The Structure of Mithramycin Reinvestigated

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A reinvestigation of the structure of mithramycin, the principal product of *Streptomyces argillaceus* ATCC 12956, is reported. The structure elucidation was carried out with mithramycin decaacetate (**4**) using 2D NMR methods, including TOCSY, HMBC, and HSQC experiments. The work resulted in structure **3** being confirmed for mithramycin.

Mithramycin (aureolic acid, plicamycin, mithracin, LA-7017, PA-144) shows remarkable cytotoxicity against a variety of tumor cell culture lines, including brain tumors and experimental animal tumors. Along with the chromomycins, chromocyclomycins, olivomycins, and UCH9, it forms the small, but distinct, group of aureolic acid antibiotics.[1−6] Mithramycin is used clinically for the treatment of certain tumors, such as disseminated embryonal cell carcinoma, as well as for Paget's bone disease.[7,8]

Since the discovery of mithramycin in 1953,[9] various partially contradictory structures of the molecule have been published, in the context of reports on the structure elucidation,[10−14] DNA interactions,[15−17] syntheses of the saccharide chains,[12,18−21] and genetic work.[22,23] For the first structure elucidation of mithramycin, Kolosov et al. used selected degradation experiments and compared the resulting structural fragments with known substances.[10] In this way, Kolosov et al. could prove that the aglycon of mithramycin is the same as that of the known chromomycin. Furthermore, the Russian investigators were able to determine the five deoxysugar moieties, assembled in two different deoxysugar chains and attached at C-2-O and C-6-O, respectively. As a result, they proposed the sugar sequences shown in formula **1** (Figure 1). The deoxysugar moieties found were D-olivose (3×), D-oliose (1×), and D-mycarose (1×).[10]

In the following work on mithramycin, neither the aglycon moiety nor the identity of the five mentioned deoxysugar building blocks was questioned. However, revisions were made regarding the sequence of the sugars within the deoxysugar side chains and their interglycosidic connectivities. By using the NMR methods available at that time and partial synthesis, in particular of the supposed disaccharide fragment, Thiem et al. revised the structure of mithramycin as **2**. In their structure, the position of the olivose building block was moved from the center of the trisaccharide to the beginning of the disaccharide chain.[11−13,20] Moreover, the interglycosidic linkages were corrected. A third structure (**3**) of mithramycin incorporating features of the Kolosov (sequence of the sugar building blocks) and the Thiem (interglycosidic bondage) structures was later used by Patel et al. in context with publications on the mithramycin−Mg^{2+} dimer/DNA complex.[15,16] However, no evidence was given by these researchers as to why this variant of the mithramycin structure was used or how the structure **3** was elucidated. Instead, the Thiem publications were referenced for the mithramycin structure.[15] Finally, erroneous mithramycin structures showing, inter alia, a fourth olivose unit instead of the oliose moiety have been published.[22,23] Various authors have used one or the other of these "revised" structures, sometimes along with controversial discussions,[18] leading to even more confusion about the correct structure of mithramycin.

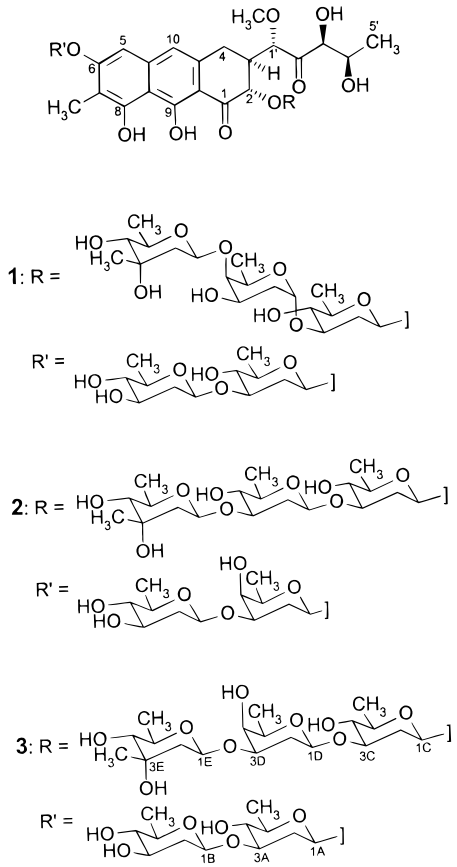

Figure 1. Structures used for mithramycin; the correct structure is **3**.

