Characterization of Moenomycin Antibiotic Complex by Multistage MALDI-IT/RTOF-MS and ESI-IT-MS

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Flavomycin is a commercially available antimicrobial growth promoter and an authorized additive for feeding stuffs in the EU and in the USA. As most antibiotically active products biosynthesized by microorganisms, it contains not only a single active compound but is a complex mixture of structurally closely related substances. Multistage matrix-assisted laser desorption/ionization-ion trap/reflectron time-of-flight mass spectrometry (MALDI-IT/RTOF-MS) and liquid chromatography-electrospray ionization-ion trap-mass spectrometry (LC-ESI-IT-MS) were utilized for a detailed analysis of the constituents of the Flavomycin complex based on low-energy collision induced dissociation (CID). An optimal sample preparation for negative ion vacuum MALDI-MS for this compound class was developed. The MALDI-IT/RTOF-MS² and -MS³ analysis starting with the precursor $[M - H]^-$ ions of these interesting phosphoglycolipids, named moenomycins, yielded a large variety of product ions that facilitated the structural characterization of this class of compounds. Based on the derived CID fragmentation pathway of the five known major constituents, namely moenomycin A, moenomycin A₁₂, moenomycin C₄, moenomycin C₃, and moenomycin C₁, four not yet described moenomycin-type constituents could be characterized. They were assigned as 4F-demethyl-6E-O-de-β-D-glucopyranosyl-moenomycin A, 6B-N-de(2-hydroxy-5-oxo-1-cyclopenten-1-yl)-moenomycin A, 6B-hydroxy-6B-de[N-(2-hydroxy-5-oxo-1-cyclopenten-1-yl)amino]-moenomycin A, and 6C-hydroxy-moenomycin A. In addition, a moenomycin A carrying an oxygen in the moenocinol-group was found, which is most probably a chemical degradation product. These new compounds were verified by LC-ESI-IT-MS. (J Am Soc Mass Spectrom 2006, 17, 1081-1090) © 2006 American Society for Mass Spectrometry

Moenomycins, alternatively named bambermycins, Flavomycin, and flavophospholipol, are a group of structurally very similar phosphoglycolipid antibiotics produced by several *Streptomyces* species, including *S. bambergensis*, *S. ghanaensis*, *S. geysiriensis*, and *S. ederensis* [1–3]. The spectrum of antibiotic activity of the Flavomycin complex covers primarily gram-positive microorganisms, such as staphylococci and streptococci [2, 4]. It is used as antimicrobial growth promoter of several farm animals [2] and is an authorized additive for feeding stuffs in the EU (registration number E 712) [5] and in the USA [6].

The antibiotic activity of the moenomycins is based

on the inhibition of the bacterial cell wall formation [2, 3, 7, 8]. The biosynthesis of the peptidoglycan in the cell wall involves the assembly of lipid II (the disaccharide pentapeptide monomer unit that is linked to an undecaprenyl phosphate-membrane carrier) from cytoplasmic intermediates, the translocation of lipid II through the cytoplasmic membrane to the outer surface followed by the incorporation of the disaccharide pentapeptide monomer units into the growing glycan chains (transglycosylation) and finally the formation of the peptide cross-bridges to the pre-existing peptidoglycans in the mature cell wall (transpeptidation) [8]. The moenomycins interfere with the transglycosylation reaction by inhibition of some membrane-bound, non-penicillin-binding, monofunctional glycosyltransferases (Mgts) and some class A high-molecular-mass penicillin-binding proteins (HMM-PBPs) [2, 3, 7–12]. Class A HMM-PBPs, such as the PBP1b from *Escherichia coli*, are

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Scheme 1. Structure of the five previously characterized moenomycin antibiotics that are known to constitute the Flavomycin complex.

two-domain proteins that catalyze both the transglycosylation reaction and the transpeptidation reaction. While β -lactam antibiotics bind covalently to an active-site serine in the transpeptidase domain of this bifunctional enzymes, moenomycins are assumed to act as competitive inhibitors at the transglycosylase domain [3, 7–10]. Although several antibiotics interfere with the transglycosylation reaction, moenomycins are the only compounds known to date that directly interact with the transglycosylases rather than bind to their substrates [3]. The moenomycins therefore represent an interesting template for the design of new antibiotic drugs [3, 7, 8, 13, 14], which are highly desired because of the problem of bacterial resistance [14–18].

