Isolation and Analysis of Moenomycin and Its Biosynthetic Intermediates from *Streptomyces ghanaensis* (ATCC 14672) Wildtype and Selected Mutants

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Streptomyces ghanaensis (ATCC 14672) produces the phosphoglycolipid antibiotic moenomycin consisting of several components. A solid phase extraction procedure was developed which allowed a rapid isolation of both moenomycin and its biosynthetic intermediates from culture filtrates. Semi-preparative high performance liquid chromatography followed by high performance liquid chromatography-mass spectrometry provided structural data on the different moenomycin components. In order to obtain initial information on the biosynthetic pathway, moenomycin non-producing mutants were isolated. They were shown to release intermediates with shorter lipid chains suggesting that the lipid chain synthesis probably takes place at a later stage of the moenomycin biosynthesis. Based on the biological activity and the analytical data, we assume that a modification and in particular a shorter lipid portion drastically influences the inhibitory activity of this antibiotic.

Introduction

Streptomycetes are interesting from a biological and pharmacological point of view in that they synthesize an unusually complex range of secondary metabolites exhibiting antibiotic activity. More than half of such compounds known today including most of the medically and agriculturally important ones are of streptomycete origin. Various Streptomycetes sps. namely *S. ghanaensis*, *S. bambergiensis*, *S. geysirensis* and *S. ederensis* produce highly active phosphoglycolipid antibiotics, the so-called moenomycins (Lindner *et al.*, 1961). Their complex structure consists of a tetrasaccharide part, phosphoglyceric acid, the C_{25}-lipid alcohol moenocinol and the UV-chromophor 1-amino-2,5-dioxocyclopentane (λ max 257–258 nm) (Huber, 1979) (Table I). These antibiotics have the unique capability to inhibit the transglycosylation reaction during cell wall peptidoglycan synthesis.

With cell free systems from *Escherichia coli* it was demonstrated that the antibiotic moenomycin A selectively inhibits the transglycosylation step by its inhibitory effect on the penicillin binding protein PBP 1b (van Heijenoort *et al.*, 1992). PBPs catalyse both transglycosylation and transpeptidation, the two last biosynthetic reactions needed for the production of crosslinked peptidoglycan from the membrane intermediate GlcNAc-MurNAc-(pentapeptide)-PP-undecaprenol. The structural resemblance between moenomycin and the disaccharide phospholipid carrier intermediate led to the speculation that moenomycin A functions as a competitive inhibitor of this enzyme. Van Heijenoort *et al.* (1992) demonstrated that an antibiotically active derivative of moenomycin A forms a reversible complex with the enzyme. By analysing the transglycosylation inhibition properties of both moenomycin degradation products (Welzel, 1993, Scherkenbeck *et al.*, 1993, Marzian *et al.*, 1994) as well as of synthetic compounds (Lüning *et al.*, 1994) it has been shown that the units E-F-G-H-I (Table I) are necessary for total antibiotic activity. Prior to this work, there was no informa-
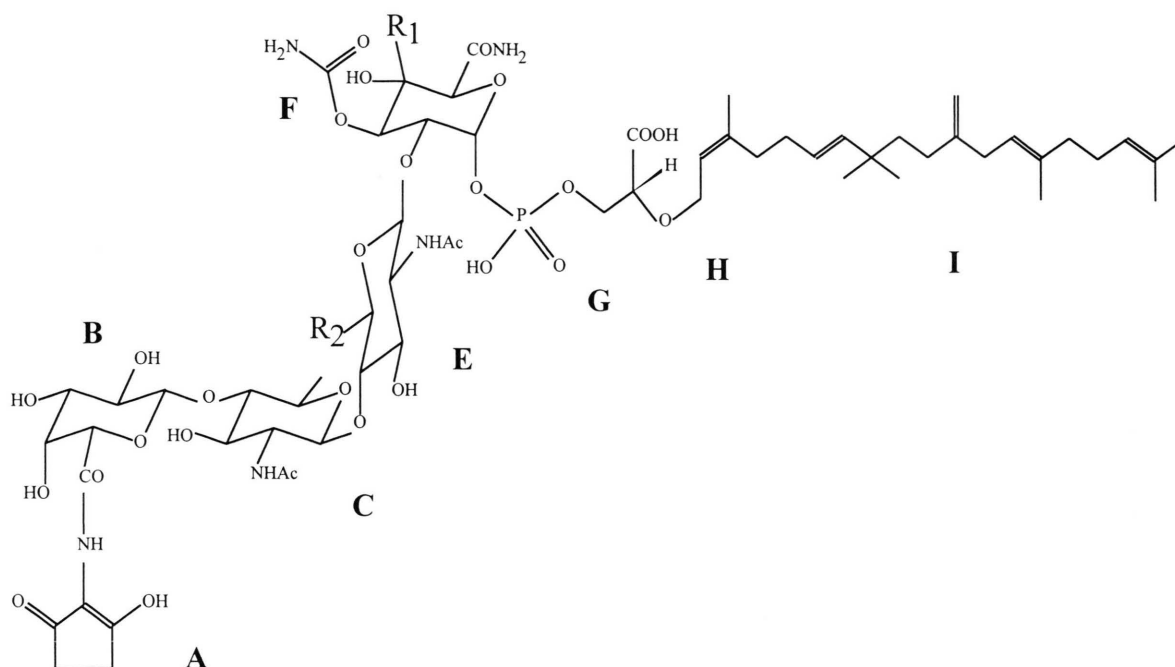
Table I. Structure of moenomycin A and proposed structures of the moenomycin components A_{12}, C_1, C_3 and C_4. Moenomycin A consists of A: 2-aminocyclopentane-1,3-dione, B: D-galacturonic acid, C: D-chinovosamine, R_2: D-glucose, E: D-glucosamine, F: moenuronamide, G,H: phosphoglyceric acid and I: moenocinol. The mass of the $(M+H)^+$ ions of the moenomycins and the difference with respect to that of the published moenomycin A data is shown in the table.