Results and Discussion

Since a knowledge of the correct structure of mithramycin is crucial for our ongoing work on the biosynthesis of

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mithramycin[24,25] and is also important in the context of using mithramycin biosynthetic genes for the generation of hybrid natural products,[26] we reinvestigated its structure using modern NMR methods. For this effort, the decaacetyl derivative **4** was prepared, since mithramycin itself is insufficiently soluble in most solvents, while the decaacetyl derivative shows reasonable solubility. Various one- and two-dimensional proton and carbon NMR spectra (^{1}H, ^{13}C, APT, H,H-COSY, HSQC, HMBC, and TOCSY)[27] were recorded. The ^{13}C NMR spectrum of **4** showed the expected 72 signals, including 21 quaternary carbon signals (10 acetate carbonyls, C-3 of the D-mycarose unit, and the 10 from the aglycon moiety). In addition, 26 CH signals (two aromatic doublets, one aliphatic CH− and 23 CHO signals), six CH_2 triplets (five C-2 methylene groups of the deoxysugar units and C-4 of the aglycon), and 19 methyl groups (10 acetate units, six from the deoxysugar moieties and the remaining three from the aglycon) were observed.

The NMR data of the aglycon moiety could be assigned unambiguously due to the distinct chemical shifts and from the couplings observed in the H,H-COSY, HSQC, and HMBC spectra. The results (Table 1) confirmed the assignments of Thiem et al.[11,12] and are not discussed here further.

The TOCSY NMR spectrum[27d] obtained for **4** correlated the complete spin systems of the five sugar building blocks and the three spin systems of the aglycon [(i) 5-H/7-CH_3, (ii) 10-H, the A-ring protons and H-1′, (iii) H-3′/H-4′/H_3-5′]. While one intact spin system could be observed for the three olivose units and for the oliose, the spin system of the mycarose unit was split into two halves (H-1E/H_2,-2E, and H-4E/H-5E/H_3-6E) and showed the CH_3-3E singlet separately. These two halves could be combined through the $^3J_{C-H}$ couplings observed in the HMBC spectrum (Figure 2), which showed long-range couplings between the protons of the $CH_3$3E group to C-2E, as well as between H-4E and the carbon of the CH_3-3 group. The TOCSY spectrum was particularly helpful for the unambiguous assignments of the sugar protons. The assignments of their directly attached carbons followed from the HSQC correlations. The coupling pattern of typical oliose signals, i.e., the broad quartet of H-5D (δ_H 3.56), the broad doublet of H-4D (δ_H 5.11), and the dt (J = 11.5, 4 Hz) of the H-3D (δ_H 3.89), is unique and facilitated the assignments within this sugar unit. The large, approximately 9−10 Hz, couplings observed for all of the anomeric sugar protons confirmed that all five sugars are β-glycosidically linked. The assignments of the NMR data of the single sugar units (Table 1) differ from those of Thiem et al.,[11,12] who could not assign all of the NMR data of the deoxysugar moieties with the NMR techniques they used. In addition, some of these proton assigments were incorrect, which ultimately led to the incorrect structure **2**. Most important were the $^3J_{C-H}$ couplings observed in the HMBC spectrum (Figure 2), which showed the interglycosidic linkages, as well as the connections of sugars A and C to the aglycon moiety. In each interglycosidic linkage, both of the $^3J_{C-H}$ couplings, namely, the one between H-1 and C-3, and the one between H-3 and C-1, showed unambiguously the sequences of the two sugar chains of mithramycin to be D-olivose-3→1-D-olivose (sugars A and B) for the disaccharide chain and D-olivose-3→1-D-oliose-D-3→1-D-mycarose (sugars C−E) for the trisaccharide chain, respectively. Furthermore, two $^3J_{C-H}$ couplings, one between the anomeric proton of the olivose A to C-6 of the aglycon and the second between H-2 and C-1C, determined unambiguously the linkage positions of the two deoxysugar chains to the aglycon, namely, C-6-O of the disaccharide and C-2-O of the trisaccharide chain.