Now, five major components of the Flavomycin complex have been structurally characterized, namely moenomycin A [19–21], moenomycin A₁₂ [22], moenomycin C₄ [23], moenomycin C₃ [23], and moenomycin C₁ [24]. They all consist of a central oligosaccharide part (B–F) that is, on the one side linked to an isoprenoid-like C₂₅-alcohol (I) via a phosphodiester group (G) and a 2,3-dihydroxypropanoic acid (glyceric acid) group (H) and, on the other side to a 2-hydroxy-5-oxo-1-cyclopenten-1-yl group (A) (Scheme 1). From structure-activity relationship analysis, it has been found that moenomycin trisaccharides containing the units C, E, F, G, H, and I are the smallest fragments that show antibiotic activity in vivo, whereas moenomycin disaccharides containing the units E–I still act as transglycosylase inhibitors in vitro [3, 7–9, 12, 25]. The isoprenoid-like C₂₅-alcohol [26], named moenocinol, seems to be required for the binding of the moenomycin antibiotics to the cytoplasmic membrane [27], whereas the units E, F, and H are concluded to form a polar binding epitope that is recognized by the donor binding site of the transglycosylases [3, 7–9, 19, 28]. In addition to the five known constituents of the Flavomycin complex, structurally related moenomycin-type antibiotics, including the prasinomycins, the diumycins (macarbomycins), ensanchomycin, prenomycin, teichomycin, pholipomy-

cin, AC326- α , 11837 RP, 8036 RP (quebemecin), and 19402 RP, have been found [3, 23, 29, 30].

The chemical structures of the five major components of the Flavomycin complex have been established by ¹³C-NMR spectroscopy and fast-atom-bombardment (FAB)-mass spectrometry (MS) of the purified compounds [20–24] and were later confirmed by ¹H-NMR spectroscopy [31]. Subramaniam-Niehaus et al. [32] have used liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) to confirm the presence of these five compounds in the culture filtrate of *S. ghanaensis*. Recently, Eichhorn and Aga [6] isolated the moenomycin antibiotics from medicated chicken feed and investigated the fragmentation behavior of the five known constituents by positive and negative ion mode LC-ESI-ion trap (IT)-MS.

The focus of our study is on the detailed mass spectrometric characterization of the Flavomycin complex and the identification of novel natural moenomycin compounds by, for the first time, matrix-assisted laser desorption/ionization-ion trap/reflectron time-of-flight multistage mass spectrometry (MALDI-IT/RTOF-MS) and additional LC-ESI-IT-MS. The applicability of the MALDI-IT/RTOF-MS technique to the structural analysis of oligosaccharides and glycopeptides has been described in previous work [33–38] and is extended to the class of medically relevant phosphoglycolipids.

Experimental

Materials

Flavomycin was obtained from Hoechst Roussel Vet (Wiesbaden, Germany). The MALDI matrix compound 2,4,6-trihydroxyacetophenone monohydrate (2,4,6-THAP) and the additive diammonium hydrogen citrate were obtained from Fluka (Buchs, Switzerland). Ammonium acetate was supplied by Sigma-Aldrich (St. Louis, MO). Sodium chloride, methanol, and water with

a specific conductivity of $\leq 1 \mu\text{Scm}^{-1}$ were purchased from Merck (Darmstadt, Germany).

MALDI Sample Preparation

Sample preparation was performed on microtiter format stainless-steel target slides from Shimadzu Biotech-Kratos Analytical (Manchester, UK). Flavomycin was dissolved in 20 mM aqueous ammonium acetate to 0.1 gL^{-1} . For negative ion mode measurements, $0.4 \mu\text{L}$ of 0.2 M aqueous diammonium hydrogen citrate, $0.6 \mu\text{L}$ of the Flavomycin solution, and $0.8 \mu\text{L}$ of a solution of 20 gL^{-1} 2,4,6-THAP in methanol were mixed on the target in the given order and dried at room temperature in a gentle stream of air. For positive ion mode measurements, either the same preparation method was used or the 0.2 M aqueous diammonium hydrogen citrate was replaced by 20 mM aqueous sodium chloride.