Substance	R_1	R_2	m/z (Da)	Δm (Da)
Moenomycin A	$-CH_3$	$-Glc$	1582	–
Moenomycin A_{12}	$-H$	$-Glc$	1568	14
Moenomycin C_1	$-H$	$-H$	1390	192
Moenomycin C_3	$-CH_3$	$-H$	1404	178
Moenomycin C_4	$-CH_3$	$-OH$	1420	162

tion available concerning the biosynthesis of the moenomycins. In this publication we describe the isolation of *S. ghanaensis* mutants, the development of a solid phase extraction method to obtain moenomycin components from the wildtype and biosynthetic intermediates from the mutants, and a HPLC-MS analysis of the moenomycin components. Furthermore, we performed structure activity studies in order to get an idea of the biosynthetic process.

Materials and Methods

Apparatus and chemicals

Fine chemicals, TLC-plates, HPLC columns, HPLC solvents and Adsorbex reversed phase 18 (RP-18) columns were obtained from (E. Merck, Darmstadt, Germany); yeast extract, tryptone and agar from Unipath (Wesel, Germany); beef extract and antibiotic medium no.1 from Difco (Norderstedt, Germany). Moenomycin standard, moenomycin A degradation product MC and genapol were kindly provided by Hoechst AG. The UV mutagenesis was carried out using a Spectroline ENF-26/F lamp. HPLC was performed using a LKB Bromma (Freiburg, Germany) HPLC system consisting of a 2157 autosampler cum injector, 2249 gradient pump and a 2151 variable wavelength monitor with a 2221 integrator. In addition a 2140 diode array detector equipped with a personal computer and LKB wavelength EG software was used. A triple-quadrupole mass spectrometer API III (Sciex, Thornhill, Ontario, Canada) equipped with a nebulizer-assisted electrospray ion source was also used.

Microorganisms

S. ghanaensis ATCC 14672 wildtype strain producing moenomycin and its UV-mutants 3, 32, and 1146 isolated within this work were used for characterization purposes. *Staphylococcus aureus* p209 (strain collection, Hoechst AG) was used as the sensitive Gram positive test organism.

UV mutagenesis

The wildtype *S. ghanaensis* was cultivated on sporulation agar plates at 30 °C for 7–10 days. The green spores were scraped off and suspended in sterile water. A 10 ml suspension with a titre of 4.55×10^8/ml was then subjected to UV light (254 nm) and samples were taken after different time intervals (0, 3, 5, 7, 10, 12, 15, 17, 20, 25 and 30 min). After a serial dilution till 10^{-4} they were plated on sporulation agar plates and incubated at 30 °C. The sporulation medium contained sucrose 3 g, dextrin 15 g, NaCl 0.5 g, $MgCl_2$ 0.5 g, KH_2PO_4 0.5 g, tryptone 5 g, beef extract 1 g and agar 23 g in 1 liter tap water.