Table 1. ^{1}H and ^{13}C NMR Data of Mithramycin Decaacetate (**4**) in Chloroform-d_1 (Relative to TMS, at 500 and 75.5 MHz, Respectively, δ in ppm, J (Hz), ^{13}C Multiplicities from APT and HSQC Experiments)[a−c]

position	δ_H	multiplicity (J, Hz)	δ_C
1			202.8 s
2	4.64	d (11)	77.0 d
3	2.75	dddd (12, 11, 4, 2)	41.9 d
4	2.61	dd (16, 4)	26.7 t
4	3.07	dd (16, 12)	26.7 t
4a			138.8 s
5	6.95	s	106.7 d
6			157.4 s
7			120.8 s
CH_3-7	2.13	s	20.9 q
8			147.4 s
8a			112.6 s
9			163.5 s
9a			109.9 s
10	6.80	s	116.5 d
10a			136.9 s
1′	4.59	d (2)	81.5 d
OCH_3-1′	3.35		58.7 q
2′			204.5 s
3′	5.23	d (3)	77.5 d
4′	5.42	dq (6.5, 3)	68.7 d
5′	1.26	d (6.5)	15.9 q
1A	5.22	dd (9, 2)	96.8 d
$2A_a$	2.04	ddd (11, 11, 9)	36.2 t
$2A_e$	2.46	ddd (11, 5, 2)	36.2 t
3A	3.99	ddd (11, 9, 5)	73.9 d
4A	4.74	dd (9, 9)	74.3 d
5A	3.67	dq (9, 6)	70.8 d
6A	1.30	d (6)	17.9 q
1B	4.62	dd (9.5, 2)	96.2 d
$2B_a$	1.68	ddd (12, 12, 9.5)	36.6 t
$2B_e$	2.27	ddd (12, 5.5, 2)	36.6. t
3B	4.93	ddd (12, 9.5, 5.5)	70.3 d
4B	4.72	dd (9.5, 9)	74.1 d
5B	3.42	dq (9, 6)	70.6 d
6B	1.21	d (6)	17.8 q
1C	5.04	dd (9, 2)	100.6 d
$2C_a$	1.71	ddd (12, 12, 9.5)	36.9 t
$2C_e$	2.64	ddd (12, 5.5, 2)	36.9 t
3C	3.97	ddd (12, 9.5, 5.5)	74.0 d
4C	4.65	dd (9.5, 9)	74.8 d
5C	3.44	dq (9, 6)	70.6 d
6C	1.21	d (6)	17.8 q
1D	4.55	dd (9.5, 2)	97.1 d
$2D_a$	1.73	ddd (12.5, 11.5, 9.5)	32.4 t
$2D_e$	1.98	ddd (12.5, 4, 2)	32.4 t
3D	3.89	dt (11.5, 4)	72.8 d
4D	5.11	dd (4, 1)	70.2 d
5D	3.56	dq (6, 1)	69.7 d
6D	1.16	d (6)	16.9 q
1E	4.89	dd (9, 2)	95.8 d
$2E_a$	1.60	dd (13.5, 9)	43.2 t
$2E_e$	1.89	dd (13.5, 2)	43.2. t
3E			70.5 s
3E-CH_3	1.10	s	27.3 q
4E	4.57	d (9.5)	77.5 d
5E	3.79	dq (9.5, 6)	68.2 d
6E	1.13	d (6)	17.6 q

[a] ^{1}H NMR signals of the acetate-CH_3 groups: δ_H 1.93, 1.95, 2.02, 2.05, 2.06, 2.09, 2.11, 2.15, 2.19, 2.37. [b] ^{13}C NMR signals of the acetyl-CO groups: δ_C 171.6, 171.4, 171,4, 171.3, 171.2, 171.2, 171.2, 171.1, 170.7, 170.5. [c] ^{13}C NMR signals of the acetyl CH_3 groups: δ_C 21.0, 21.1, 21.2, 21.2, 21.2, 21.3, 21.4, 21.5, 21.6, 21.6.

In summary, the structure of the mithramycin aglycon and its NMR assigments were confirmed. However, the NMR data of the sugar units must be reassigned. The resulting correct structure of mithramycin is consistent

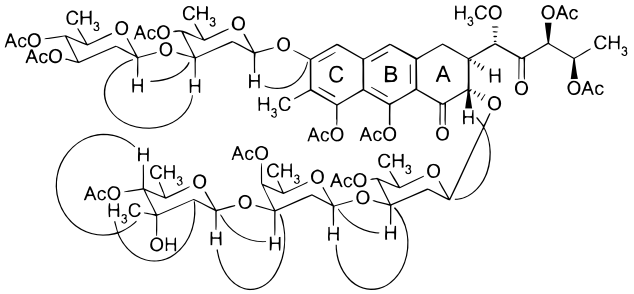

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Figure 2. Most important $^3J_{C-H}$ couplings visible in the HMBC spectrum of mithramycin decaacetate (**4**).