MALDI-IT/RTOF Mass Spectrometry

The MALDI-MS¹ and MALDI-low-energy CID-MSⁿ ($n = 2-3$) measurements were performed on an AXIMA-QIT (Shimadzu Biotech-Kratos Analytical). This hybrid-instrument consists of a 3D quadrupole ion trap coupled to a double-stage gridless reflectron TOF analyzer [39, 40]. The AXIMA-QIT is equipped with a pulsed nitrogen laser, having a wavelength of 337 nm and a pulse width (FWHM) of about 4 ns, and an integrated 1 GHz recorder. The total acceleration voltage in negative and positive ion mode was $\pm 4 \text{ kV}$. The ion trap was continuously supplied with helium at a pressure of $5 \times 10^{-5} \text{ mbar}$ to allow collisional cooling of the trapped ions. In addition, a short pulse of argon was injected into the IT immediately before the ion introduction to enhance the ion-cooling efficiency and immediately before the radio frequency-induced precursor ion excitation to enhance the fragmentation efficiency during CID in MSⁿ-mode. CID spectra were acquired by averaging 1024–5041 unslected single laser shots using a precursor ion selection width of 1/70, 1/250, or 1/1000 of the precursor ion mass in MS² and 1/70 or 1/250 of the precursor ion mass in MS³. External mass calibration using fullerite (Shimadzu Biotech-Kratos Analytical) or the synthetic peptide P₁₄R (Sigma-Aldrich) was performed for mass assignment.

LC-ESI-IT Mass Spectrometry

The measurements were carried out on an Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) coupled to a ThermoFinnigan LCQ Deca XP ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) fitted with an orthogonal ESI ion source. Chromatographic separation of the Flavomycin constituents was performed with a Hypersil ODS-column of $200 \times 2.1 \text{ mm}$ dimension having a particle size of $5 \mu\text{m}$ (Thermo Electron, Waltham, MA), and at a column temperature

Figure 1. Negative ion mode MALDI-IT/RTOF mass spectrum of the Flavomycin complex showing the $[M - H]^-$ ions of the five known major constituents, namely moenomycin A, moenomycin A₁₂, moenomycin C₄, moenomycin C₃, and moenomycin C₁, and five not yet characterized compounds.

of 50°C . The solvents used were 10 mM ammonium acetate in doubly distilled water (Solvent A) and 10 mM ammonium acetate in methanol (Solvent B). The gradient was performed from 75–95% Solvent B for 25 min at a flow rate of $250 \mu\text{Lmin}^{-1}$. The ESI source was operated in negative ion mode with a source temperature of 200°C and a spray voltage of 4 kV. The instrument was tuned for maximum transmission of doubly-charged negative ions ($[M - 2H]^{2-}$) and the scan-range was set to m/z 300–1650.

Results and Discussion

MALDI-IT/RTOF-MS¹

Positive ion mode MALDI mass spectra of the Flavomycin complex turned out to be of limited use for the characterization of the individual constituents. In contrast to ESI measurements, $[M + H]^+$ ions could not be observed with MALDI, which is related to their low stability [6]. Series of alkali adduct ions ranging from $[M + \text{Na}]^+$ up to $[M + 2\text{Na} + 2\text{K} - 3\text{H}]^+$ were detected instead, which resulted in a complicated peak pattern and the overlapping of the isotopic distributions of ions from different compounds. The addition of small amounts of sodium chloride (20 mM aqueous sodium chloride was added instead of the 0.2 M aqueous diammonium hydrogen citrate) to suppress potassium adduct ion formation did not improve the situation sufficiently. However, it has to be mentioned that MSⁿ experiments of the $[M + 2\text{Na} - \text{H}]^+$ ion of moenomycin A, which was the most abundant species, have yielded a large variety of highly abundant fragment ions that would in principle facilitate the structural characterization of this class of compounds (data not shown).

Negative ion mode MALDI measurements using the matrix substance 2,4,6-THAP and diammonium hydrogen citrate as additive (to suppress alkali ad-

Figure 2. Negative ion mode MALDI-IT/TOF product ion spectra of moenomycin A: (a) MS² spectrum of the $[M - H]^-$ ion (m/z 1581 $\rightarrow$); (b) same spectrum amplified by a factor of 42 to show the low abundant product ions; (c) MS³ spectrum of the main product ion (m/z 1581 $\rightarrow$ 1152 $\rightarrow$).

duct ion formation), on the other hand, gave rather clean mass spectra showing nearly exclusively $[M - H]^-$ ions (Figure 1). Beside the $[M - H]^-$ ions of the five known major components of the Flavomycin complex, several other ions were detected, e.g., at m/z 1404.61, m/z 1484.64, m/z 1485.63, and m/z 1596.66. A consequence of the coupling of vacuum MALDI with an ion trap device is the enhanced probability of post source decay in the larger time frame inherent to the trapping process, resulting in the occasionally observed fragmentation of the precursor ions already in MS¹ [33, 41]. Indeed, the rather labile phosphoglycolipids exhibited prompt decay in MS¹ as well (most abundant fragment ion/deprotonated molecular ion ratio of approximately 2/1). However, in the case of the moenomycins, all abundant fragment ions were below m/z 1200 and, thus, did not interfere with the analysis of the intact species.