Isolation of stable non-producing mutants

Colonies appearing on selected sporulation agar plates (= 0.7% survival rate after UV mutagenesis) were transferred onto sporulation agar plugs in sterile petri plates and incubated further at 30 °C for 7–10 days. These were then placed on test agar plates seeded with *Staph. aureus* and incubated overnight at 37 °C. The test agar contained 30.5 g antibiotic medium no.1 in 1 liter tap water. *Staph. aureus* was cultivated in Luria broth, overnight, at 37 °C. This medium consisted of tryptone 10 g, yeast extract 5 g, NaCl 0.5 g, glucose 1 g, tap water 1 liter. The cells were centrifuged and the pellet washed twice and resuspended in sterile normal saline. A difference in the type and size of the inhibition zones led to the selection of desired mutant colonies for further analysis.

Cofeeding tests

Stable mutants were grown in parallel, on sporulation agar plates, two at a time, with a gap of about 2 mm. These plates were then incubated at 30 °C. After 10 days, agar strips, each carrying 2 mutants were placed on bioassay plates containing *Staph. aureus* for detecting a cofeeding reaction if any. These strains were also simultaneously cocultivated pairwise in shake flasks for 10 days at 30 °C for the same purpose.

TLC

Pre-coated high performance TLC (HPTLC) RP-18 plates (silica gel, 0.25 mm) were cut into 1 cm strips and spotted with a 3 µl moenomycin solution (1 mg/ml in 20 mM potassium phosphate buffer, pH 7.2) and eluted in different solvent combinations (Table II). Detection was by charring at 110 °C after spraying with 5% H_2SO_4 in absolute EtOH.

Acid extraction

The extraction was carried out as described by Lindner *et al.* (1961).

Solid phase extraction (SPX)

Adsorbex RP-18 columns (100 mg) were used for selective sample enrichment. The columns were activated with each 2 ml of isopropanol, MeOH and 20 mM potassium phosphate buffer, pH 7.2. Then 10 ml of centrifuged culture supernatants were applied, followed by a wash using 2 ml of the same phosphate buffer, 1 ml acetonitrile and 2 ml isopropanol. Selective elution was done with 2 ml of a 50:50 acetonitrile: phosphate buffer mixture. The eluate was vacuum dried and dissolved in 100 µl phosphate buffer. For this purpose the wildtype and the mutants were cultivated for 10 days at 30 °C in 30 ml sporulation media containing 0.5% genapol. The mycelia were additionally homogenised manually together with the culture fluid and centrifuged at 11,000 x *g* for 30 min. 10 ml supernatant were used for SPX.

HPLC

Analytical HPLC was performed using a LiChroCart 250-4 HPLC cartridge (Superspher-100, RP-18 endcapped). Phosphate buffer was prepared by dissolving 10 mg KH_2PO_4 in 1 l demineralised water and adjusted to pH 7.8 with K_2HPO_4. HPLC grade MeOH, acetonitrile plus phosphate buffer (35:25:40 v/v) were degassed with helium and comprised the mobile phase. The flow rate of the mobile phase was 0.4 ml/min. In addition to a UV detector, a diode array detector was em-

ployed. For detection purposes, a wave-length range between 180–300 nm was used. The lipid portion of the moenomycins absorbs strongly below 220 nm due to the presence of double bonds, and the UV chromophore at 258 nm. Semi-preparative HPLC was carried out by injecting 60 μl of an 1 mg/ml moenomycin standard solution into a Lichrosorb RP-18 column (7 μm, 250 x 10 i.d.) and separation was performed at a flow rate of 3 ml/min. The mobile phase contained phosphate buffer (pH 7.8), MeOH and acetonitrile (40:35:25 v/v). Detection wavelength was 258 nm. The separated moenomycin fractions were dried in vacuo and then dissolved in a small volume of phosphate buffer.

Fractions isolated by semi-preparative HPLC were analysed by HPLC-MS on a Nucleosil C-18 column (5 μm, 100 x 2 i.d.) with a linear gradient elution within 10 min from solvent A (0.09% TFA/acetonitrile) to solvent B (0.1% TFA) at a flow rate of 200 μl/min. Electrospray mass spectra were recorded on-line with a triple-quadrupole mass spectrometer equipped with a nebulizer assisted electrospray (= ion spray) source.

