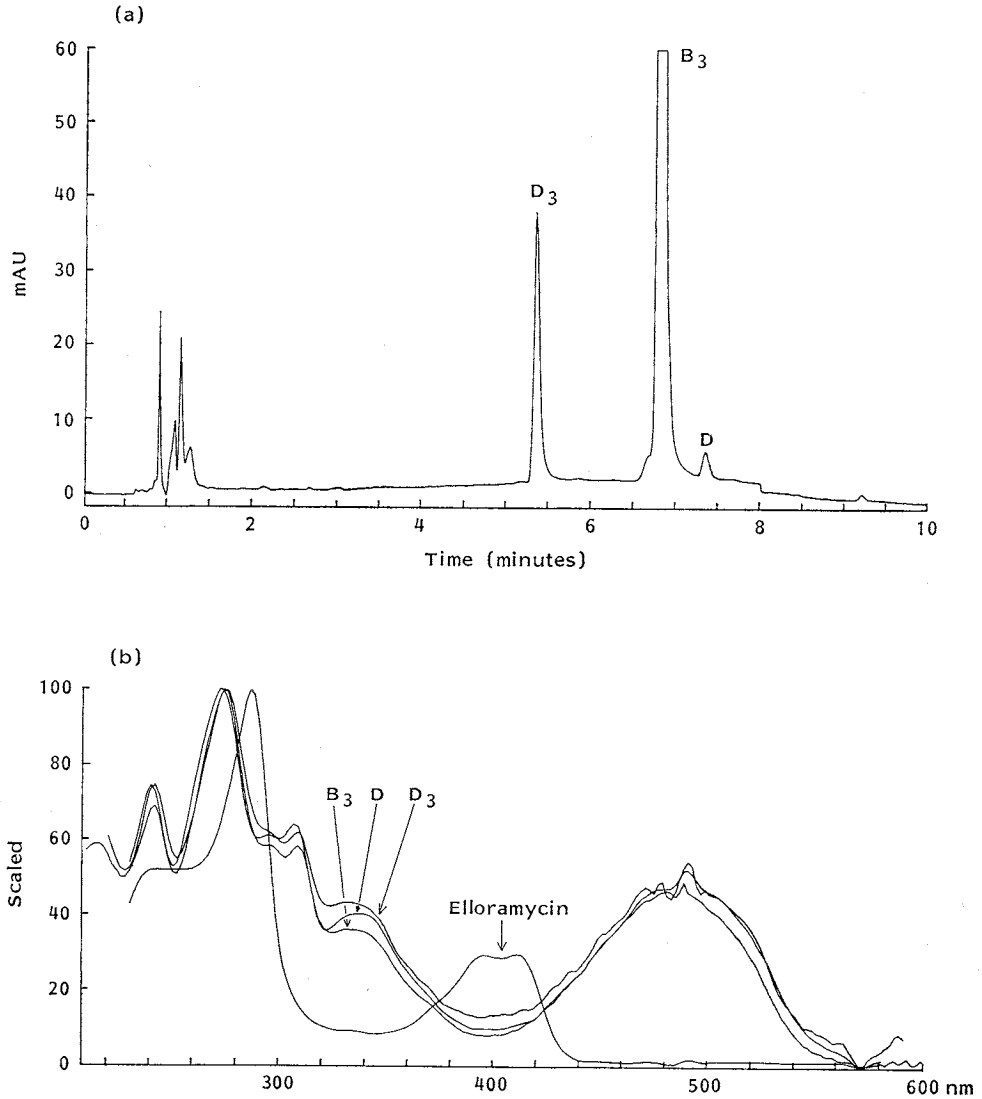
Producing Organism

The tetracenomycins B_3, D and D_3 producing strain TÜ 2353-R was obtained by UV mutagenesis

† See ref 1.

†† The numbers indicate the structures shown in Fig. 2.

Fig. 1. HPLC elution profile of the cell extract of strain TÜ 2353-R.



(a) Monitored at 230 nm, and (b) UV/VIS spectra of tetracenomycins B_3, D and D_3, recorded during the HPLC and compared with the spectrum of elloramycin.

from the elloramycin producer *S. olivaceus* TÜ 2353.

Strain TÜ 2353-R is characterized by a dark red discoloration of the growing medium. The analysis of the mycelium extract and culture filtrate by HPLC and diode array detection showed three peaks that were not identical with elloramycins in their retention times and UV/VIS spectra. The similarity of the UV/VIS spectra of all three peaks (Fig. 1) led to the assumption that the compounds were structurally related.