Moenomycin A

Moenomycin A [21], the main constituent of the Flavomycin complex (40–80%), contains a pentasaccharide core (B, C, D, E, F) with a glucopyranosyl residue (D) that is $\beta(1\rightarrow6)$ -linked to the 2-(acetyl-amino)-2-deoxyglucopyranosyl group (E) (Scheme 1). The corresponding $[M - H]^-$ ion was detected at m/z 1580.66 (calc. m/z 1580.65) representing the most abundant species (Figure 1). The low-energy CID spectrum of the precursor ion m/z 1581 shows one predominant product ion at m/z 1152 representing the loss of the moenocinol group (I) and the 2,3-dihydroxypropanoic acid group (H) (Figure 2a and Scheme 2). In addition, only four other ions were detected with a relative intensity $>1\%$ of the base peak. These ions have all lost the units H and I, and, in addition, a $\text{HN}=\text{C}=\text{O}$ group (m/z 1109), the carbamate group (NH_2COOH) from unit F (m/z 1091), a $\text{HN}=\text{C}=\text{O}$ as well as the PO_3H group (m/z 1029), or the whole uronamide-derived unit F (m/z 902). The formation of the latter ion by an internal residue loss requires a rearrangement reaction, which has been described for several compounds [42–46]. One possible structure of the ion at m/z 902, which is an alternative to the one proposed by Eichhorn and Aga [6], is given in Scheme 2. Most of the interesting product ions in MS² were

Scheme 2. Proposed low-energy CID fragmentation pathway for the $[M - H]^-$ ion of moenomycin A (MS²; m/z 1581 $\rightarrow$). Additional water and ammonia losses have been omitted.

Scheme 3. Proposed low-energy CID fragmentation pathway for the main product ion of moenomycin A (MS^3 ; m/z 1581 $\rightarrow$ 1152 $\rightarrow$). Additional water and ammonia losses have been omitted.

observed in the intensity-range below 1% of the base peak, but still with a good S/N ratio (Figure 2b and Scheme 2). The MS^3 -spectrum of the main product ion at m/z 1152 showed mainly the same ions that were already present in MS^2 , with the exception of a product ion at m/z 962, which is assumed to originate from a $^{0,2}X$ -type cross-ring cleavage of unit F, and a low mass product ion at m/z 329, assigned to the phosphorylated unit F (Figure 2c and Scheme 3). The proposed fragmentation pathways given in Scheme 2 and Scheme 3 have been corroborated by additional MS^3 experiments of the precursor ions at m/z 1109, m/z 1091, m/z 1029, and m/z 902 (data not shown).

From the whole variety of observed fragmentations, ten characteristic product ions have been selected that allow the mass determination of each individual building unit of the moenomycin antibiotics (Scheme 4 and Table 1). The discussion of the CID-behavior of the other compounds of the Flavomycin complex is mainly restricted to these selected product ions to reduce redundancy.

Moenomycin A_{12}

Moenomycin A_{12} [22] also contains the pentasaccharide core with the glucopyranosyl residue (D), but in contrast to moenomycin A, the 4-C-methyl group in the uronamide-derived unit F is absent (Scheme 1). The corresponding $[M - H]^-$ ion was detected with low abundance at m/z 1566.66 (calc. m/z 1566.64) (Figure 1). The CID-behavior observed in MS^2 and in MS^3 of the main product ion P1 (m/z 1138) was very similar com-

pared to moenomycin A. All product ions containing the unit F (P1, P3, P7, P8, P9, and P10; P5 was only observed with $S/N < 3$), were detected at -14 Da, whereas P4 and P6 had identical masses compared to moenomycin A (Table 1). However, the influence of the 4-C-methyl group or, more probably, of the different configuration of unit F (D-galacto rather than D-gluco) [31], was apparent from significantly increased relative intensities of those product ions originating from the cleavage of bonds around unit F, such as product ions of the type P1-HN=C=O, P1-NH₂COOH, and P6 (but not P4).