Results

Analysis of the moenomycin production pattern of the wildtype Streptomyces ghanaensis (ATCC 14672) using solid phase extraction, HPLC and HPLC-MS

In order to analyse the biosynthesis of an antibiotic, techniques are required for a rapid and reliable isolation and structure determination of biosynthetic intermediates. We therefore developed a solid phase extraction (SPX) protocol for the isolation of moenomycin and moenomycin-like products from culture broths. The hydrophobic RP-18 material was chosen as column material since the lipid portion of moenomycin binds effectively to this matrix in polar solutions, (e.g in buffer) and unbound matter can be simply washed off. RP-18 coated TLC plates were used in a preliminarily way to test the mobility of moenomycin in different solvent systems (Table II). The method was optimised using a moenomycin standard. Using this method, substance recovery after extraction was nearly 100%. With this technique, it was possible to remove constituents of the sporulation medium effectively. The established method is sensitive (only 10 ml sample required), specific and reproducible.

Table II. RP-TLC analysis of the mobility of moenomycin and its R_f values in different solvent systems.

Solvent System	Rf Value on RP18-TLC
Phosphate buffer pH 7.8	0
Methanol	0.95
Acetonitrile	0
Isopropanol	0
Methanol:Acetonitrile:Phosphate buffer 40:20:40 (v/v)	0.75
Methanol:Phosphate buffer 90:10	0.7
Methanol:Phosphate buffer 50:50	0.2
Methanol:Phosphate buffer 10:90	0
Acetonitrile:Phosphate buffer 90:10	0
Acetonitrile:Phosphate buffer 50:50	0.9
Acetonitrile:Phosphate buffer 10:90	0.1
Methanol:Acetonitrile 90:10	0.98
Methanol:Acetonitrile 50:50	0.3
Methanol:Acetonitrile 10:90	0

The culture eluates of the wildtype *S. ghanaensis* obtained after acid extraction and SPX were separated on a RP-18 HPLC column and compared to a 1 mg/ml standard moenomycin solution (Fig. 1). The SPX eluate showed the characteristic elution
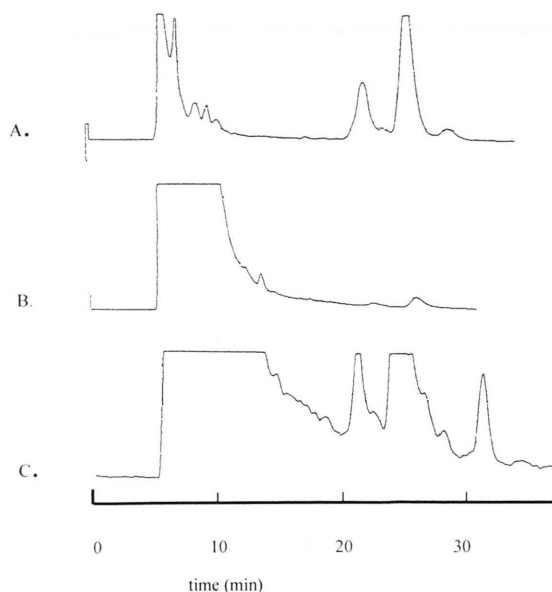

Fig. 1. HPLC profiles of a moenomycin standard solution (A), moenomycin extracts obtained after acid extraction (B) and solid phase extraction (SPX) (C). In each chromatogram UV detection was at 258 nm and the detector sensitivity was 0.02 absorbance units for full scale deflection (AUFS). Profile B corresponds to 200 ml and profile C to 10 ml extracted volume.

profile for the moenomycins (Fig. 1C) as in the case of the standard (Fig. 1A). In the case of the acidic extraction sample, the moenomycin peaks were very small (Fig. 1B). A diode array detector was used, which allowed several wavelengths to be scanned simultaneously and to obtain a UV spectrum of every single detected peak. Using a diode array detector it was found that the moenomycin components A_{12}, C_1, A , C_3 and C_4 absorb both at 258 and below 220 nm and therefore possess both the UV chromophor and the lipid portion.