Fermentation and Isolation

To produce larger amounts of the tetracenomycins, 10-liter fermentations were performed. After drifting slightly into the acidic range, the pH rose to 8.25. The maximal tetracenomycin production

was reached after 140 hours incubation time, at which the concentrations of the tetracenomycins were B_3 (**2**) 555 mg/liter, D (**3**) 15 mg/liter and D_3 (**1**) 134 mg/liter. Sixty % of the compounds was accumulated in the mycelium whereas the rest was secreted into the culture medium.

The combined extracts from the mycelium (methanol - ammonium hydroxide) and culture filtrate (butanol) contained 40 g of dry substance, which consisted of tetracenomycin B_3 (**2**) 5.55 g, D (**3**) 0.15 g and D_3 (**1**) 1.34 g, representing 17.6% of the crude material. The concentration of the combined extracts to the aqueous residue and extraction with petroleum benzene resulted in a reduction of the dry substance from 40 to 34 g without any loss of the tetracenomycins, which remained in the aqueous phase.

The isolation of **1** and **2** was achieved by chromatography of the crude material on Amberlite XAD-1180. After concentration and freeze-drying of the effluent, 27 g of a crude powder was obtained that contained 4.43 g of **2** and 1.13 g of **1**. These two compounds were separated and purified by preparative medium-pressure liquid chromatography (MPLC) using a reversed-phase packing (particle size 25~40 μm). Per injection, 250 mg of the crude product were separated without loss of tetracenomycins into 41 mg of **2** and 10 mg of **1**. After concentration and freeze-drying of the effluent, the two substances were obtained as red powders.

To isolate **3**, the aqueous residue of the petroleum benzene extraction was extracted with butanol and the residue from this extract was chromatographed on silica gel. The crude powder (507 mg) contained 100 mg of **3** and was further purified by preparative reversed-phase MPLC. Per injection, 25 mg of the crude product could be separated without loss of tetracenomycin. After concentration and freeze-drying the compound was obtained as red powder.

Physico-chemical Properties

The molecular formula of tetracenomycin B_3, $C_{21}H_{14}O_8$, was determined by high resolution analyses of the molecular ion (*m/z* 394) in its electron impact mass spectrum (EI-MS). Its structure is in accordance with formula **2** as proven by its NMR spectra (Tables 1 and 2) and by the fact that treating **2** with diazomethane at 0°C yields tetracenomycin A_2 (**4**, TLC control). Both chelated OH groups can be observed in the ^{1}H NMR spectrum (δ 12.30, 14.95) as well as the four aromatic protons, of which 2-H (δ 6.72) and 4-H (δ 7.08) show a *meta* coupling constant of 2.5 Hz. The free carboxyl group (δ 187.8 in the ^{13}C NMR spectrum) is also indicated by the broad absorption band between

Table 1. ^{1}H NMR data in DMSO-d_6 (δ in ppm relative to internal TMS), *J* (Hz) in brackets.

	B_3 (**2**)	D_3 (**1**)	D (**3**)	A_2 (**4**)[a]
10-CH_3	3.00 s	3.12 s	2.82 s	2.86 s
OCH_3	3.90 s	—	—	3.93 s, 3.96 s, 3.97 s
2-H	6.72 d (2.5)	6.61 d (3)	6.58 d (2.5)	6.68 d (2.5)
4-H	7.08 d (2.5)	7.17 d (3)	7.15 d (2.5)	7.36 d (2.5)
6-H	7.78 s	7.88 s	7.91 s	8.03 s
7-H	7.12 s	7.14 s	7.01 d (2)	7.09 s
9-H	—	—	7.19 d (2)	—
1-OH[b]	12.30 s	12.33 s	12.20 s	12.44 s
11-OH[b]	14.95 s	15.16 s	14.60 s	14.74 s

[a] In $CDCl_3$.

[b] Exchangeable with D_2O.

Table 2. ^{13}C NMR data of tetracenomycin B_3 (**2**) in comparison to those of tetracenomycin A_2 (**4**) and tetracenomycin D (**3**), δ in ppm relative to internal TMS.