Moenomycin C_4 , C_3 , and C_1

In moenomycin C_4 [23], the glucopyranosyl residue (D) is absent, whereas the remaining structure is identical to moenomycin A. Moenomycin C_3 [23] and moenomycin C_1 [24] both contain a 2-(acetylamino)-2,6-dideoxyglucopyranosyl group as unit E and moenomycin C_1 furthermore lacks the 4-C-methyl group in unit F (Scheme 1). The corresponding $[M - H]^-$ ions were detected at m/z 1418.62 (calc. m/z 1418.60), 1402.61 (calc. m/z 1402.61), and 1388.60 (calc. m/z 1388.59), respectively (Figure 1). The CID behavior observed in MS^2 and in MS^3 was in all three cases very similar to moenomycin A, although the main product ions (P1) were not that much predominant in MS^2 . Moenomycin C_4 and moenomycin C_3 yielded product ions P2, P3, and P5 with identical masses compared to moenomycin A. All other product ions contain the unit E and were

Scheme 4. Ten selected key product ions of moenomycin-type structures.

Table 1. Observed monoisotopic m/z -values for the $[M - H]^-$ ions and selected low-energy CID product ions (defined in Scheme 4) of the moenomycin antibiotics

Compound	$[M - H]^-$	m/z									
		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Moenomycin A	1580.66	1152	507	757	902	329	822	694	881	1039	990
Moenomycin A ₁₂	1566.66	1138	n.d.	743	902	n.d.	822	680	867	1025	976
Moenomycin C ₄	1418.62	990	507	757	740	329	660	532	719	877	-
Moenomycin C ₃	1402.61	974	507	757	724	329	644	516	703	861	-
Moenomycin C ₁	1388.60	960	507	743	724	315	644	502	689	847	-
m/z 1405	1404.61	976	n.d.	743	740	n.d.	660	n.d.	705	863	-
m/z 1485	1484.64	1056	507	757	806	329	726	694	881	-	n.d.
m/z 1486	1485.63	1057	507	757	807	329	727	694	881	-	895
m/z 1597a	1596.66	1168	n.d.	757	918	n.d.	838	694	897	1055	1006
m/z 1597b	1596.66	1152	n.d.	n.d.	902	n.d.	822	694	n.d.	1039	n.d.

n.d., not detected; -, not possible.

therefore detected at -162 Da ($-C_6H_{10}O_5$) and -178 Da ($-C_6H_{10}O_6$), respectively (Figure 3a and Table 1). Those product ions of moenomycin C₁ containing unit F (P3 and P5) were shifted by -14 Da due to the missing 4-C-methyl group, those containing unit E (P4 and P6) were observed at -178 Da, and those possessing both units (P1, P7, P8, and P9) were detected at -192 Da (Figure 3b and Table 1). The product ions P1-HN=C=O, P1-NH₂COOH and P6 were found with higher relative intensity in moenomycin C₁ compared to moenomycin C₃, showing again the effect of the different configuration of unit F. The product ion P4, on the other hand, was observed to be much less prominent in moenomycin C₁ and C₃ compared to the other three moenomycins, indicating that the oxygen at position 6 of unit E favors the rearrangement reaction that causes the internal loss of unit F.

4F-Demethyl-6E-O-de- β -D-Glucopyranosyl-Moenomycin A

The measured isotopic pattern of moenomycin C₃ has been found to strongly differ from the theoretical distribution, which could only be explained by the presence of a second component, whose monoisotopic peak overlaps with the third isotopic peak of moenomycin C₃ at m/z 1404.61 (Figure 1). Indeed, product ions not related to moenomycin C₃ were observed in MS² and MS³ spectra obtained with a precursor ion selection width in MS² of 1/250 of the precursor ion mass, corresponding to 5.6 Th (data not shown). These product ions disappeared when the precursor ion selection width had been reduced to 1/1000 of the precursor ion mass (corresponding to 1.4 Th), which is feasible with the applied instrument at the expense of sensitivity, so that only the first two isotopes of moenomycin C₃ were isolated (Figure 3a). The relative abundance of these product ions was, on the other hand, strongly enhanced when only the isotopes at 1405.60 and 1406.58 were isolated and fragmented (Figure 3c). This way, several product ions belonging to the component at m/z 1404.61

Figure 3. Negative ion mode MALDI-IT/RTOF product ion spectra (MS³) of (a) moenomycin C₃ (m/z 1403 $\rightarrow$ 974 $\rightarrow$); (b) moenomycin C₁ (m/z 1389 $\rightarrow$ 960 $\rightarrow$); (c) the new compound assigned as 4F-demethyl-6E-O-de- β -D-glucopyranosyl-moenomycin A (m/z 1405 $\rightarrow$ 976 $\rightarrow$). The latter spectrum (c) was obtained from the second and third isotopes of 4F-demethyl-6E-O-de- β -D-glucopyranosyl-moenomycin A, but also contains product ions derived from the fourth and fifth isotopes of moenomycin C₃.