For a more detailed analysis, the moenomycin components were first separated by semi-preparative HPLC (Fig. 2A), and then the fractions were analysed using HPLC-MS. Since the separation of the components by semi-preparative HPLC was not as good as on an analytical column, only 3 different fractions were collected. These were then checked with an analytical HPLC column in order to determine their purity, and compared with a moenomycin standard. It was found that fraction 1 contained moenomycin A_{12} and minor amounts of the moenomycins C_1, A and C_3 (Fig. 2B). Fraction 2 consisted of a mixture of moenomycin A

and C_3 and fraction 3 contained moenomycin C_4 (Figs. 2C and 2D). These fractions were investigated by HPLC-MS. The individual components of the purified fractions were clearly separated under the selected conditions (Fig. 3). The electrospray mass spectra obtained by HPLC-MS showed the $(M+H)^+$ ions of the different moenomycin components. Fraction 2 was separated on the nucleosil C-18 column into two components with retention times of 8.2 and 8.5 min (Fig. 3A1). The mass spectrum of the first signal had its main mass peak at m/z 1582 (Fig. 3A2) and this result correlated well with that of moenomycin A, which was determined as m/z 1581.7 (Fehlhaber *et al.*, 1990). Interestingly, an additional signal was detected at m/z 1599, which was assigned to a $(M+NH_4)^+$ ion observed also in the case of the other moenomycin components. The component eluting at 8.5 min corresponds to moenomycin C_3 and showed a molecular mass peak at m/z 1404 (Fig. 3A3).

The HPLC-MS analysis of fraction 3 showed that beside moenomycin C_4 it still contained traces of moenomycin A (Fig. 3C1). The TIC showed several peaks. The main mass signal at m/z 1420 belongs to moenomycin C_4 (Fig. 3C2). As previously stated, moenomycin A_{12} (Figure 2B) could only be partially separated. Two major signals at 8.9 and 9.1 min were seen in the total ion chromatogram (Fig. 3B1). The fraction eluting at 8.9 min consisted of two compounds with $(M+H)^+$ ions at m/z 1582 and 1568, respectively (Fig. 3B2). The former as already quoted, corresponds to moenomycin A and the latter to moenomycin A_{12}. The mass difference of only 14 showed that the two components are very similar in their structure. The major compound eluting after 9.1 min showed a protonated molecular ion at m/z 1390. It has the greatest mass difference of 192 with respect to moenomycin A and represents moenomycin C_1 (Fig. 3B3). After determining the moenomycin production pattern of the wildtype it was possible to take a closer look at the biosynthesis of this antibiotic.

Fig. 2. Semi-preparative HPLC separation of the moenomycin complex into its individual components A_{12}, C_1, $A+C_3$ and C_4 (A). The collected fractions were concentrated and checked for their purity on an analytical RP-18 column. Fraction 1 still contained some amounts of C_1, A and C_3 (B). Fractions 2 (C) and 3 (D) were nearly pure. The bar represents a period of 10 min.