	2 (DMSO-d_6)	**3** (DMSO-d_6)	**4** ($CDCl_3$)
Quinone-CO	187.9 s	187.8 s	189.8 s
	179.9 s	180.4 s	181.6 s
9-CO	187.8 s	—	168.1 s
Aromatic C	169.4 s	165.8 s	167.2 s
(OH)	169.3 s	165.1 s	166.3 s
	165.4 s	164.1 s	165.2 s
	164.0 s	159.4 s	157.5 s
Aromatic C	121.5 d	123.0 d	121.7 d
(H)	112.5 d	121.1 d	107.5 d
	106.6 d	108.0 d	107.4 d
	106.6 d	107.7 d	107.1 d
Aromatic C	139.3 s	141.1 s	140.0 s
	139.3 s	139.6 s	138.6 s
	135.4 s	135.4 s	135.9 s
	126.4 s	127.5 s	129.4 s
	121.4 s	119.2 s	129.0 s
	112.5 s	111.3 s	121.5 s
	110.6 s	109.2 s	111.1 s
	106.5 s	106.2 s	108.4 s
OCH_3	56.1 q	—	56.1 q
			56.0 q
			52.5 q
10-CH_3	20.8 q	24.1 q	20.9 q

Table 3. Antibacterial spectrum of tetracenomycin D_3 (**1**).

Test organism	MIC[a] (μg/ml)	Inhibitory diameter[b] (mm)
Bacillus subtilis[c]	100	—
B. subtilis[d]	ND	21
Micrococcus luteus	>100	—
Arthrobacter aurescens	100	13
Streptomyces olivaceus	>100	—
S. prasinus	>100	—
S. violaceus-niger	>100	—
S. viridochromogenes	>100	—

[a] Broth dilution method.
[b] Agar-plate diffusion assay, concentration of 2 mg/ml.
[c] Complex medium.
[d] Chemically defined medium.
ND: Not determined.
—: No inhibition.

2400 and 3400 cm^{-1} and by the valence vibration at 1715 cm^{-1} in the IR spectrum of **2**. After treatment with diazomethane, the broad absorption band disappears and the carbonyl vibration band moves to 1740 cm^{-1}, which is typical for esters.

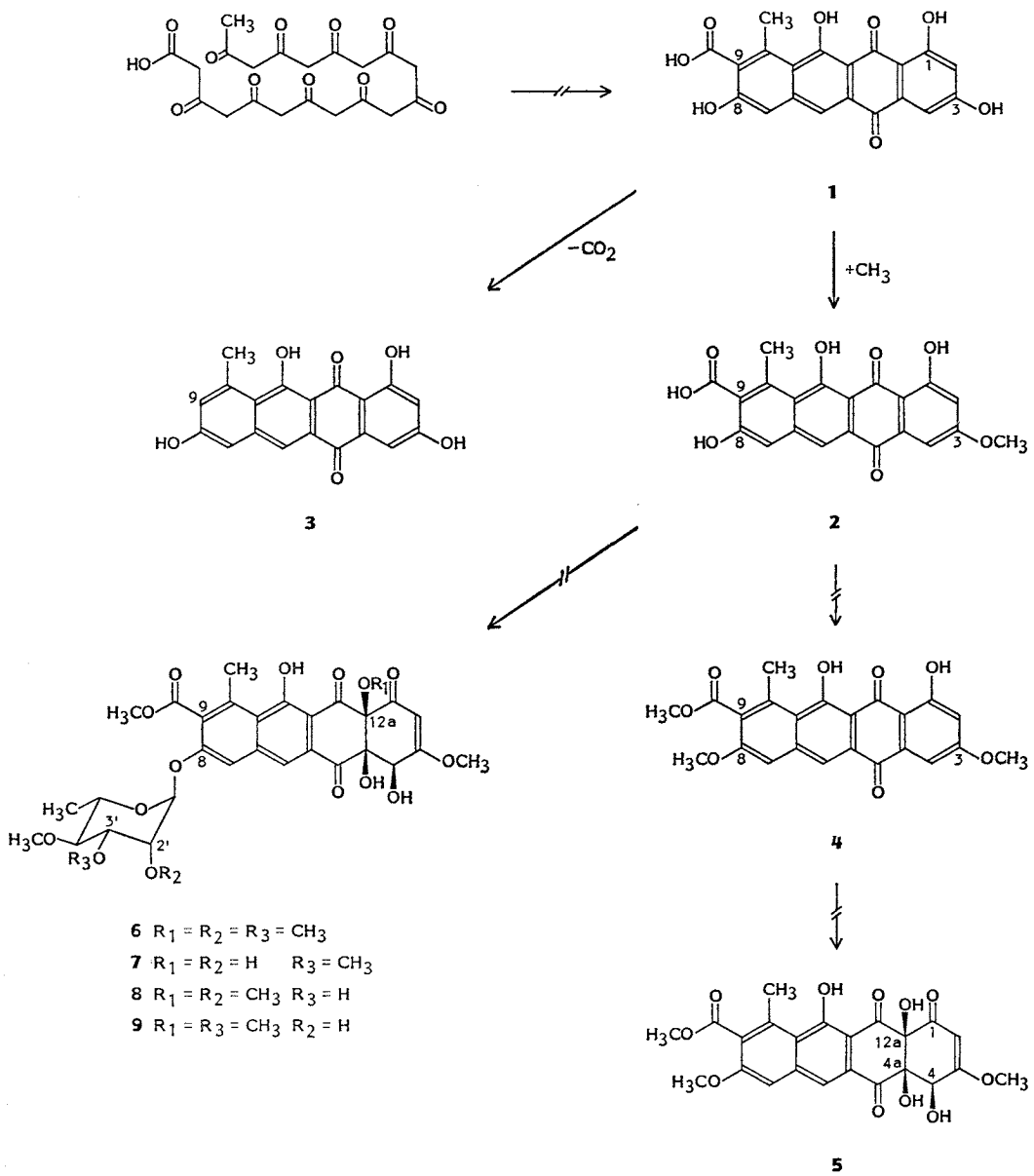
The ^1H NMR spectrum of tetracenomycin D_3 (**1**) shows a similar proton pattern as the one of **2**, *i.e.* two chelated OH groups at δ 12.33 and δ 15.16 and four aromatic protons, two of them (2-H δ 6.61, and 4-H δ 7.08) with a *meta* coupling of 3.5 Hz. But in contrast to **2**, no OCH_3 signal is observable. The IR spectrum also shows a free carboxyl group as a broad absorption band between 2400 and 3400 cm^{-1}. Because the substance is non-volatile, no molecular ion was detected in the EI-MS, but a peak *m/z* 318 indicated $M^+ - CO_2 - H_2O$ from which a molecular formula of $C_{20}H_{12}O_8$ for **1** was deduced. Treatment of **1** with diazomethane also yields tetracenomycin A_2 (**4**). This was confirmed by the ^1H NMR spectrum, which showed three OCH_3 signals; and by the EI-MS, which showed a molecular ion at *m/z* 422, of which high resolution analysis proved the expected molecular formula of $C_{23}H_{18}O_8$ (422.1002).