Figure 4. Mixed negative ion mode MALDI-IT/TOF product ion spectrum (MS^2) of the new compounds assigned as 6B-N-de(2-hydroxy-5-oxo-1-cyclopenten-1-yl)-moenomycin A and 6B-hydroxy-6B-de[N-(2-hydroxy-5-oxo-1-cyclopenten-1-yl)amino]-moenomycin A (m/z 1485/1486 $\rightarrow$).

could be unambiguously assigned, although CID spectra of the pure compound were not obtained. The product ions **P1**, **P8**, and **P9** were detected at +2 Da compared to moenomycin C_3 , indicating that groups **A**, **B**, **H**, and **I** are identical. **P3** was found at m/z 743 instead of m/z 757, which points out the absence of the 4-C-methyl group in unit **F**, such as in the case of moenomycin A_{12} and moenomycin C_1 . Consequently, **P4** and **P6** had a mass shift of +16 Da compared to moenomycin C_3 (Figure 3c and Table 1). Although **P7** could not be detected with a sufficient S/N-ratio, the product ion **P7**-HN=C=O was observed at m/z 475, which allows the localization of the additional oxygen at unit **E**. This lets us assume the presence of the 2-(acetylamino)-2-deoxyglucopyranosyl group as unit **E**, such as in the case of moenomycin C_4 . Thus, the new compound at m/z 1404.61 is assigned as 4F-demethyl-6E-O-de- β -D-glucopyranosyl-moenomycin **A**. This compound is a very likely (by)product of the moenomycin biosynthesis considering the known variability of both, the 4-C-methyl group in unit **F** and the 6-hydroxy group in unit **E**.

Figure 5. Mixed negative ion mode MALDI-IT/TOF product ion spectrum (MS^2) of the two new isobaric compounds assigned as 6C-hydroxy-moenomycin **A** (product ions labeled Pxa) and oxidized moenomycin **A** (product ions labeled Pxb) (m/z 1597 $\rightarrow$). Only the m/z -range 600–1200 is shown.

Figure 6. Negative ion mode LC-ESI-IT-MS (a) total ion chromatogram (TIC) of the Flavomycin complex and (b)–(j) extracted ion chromatograms (EIC) of the $[M - 2H]^{2-}$ ions of the 10 analyzed constituents. The displayed m/z -traces correspond to (b) moenomycin **A**, (c) moenomycin A_{12} , (d) moenomycin C_4 , (e) moenomycin C_3 , (f) moenomycin C_1 , (g) 4F-demethyl-6E-O-de- β -D-glucopyranosyl-moenomycin **A** (RT $\sim$ 11.00 min), and moenomycin C_3 (RT = 11.25 min), (h) 6B-N-de(2-hydroxy-5-oxo-1-cyclopenten-1-yl)-moenomycin **A**, (i) 6B-hydroxy-6B-de[N-(2-hydroxy-5-oxo-1-cyclopenten-1-yl)amino]-moenomycin **A**, and (j) 6C-hydroxy-moenomycin **A** (RT = 11.28 min) and oxidized moenomycin **A** (RT = 4.18 min).

6B-N-de(2-Hydroxy-5-oxo-1-Cyclopenten-1-yl)-Moenomycin **A** and 6B-Hydroxy-6B-de[N-(2-Hydroxy-5-oxo-1-Cyclopenten-1-yl)Amino]-Moenomycin **A**

Two other novel components of the Flavomycin complex were detected at 1484.64 Da and 1485.63 Da (Figure 1). Due to the strong overlap of the isotopic patterns, both components have been isolated and subjected to CID experiments at once (Figure 4). They yielded product ions of the type **P2**, **P3**, **P5**, **P7**, and **P8** that had identical masses compared to moenomycin **A**. Only product ions containing the unit **B**, namely **P1**, **P4**, and **P6**, were detected at -96 Da and -95 Da (Table 1). From these results, it was concluded that the new compounds are moenomycin **A**-like structures that lack the chromophore unit **A**. In the less abundant compound at m/z 1484.64 Da, unit **B** remains as a galactopyranuronamid. This 6B-N-de(2-hydroxy-5-oxo-1-cyclopenten-1-yl)-moenomycin **A** has been previously

Scheme 5. Structure of the ten characterized moenomycin antibiotics that constitute the Flavomycin complex. The nature of the six variable positions (R_1 – R_6) is given in Table 2.