with structure **3**, which was first introduced by Patel et al.[15,16] and has since been used also by others.[18,21,24]

Experimental Section

General Experimental Procedures. NMR spectra were recorded in $CDCl_3$ on a Varian Unity 500 NMR instrument at 500 MHz for ^1H and 125.7 MHz for ^{13}C, using standard Varian pulse sequences. The CD and the UV spectra were determined using a JASCO J-500 A and a Kontron Uvikon 860 spectrometer, respectively. Optical rotations were measured using a Perkin-Elmer 241 polarimeter, and the melting points were determined using a Büchi SMP-20 instrument. The *S. argillaceus* cultures were grown in a Sanyo MIR 1520 incubator.

Bacterial Strain. Mithramycin (**3**) was produced by the wild-type strain *Streptomyces argillaceus* ATCC 12956.

Cultivation and Fermentation. *S. argillaceus was* incubated on agar plates (R2YENG-medium)[28] for 6 days at 30 °C.

Isolation of Mithramycin (3). The agar with the incubated *S. argillaceus* was cut into pieces, and the cells were lysed using a H_2O−acetone mixture (50:50) in an ultrasonic bath for 15 min. Afterward, this mixture was extracted six times at pH 5.5 (adjusted with citric acid), each time with 500 mL of ethyl acetate per liter of agar. The organic phase was evaporated to dryness, the crude extracts were chromatographed two times on silica gel (column 30 × 3 cm, CH_2Cl_2−MeOH 4:1), and the resulting fraction was finally purified on Sephadex LH-20 (MeOH); yield: 10 mg/L mithramycin (**3**).

Acetylation of Mithramycin (3) to Mithramycin Decaacetate (4). Acetic anhydride (2.4 mL) was added to a solution of mithramycin (**3**) (24 mg, 22 μmol) in pyridine, and the mixture was stirred for 20 h at room temperature. The solution was then poured on ice and extracted with CH_2Cl_2, and the organic layer concentrated in vacuo. The remaining pyridine was removed with toluene in vacuo. The residue was chromatographed by semipreparative HPLC (flow rate 5 mL/min; mobile phase, acetonitrile−H_2O 8:2; solid phase, Kontrosorb 10 C_{18}; retention time, 8 min), followed by gel filtration on Sephadex LH-20 (column 100 × 2.5 cm; solvent, CH_2Cl_2).

Mithramycin decaacetate (4) was obtained as an amorphous powder: mp 158 °C; $[\alpha]^{20}_D$ 143° (*c* 0.0021, $CHCl_3$); UV ($CHCl_3$) λ_{max} (ϵ) 238 (20 800), 248 (15 800), 270 (51 000), 277 (74 100), 295 (6500), 317 (13 700), 322 (11 500), 327 (13 700), 345 (2900), 392 (8600) nm; ($CHCl_3$ + 1 drop 37% HCl) λ_{max} (ϵ) 238 (20 800), 248 (15 800), 270 (51 000), 277 (74 100), 295 (6500), 317 (13 700), 322 (11 500), 327 (13 700), 345 (2900), 392 (8600) nm; ($CHCl_3$ + 1 drop 1 N NaOH) λ_{max} (ϵ) 238 (20 800), 248 (15 800), 270 (51 000), 277 (74 100), 295 (6500), 317 (13 700), 322 (11 500), 327 (13 700), 345 (2900), 392 (8600) nm; IR (KBr) ν_{max} 3436, 2931, 1743, 1625, 1431, 1367, 1232, 1232, 1162, 1044 cm^{-1}; CD (*c* 1.5·10^{-5}mol/L, $CHCl_3$) λ ($[\theta]^{26}$) 223 (−2500), 240 (3700), 257 (−7300), 266 (14 900), 275 (3000), 279 (3400), 284 (1000), 294 (10 800), 308 (−3400), 320 (9600), 326 (7500), 333 (11 700), 357 (−14 600), 382 (700), 394 (−2400), 417 (2600), 432 (−600), 450 (3600) nm; NMR data see Table 1; mixing time used for the TOCSY experiment 120 ms; R_f 0.92 (CH_2Cl_2/CH_3OH 9:1).

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Supporting Information Available: The ^1H, ^{13}C NMR, H,H COSY, TOCSY, HMBC, and HSQC spectra and the formula for **4** in which all long-range C,H couplings are drawn (8 pages). Ordering information is given on any current masthead page.

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