The third product was identified as tetracenomycin D (**3**) by high resolution analysis of its molecular ion in the EI-MS (*m/z* 336.0631, *i.e.* $C_{19}H_{12}O_6$), by its NMR spectra (Tables 1 and 2), and by the IR and the UV spectra, which were identical with those of an authentic sample.

Biological Activity

2 and **3** showed antimicrobial activity neither against Gram-positive nor Gram-negative bacteria tested by the broth dilution method and by an agar-plate diffusion assay. The antimicrobial spectrum of **1** is limited to an activity against *Bacillus subtilis* and *Arthrobacter aurescens*, as shown in Table 3. In contrast to tetracenomycin C[4], the elloramycins[2,3] and the urdamycins[7], the growth of streptomycete strains, including the producing organism, was insensitive to **1**.

Fig. 2. Biosynthetic pathway to the elloramycins and tetracenomycin C.



Tetracenomycin B_3 (**2**), the main compound of the tetracenomycin producing strain TÜ 2353-R was tested in the proliferation assay and the stem cell assay against L1210 leukemia cells, where a weak cytostatic effect was observed.

Discussion

As described in previous publications, the coupling of high-performance liquid chromatography with computer assisted diode array detection (HPLC/DAD) is a powerful tool for the identification of new microbial metabolites[8~10]. The comparison of the retention times and UV/VIS spectra of the elloramycins, stored in an HPLC/DAD computer library, with those of the compounds produced by

a blocked mutant of the elloramycin producer *S. olivaceus* confirmed the presence of three structurally related compounds showing distinct differences from the elloramycins.