synthesized by $K_3[Fe(CN)_6]$ oxidation of moenomycin A and was shown to be antibiotically active [47]. In the compound at m/z 1485.63 Da, unit **B** is assumed to be converted to a galactopyranuronic acid. Although the fragmentation pathway of these compounds is still nearly identical compared to moenomycin A, the missing 2-hydroxy-5-oxo-1-cyclopenten-1-yl-group has a strong effect on the relative intensities of some product ions (Figure 4). The most prominent effect was observed for **P8** and other product ions originating from the loss of unit **B** (e.g., $P4 - B$ at m/z 631 and $[M - B - H]^-$ at m/z 1310), which were detected with strongly enhanced relative intensities. Surprisingly, the presence of the terminal galactopyranuronic acid, but not the galactopyranuronamid group, had also a strong favoring effect toward the loss of unit **D**, which can be seen for **P10** or the loss of unit **D** from the intact molecule ($[M - D - H]^-$ at m/z 1324).

The presence of the two moenomycin A-like compounds lacking the chromophore unit **A**, which might either originate from an incomplete biosynthesis or from a degradation of the mature moenomycin A, suggests the presence of the corresponding compounds from the other known moenomycins as well. Indeed, very small peaks could be found at $-96/-95$ Da of moenomycin A_{12} , moenomycin C_4 , moenomycin C_3 , and moenomycin C_1 in MS^1 , but the abundance of these ions was too low to obtain useful MS^2 spectra.

6C-Hydroxy-Moenomycin A and Oxidized Moenomycin A

Two other novel, isobaric compounds of the Flavomycin complex were discovered at 1596.66 Da (Figure 1). The more abundant of these two constituents yielded only two product ions with identical masses compared to moenomycin A, namely **P3** and **P7**, which contain the units **D**–**I** (**P2** and **P5** were not detected with sufficient S/N). All other product ions, including **P9** and **P8**, had a mass shift of +16 Da compared to moenomycin A (see Figure 5 and m/z 1597a in Table 1), which means that units **A** and **B** also have the same mass as in moenomycin A. Consequently, unit **C** must carry a modification that causes the observed mass shift of +16 Da. This finding is also corroborated by a number of other product ions, such as the one originating from further fragmentation of **P4** (analogous to Scheme 2). Although a more exact localization was not possible from the observed low-energy CID product ions, we assume that the unit **C** in this component carries a hydroxy-group in position 6. The presence of 6C-hydroxy-moenomycin A in the Flavomycin complex is also not completely surprising, taking into account the similarity of unit **C** to unit **E**, and the presence of a 2-(acetylamo)-2-deoxyglucopyranosyl group as unit **C** in the moenomycin-type antibiotics pholipomycin (6E-O-de- β -D-glucopyranosyl-6C-hydroxy-moenomycin A) from *Streptomyces lividoclavatus* [23, 29] and AC326- α from *Actinomyces* AC326 [30]. The latter is structurally identical to the proposed 6C-hydroxy-moenomycin A except that it possesses a cyclized moenocinol derivative.

The second compound at m/z 1596.66 Da yielded a series of product ions in MS^2 , namely **P1**, **P4**, **P6**, and **P9**, which cannot originate from 6C-hydroxy-moenomycin A but are identical to moenomycin A (see Figure 5 and m/z 1597b in Table 1). These product ions clearly show that the units **A**–**G** have the same mass as in moenomycin A and, although **P2** and **P3** could not be detected due to the low abundance of this constituent, suggest that unit **H** or **I** are modified to give a mass shift of +16 Da. We assume that this compound is a chemical

Table 2. The nature of the six variable positions (R_1 – R_6 in Scheme 5) in the structure of the ten characterized moenomycin antibiotics that constitute the Flavomycin complex

Compound	R_1	R_2	R_3	R_4	R_5	R_6
Moenomycin A	HI	CH ₃	OH	D	H	A
Moenomycin A ₁₂	HI	OH	H	D	H	A
Moenomycin C ₄	HI	CH ₃	OH	OH	H	A
Moenomycin C ₃	HI	CH ₃	OH	H	H	A
Moenomycin C ₁	HI	OH	H	H	H	A
m/z 1405	HI	OH	H	OH	H	A
m/z 1485	HI	CH ₃	OH	D	H	NH ₂
m/z 1486	HI	CH ₃	OH	D	H	OH
m/z 1597a	HI	CH ₃	OH	D	OH	A
m/z 1597b	oxidized HI	CH ₃	OH	D	H	A

degradation product of moenomycin A, originating from oxidation of the moenocinol group (I) [48].