Isolation and analysis of moenomycin biosynthesis mutants

To obtain information on the biosynthesis of moenomycin and the part of the molecule relevant
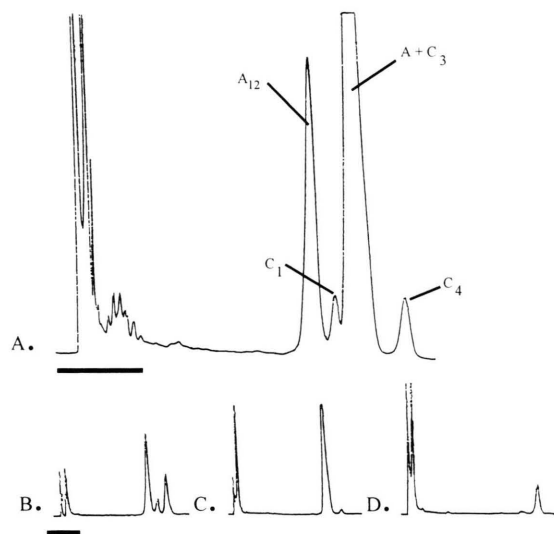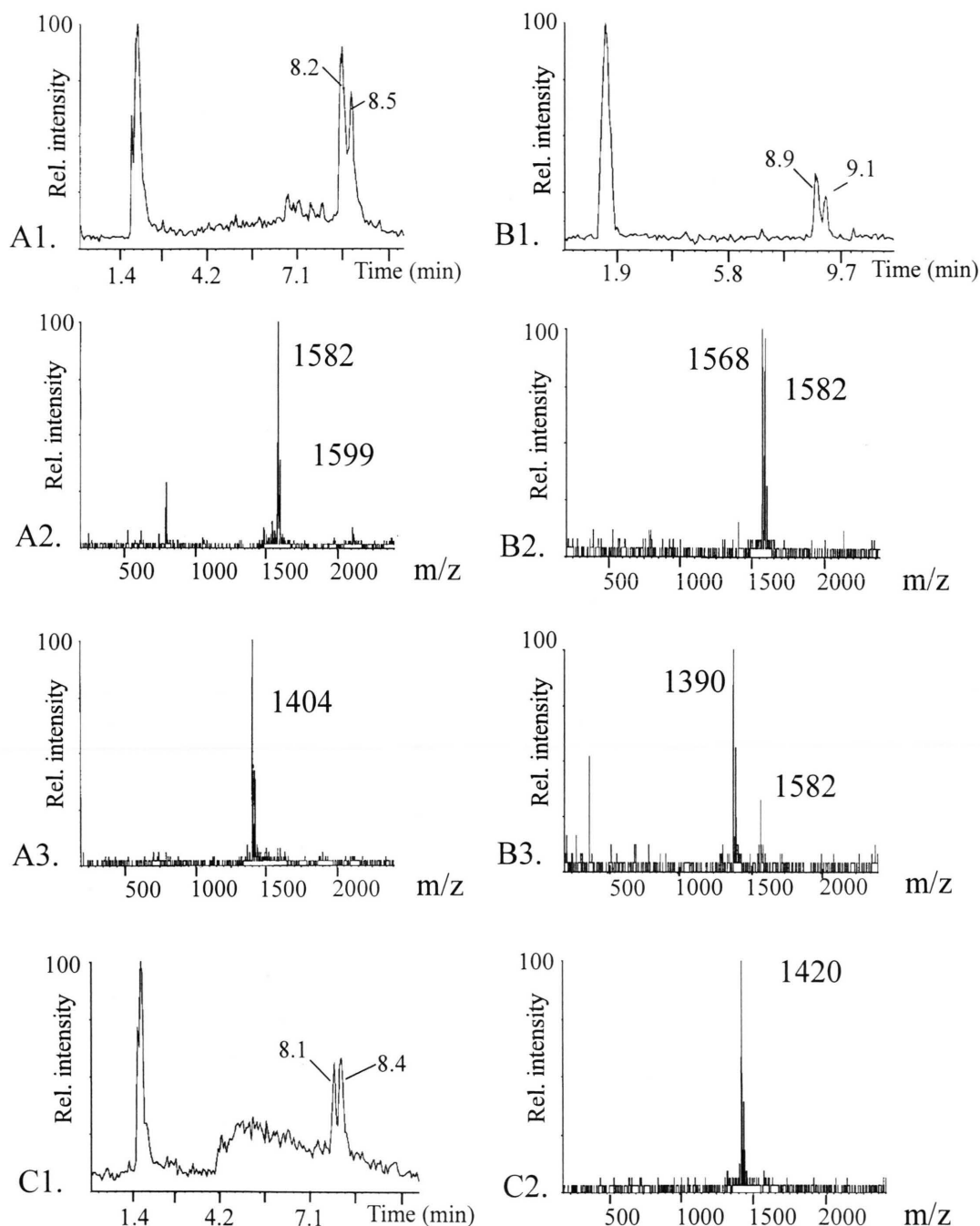
Fig. 3. HPLC-electrospray-MS analysis of the collected moenomycin component fractions. (A1) Total ion current chromatogram of fraction 2. The components A (8.2 min) and C_3 (8.5 min) were separated under these conditions. (A2 & A3) Electrospray mass spectra of the moenomycins A and C_3. The mass of the $(M+H)^+$ ions are indicated. (B1) Total ion current chromatogram of fraction 1. (B2) Electrospray mass spectrum of moenomycin A_{12}. The mass spectrum of the signal at 8.9 min (B1) shows two mass peaks, that of component A_{12} (m/z 1568) and moenomycin A (m/z 1582). (B3) The signal at 9.1 min (B1) also shows two mass peaks in its mass spectrum, that of moenomycin C_1 (m/z 1390) and moenomycin A. (C1) Total ion current chromatogram of fraction 3. Moenomycin C_4 (8.4 min) is well separated from moenomycin A (8.1 min). (C2) Electrospray mass spectrum of moenomycin C_4.

for biological activity, mutants of *S. ghanaensis* were isolated which were blocked in moenomycin biosynthetic steps. Therefore *S. ghanaensis* was irradiated by UV. A survival rate of 0.1%-1% which has been proven to be optimal for the isolation of mutants with single phenotypical mutations (Hopwood, 1970) was achieved after 20–30 min of irradiation in the case of *S. ghanaensis*.