Tetracenomycins B_3 (**2**) and D_3 (**1**) are two common key intermediates of the biosynthetic pathways to tetracenomycin C (**5**) and to the elloramycin family (**6**~**9**, Fig. 2). This confirms a biosynthetic hypothesis in which both compounds were suggested to be intermediates[6]. The branch leading to the elloramycins probably takes place on the stage of **2**, because we found this compound as a product of a blocked mutant of an elloramycin producer. The methylation sequence for the tetracenomycin C (**5**) biosynthesis leading from **1** was proposed to be first C-3 then C-8[6]. But one cannot absolutely distinguish with physico-chemical methods between a methylation of 3-OH and 8-OH. Thus tetracenomycin D_3 (**1**) and not B_3 (**2**) might be the last common precursor of both pathways, if the structure of **2** were an 8-*O*-methyl ether instead of an 3-*O*-methyl ether. The latter assignment is more likely, however, because of the chemical shifts in the ^{1}H NMR spectrum of **2** (Table 1). The biosynthetic sequence of the methylation reactions in case of the elloramycins (**6**~**9**) is impossible to establish because *S. olivaceus* TÜ 2353 produces elloramycins B (**7**), C (**8**) and D (**9**) side by side, which cannot derive from each other. For solving the methylation sequence problem of the tetracenomycin C (**5**) biosynthesis as well as for confirming all biosynthetic hypotheses in this context, we suggest to feed ^{14}C labeled **1** and **2** to growing cultures of *S. olivaceus* TÜ 2353 and *S. glaucescens* TÜ 49. The blocked mutant TÜ 2353-R seems to be an ideal strain to produce these ^{14}C enriched precursors *via* their biosynthesis from [^{14}C]acetate.

Experimental

Bacterial Strains

The producing organism TÜ 2353-R was obtained by UV mutagenesis from the elloramycin producing organism *S. olivaceus* TÜ 2353. The strain was cultivated at 27°C in a medium containing soy bean meal 2% and starch 2% (pH 7.0).

The standard strains for testing the biological activity spectrum were obtained from the stock culture collection of our laboratory or from ATCC.

Fermentation

Strain TÜ 2353-R was cultivated in a 10-liter fermentor (NB 10, New Brunswick). Nine liters of medium containing soy bean meal 2% and starch 2% in tap water (pH 7) were inoculated with 1 liter of shaking cultures grown for 48 hours in the same medium. The fermentor was kept at 27°C and agitated at 220 rpm, with an aeration rate of 0.4 v/v/m.

Isolation

Hyphlo super-cel (3%) was added to the fermentation broth (10 liters) which was then separated by multiple sheet filtration into biomass and culture filtrate. The mycelium was extracted 4 times with MeOH - 25% ammonium hydroxide (95 : 5), and the culture filtrate was extracted 4 times with butanol. The combined extracts from mycelium and culture filtrate were concentrated and extracted 3 times with petroleum benzene to remove lipophilic substances.

To isolate **1** and **2**, the aqueous residue was chromatographed on a column containing Amberlite XAD-1180 (70×8 cm i.d.). Impurities were washed out with acidic water (pH 4.5) and the tetracenomycins were eluted with MeOH. The separation of **1** and **2** was achieved by preparative MPLC using glass columns (44×2.6 cm i.d., Büchi, Göppingen, FRG) filled with the reversed-phase packing HD-Sil 18-30-100 (Orpegen, Heidelberg, FRG). Both compounds could be quantitatively separated by linear gradient elution with 0.1% aqueous acetic acid as solvent A and MeOH as solvent B, using a gradient profile from 50% B to 100% B within 45 minutes and flow rate of 30 ml/minute (Liquid Chromatograph SP-8000, Spectra-Physics, Darmstadt, FRG). The UV absorbance of the eluate was monitored at 300 nm using a spectrophotometer equipped with a preparative cell (Knauer, Berlin, FRG).

To isolate **3**, the aqueous residue of the petroleum benzene extraction was extracted 3 times with butanol. After concentration, the extract was chromatographed on a silica gel column (40×5 cm

i.d., SI 60, 63~200 μm, Merck, Darmstadt, FRG). **3** was eluted with dichloromethane - MeOH (8 : 2). Under these conditions, the compounds **1** and **2** were adsorbed on the silica gel and could be eluted by MeOH - ammonium hydroxide (95 : 5). **3** was purified by preparative MPLC using the same column as described for the compounds **1** and **2** by gradient elution from 70% aqueous MeOH to 100% MeOH within 28 minutes.

Biological Assays

The broth dilution method was used to determine the MIC of tetracenomycins. 10^5 cells/ml were used as inoculum, and growth inhibition was evaluated after incubation for 24 hours at 37°C on a rotary shaker.

The agar-plate diffusion assay was used to determine the antibacterial spectrum of tetracenomycins. The test solutions were applied to filter discs (6 mm diameter) and the test plates were incubated for 24 hours at 37°C.