LC-ESI-IT Mass Spectrometry

The negative ion mode LC-ESI-IT-MS total ion chromatogram (TIC) of the Flavomycin complex and the extracted ion chromatograms (EIC) of the $[M - 2H]^{2-}$ ions of the 10 analyzed constituents are shown in Figure 6. The different components of the mixture were only partially separated by C_{18} -RP-chromatography under the given conditions. The major component, moenomycin A, eluted at 11.06 min (Figure 6b). Moenomycin C_3 (Figure 6e, 11.28 min), 4F-demethyl-6E-O-de- β -D-glucopyranosyl-moenomycin A (Figure 6g, ~11.00 min), 6B-hydroxy-6B-de[N-(2-hydroxy-5-oxo-1-cyclopenten-1-yl)amino]-moenomycin A (Figure 6i, 11.30 min), and 6C-hydroxy-moenomycin A (Figure 6j, 11.28 min) were barely separated from moenomycin A and from each other. Moenomycin C_4 (Figure 6d, 11.83 min) and 6B-N-de(2-hydroxy-5-oxo-1-cyclopenten-1-yl)-moenomycin A (Figure 6h, 13.84 min) were more strongly retained in the LC column than the above mentioned compounds. Moenomycin A_{12} (Figure 6c, 10.50 min) and moenomycin C_1 (Figure 6f, 10.60 min) (co)-eluted before moenomycin A and moenomycin C_3 , respectively, which was expected due to the missing 4F-methyl-group. The same effect was observed for 4F-demethyl-6E-O-de- β -D-glucopyranosyl-moenomycin A, which eluted before moenomycin C_4 . The low abundant compound eluting at 4.18 min (Figure 6j) was subjected to MS^2 and this experiment confirmed that this peak represents the oxidized moenomycin A. The large reduction in the retention time observed for this compound further corroborates that this constituent possesses a free hydroxy-group in the otherwise hydrophobic isoprenoid-like substructure (unit I). Interestingly, an additional hydroxy-group in the per se polar part of this amphiphilic molecule, either on unit E (moenomycin C_1 versus 4F-demethyl-6E-O-de- β -D-glucopyranosyl-moenomycin A and moenomycin C_3 versus moenomycin C_4) or on unit C (moenomycin A versus 6C-hydroxy-moenomycin A), causes a stronger retention.

Although not all components could be baseline-separated under the applied conditions, the observed elution order of the constituents of the moenomycin complex on the reversed-phase column might be helpful in future studies aiming on the isolation and further characterization of these structures. The differences in the retention time of the postulated new constituents compared to the known constituents give clear evidence that the corresponding MALDI-generated ions represent true deprotonated molecular ions instead of fragment ions originating from the in- or post-source decay of moenomycins with higher molecular masses.

Conclusions

Negative ion mode multistage low-energy CID using a MALDI-IT/RTOF-MS, which allows detection of the fragment ions in the last CID stage in the high-resolution mode, and negative ion mode LC-ESI-IT-MS were used for the analysis of the constituents of Flavomycin, which is a mixture of structurally closely related substances. Particularly, the negative ion MALDI-MS mode turned out to be extremely useful for the characterization of the very complex phosphoglycolipids. Based on the derived fragmentation pathway of the $[M - H]^-$ ions of the five known compounds constituting the Flavomycin complex, namely moenomycin A, moenomycin A_{12} , moenomycin C_4 , moenomycin C_3 , and moenomycin C_1 , four not yet described constituents could be characterized (Scheme 5 and Table 2). 4F-demethyl-6E-O-de- β -D-glucopyranosyl-moenomycin A and 6C-hydroxy-moenomycin A are most probably (by)products of the moenomycin biosynthesis. They might be of interest in the elucidation of the enzymatic assembly of this class of compounds. In the case of the two structures lacking the chromophore unit A, namely 6B-N-de(2-hydroxy-5-oxo-1-cyclopenten-1-yl)-moenomycin A and 6B-hydroxy-6B-de[N-(2-hydroxy-5-oxo-1-cyclopenten-1-yl)amino]-moenomycin A, it cannot be unambiguously stated whether this compounds are biosynthetic intermediates or degradation products. Chemically derived 6B-N-de(2-hydroxy-5-oxo-1-cyclopenten-1-yl)-moenomycin A has already been shown to be antibiotically active [47]. From structure-activity relationship analysis of the moenomycins [3, 7-9, 12, 25] and the activity of pholipomycin [23, 29] and AC326- α [30] it can be concluded that the other three above mentioned substances are potent antibiotics as well. Furthermore, a moenomycin A carrying an oxygen in the moenocinol-group was found, which is most probably a chemical degradation product. This compound is assumed to have a comparable low, if any, antibiotic activity [48].

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