After UV mutagenesis, 16,043 potential mutant colonies were screened for antibiotic production. 83 of them showed inhibition zones different to that of the wildtype strain, which produced a very clear zone. 57 mutants produced clear, but tiny, 2 turbid and 24 null mutants absolutely no inhibition zones. This bioassay procedure was repeated several times using both plate and shake flask cultures of the mutants and they proved to be stable. The null-mutants and those with turbid zones were then subjected to cofeeding experiments. However, none of the mutant combinations lead to a wildtype zone of clearance.

Cultures of three selected mutants were subjected to SPX and the samples were analysed with HPLC. The detection was carried out at 258 nm, specific for the UV chromophore. The mutants 3, 32 (both produced turbid inhibition zones) and 1146 (produced no inhibition zone) showed the presence of new signals in their HPLC profiles (Figs. 4B, 4C and 4D). These mutant products eluted earlier than the components of the moenomycin standard (Fig. 4A) and are therefore more hydrophilic. However the main products synthesized by the mutants 3, 32 and 1146 (Figs. 4B, 4C and 4D) showed longer retention times than the reference substance MC. MC is an enzymatic degradation product of moenomycin A containing only the oligosaccharide and the chromophore (Metten *et al.*, 1992). Since MC lacks the moenocinol portion it is very hydrophilic, indicated by a short retention time in the HPLC system (Table III).

The UV spectra of the major, novel compounds synthesized by the mutant strains obtained after diode array detection revealed similar absorbance characteristics like those of the wildtype moenomycins (data not shown). The great similarity but also the definite differences between these spectra
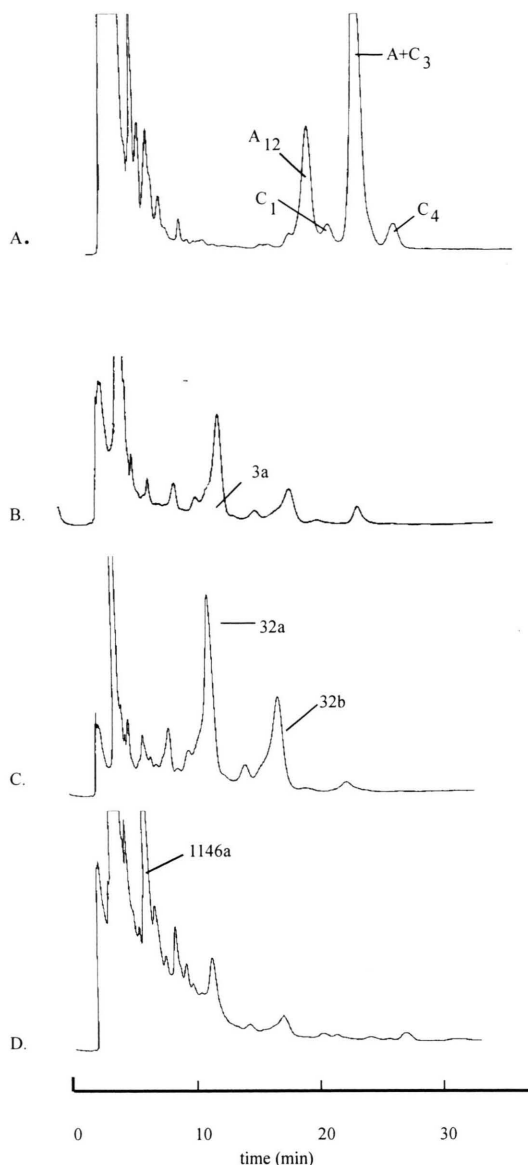

Fig. 4. Comparison of the production spectra of the moenomycin biosynthesis mutants and the wild-type using HPLC. UV detection was at 258 nm and sensitivity was 0.02 absorbance units for full scale deflection (AUFS). HPLC profiles correspond to equal amounts of extracted culture supernatants. The elution profile of the wildtype *S. ghanaensis* (A) was compared with those of mutants 3 (B), 32 (C) and 1146 (D). The moenomycin components (A) and the major novel compounds synthesized by the mutants (B,C,D) are indicated.

Table III. Comparison of the retention times of the moenomycin A derivative MC with the mutant intermediates and the moenomycin components.