HPLC Analysis

The chromatographic system consisted of a HP-1090M liquid chromatograph equipped with a diode array detection system (Hewlett-Packard, Waldbronn, FRG); a detection wavelength of 280 nm was used. 10 μl-samples were injected onto an HPLC column (125×4.6 mm i.d.), fitted with a guard column (20×4.6 mm i.d.) which were packed with 5 μm Shandon Hypersil ODS (Grom, Ammerbuch, FRG). The samples were separated by gradient elution; solvent A was 0.1% phosphoric acid, solvent B was acetonitrile. The linear gradient was from 20 to 100% solvent B in 10 minutes at a flow-rate of 2 ml/minute.

Structure Elucidation

The IR spectra were obtained in pressed KBr discs using a Perkin-Elmer Model 298 spectrometer. The NMR spectra were determined with a Varian FT 80 (1.9 T) or XL 200 (4.7 T), respectively. Chemical shifts (δ in ppm) are reported relative to internal TMS. The multiplicities of the ^{13}C NMR spectra were assigned by attached proton test (APT). The EI-MS (70 eV) were taken by a Varian MAT-311A mass spectrometer using a direct probe insert and high resolution analysis was done with perfluorokerosine as a standard. The UV spectrum was recorded using a Zeiss DMR 21 spectrometer. TLC was performed on silica gel plates (Macherey & Nagel Sil G/UV 254+366, 0.25 mm silica gel on glass).

Tetracenomycin B_3 (**2**): IR (KBr) cm^{-1} 3420, 3400~2400 (br), 2920, 2850, 1715 (sh), 1670, 1620, 1585; EI-MS (70 eV) *m/z* 394 (49%, M^+, high resolution calcd for $C_{21}H_{14}O_8$ and found: 394.0689), 376 (100%, $M-H_2O$), 350 (45%, $M-CO_2$), 320 (18%), 174 (11%), 44 (96%); ^1H NMR (200 MHz, DMSO-d_6) see Table 1; ^{13}C NMR (50.3 MHz, DMSO-d_6) see Table 2.

Tetracenomycin D_3 (**1**): IR (KBr) cm^{-1} 3440, 3400~2400 (br), 2930, 2860, 1665 (sh), 1625, 1590 (sh), 1575; EI-MS (70 eV) *m/z* 318 (2%, $M-H_2O-CO_2$), 162 (2%), 147 (2%), 136 (3%), 108 (5%), 93 (4%), 85 (11%), 83 (16%), 70 (12%), 55 (20%), 44 (100%); ^1H NMR (200 MHz, DMSO-d_6) see Table 1.

Tetracenomycin A_2 (**4**) from Treatment of **1** with Diazomethane: 10 mg of tetracenomycin D_3 were dissolved in 25 ml ethylene glycol monomethyl ether and cooled to 0°C. The solution was treated with an excess of diazomethane (5 equivalents of an etheric solution). The reaction started at once (discoloration from red to light orange) and was complete after 5 minutes. The only product **4** was purified by chromatography on Sephadex LH-20 (column 50×2.5 cm, $CHCl_3$). Yield 9 mg: IR (KBr) cm^{-1} 3440, 2960, 2930, 2860, 1738, 1681, 1619, 1590; EI-MS (70 eV) *m/z* 422 (100%, M^+, high resolution calcd for $C_{23}H_{18}O_8$ and found: 422.1002), 391 (21%), 361 (24%), 111 (18%), 97 (27%), 85 (25%), 83 (22%), 71 (30%), 69 (35%), 59 (21%), 44 (72%), 43 (75%); ^1H NMR (80 MHz, $CDCl_3$) see Table 1.

Tetracenomycin D (**3**): IR (KBr) cm^{-1} 3400, 2950, 2840, 1665, 1620 (sh), 1600, 1575 (sh); UV λ_{max} (MeOH and MeOH - HCl) nm (ε) 480 (14,100), 336 (12,000), 307 (21,700), 294 (21,700), 273 (29,300), 240 (24,000), 215 (22,400); λ_{max} (MeOH - NaOH) nm (ε) 550 (13,000), 371 (23,500), 290 (24,600), 240 (23,000), 232 (sh, 22,800); EI-MS (70 eV) *m/z* 336 (100%, M^+, high resolution calcd for $C_{19}H_{12}O_6$ and found: 336.0631), 318 (6%, $M-H_2O$), 290 (6%), 168 (4%), 87 (4%), 69 (3%), 44 (43%); ^1H NMR (200 MHz, DMSO-d_6) see Table 1; ^{13}C NMR (50.3 MHz, DMSO-d_6) see Table 2.

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