Compound	Retention time using RP18-HPLC [min]
Moenomycin A12	21.0
Moenomycin C1	22.3
Moenomycin A+C3	24.6
Moenomycin C4	28.0
Fragment MC	2.3
Compound 3a (mutant 3)	11.4
Compound 32a (mutant 32)	11.4
Compound 32b (mutant 32)	17.2
Compound 1146a (mutant 1146)	6.5

indicated that the mutant peaks originated from the moenomycin pathway.

Discussion

Structural evaluation of the moenomycins produced by S. ghanaensis (ATCC 14672)

The biosynthesis of moenomycin is supposed to be very complex, since this antibiotic is not one single, chemically well defined end product, but composed of structurally similar components (Huber, 1979; Heßler-Klintz *et al.*, 1993; Scherkenbeck *et al.*, 1993; Donnerstag *et al.*, 1995). It was therefore initially important to develop methods for analysing the production spectrum of *S. ghanaensis*, which could then provide a basis, for studying the biosynthetic pathway of this antibiotic. An acidic extraction procedure was employed initially for the isolation of moenomycin from culture extracts (Lindner *et al.*, 1961). This method required a large amount (200 ml) of culture fluid and was very insensitive. Furthermore, moenomycin biosynthetic intermediates labile at lower pH levels would have disintegrated during this process. Especially the allyl ether bond in moenomycin is sensitive to acids (Marzian *et al.*, 1994, and references therein). Therefore, we developed a solid phase extraction (SPX) protocol. This method was optimised using RP-18 TLC plates for the specific isolation of moenomycin and its intermediates. The optimization of the widely applied SPX, using the same adsorbent on TLC plates and SPX columns turned out to be highly efficient. The appropriate solvent system determined by TLC was then used with RP-18 columns. The extraction

proved to be very sensitive and reproducible. It removed disturbing impurities and thus facilitated HPLC analysis. Also, the partial antibiotic activity of the mutants 3 and 32, showing turbid inhibition zones, remained intact after this extraction. To our knowledge none of the literature on SPX to date has applied this simple but very useful time- and material-saving step employing RP-18 TLC plates.

On the basis of degradation and spectroscopic studies a structure for moenomycin was proposed (Welzel *et al.*, 1981); this was confirmed by ^{13}C NMR and fast atom bombardment mass spectrometry (FAB-MS) data (Welzel *et al.*, 1983). A structural revision after a detailed FAB-MS analysis combined with NMR and chemical results was made. This demonstrated that unit F contained a second nitrogen function, a carboxamide group instead of the carboxylic acid (Fehlhaber *et al.*, 1990) (Table I). Using the semi-preparative RP-18 HPLC column, in combination with an analytical RP-18 column from a different manufacturer, a separation of all the moenomycins was possible. The exact reproduction of the published moenomycin A molecular mass which is 1581.7 (Fehlhaber *et al.*, 1990) was used here as a positive, internal control. The spectrum of moenomycin C_3 showed a mass peak at m/z 1404 making a mass difference of 178 between moenomycin A and C_3. Since the UV chromophore is still present in moenomycin C_3 as shown by HPLC-DAD, it is probable that the glucose unit D (Table I) is missing.

The mass peak at m/z 1420, corresponding to moenomycin C_4, shows a difference of 16 from that of moenomycin C_3; this correlates with a single oxygen atom (Fig. 3C2). These results indicate the proposed structures for moenomycin C_3 and C_4 based on FAB-MS and ^{13}C NMR methods, after chemical degradation of moenomycin C_3 (Scherkenbeck *et al.*, 1993). The structure for moenomycin C_4 has not been proved rigorously by this means.

Component A_{12} appeared at m/z 1568 (Fig. 3B2) and this slight difference of 14 u from moenomycin A suggested that the two components are structurally very similar, with the difference due to the lack of a methyl group. This correlated with the structure proposal for moenomycin A_{12} (Donnerstag *et al.*, 1995). Most likely the substance eluting at 9.2 min is moenomycin C_1. The observed mass difference from that of moeno-

mycin A (Fig. 3B3) is in agreement with a structure in which both a glucose (-178) and a methyl group (-14) are absent. This is plausible, since moenomycin C_3 lacks a glucose molecule and moenomycin A_{12} a methyl group. Our results are based on a semi-preparative HPLC separation of the moenomycin components obtained after SPX from the wildtype, and subsequent HPLC-MS analyses.

Structural requirements for the antibiotic activity of moenomycin determined by S. ghanaensis mutants

Biosynthetic pathways of secondary metabolites can be understood much better with the help of mutants blocked at different stages. For example, different antibiotic biosynthetic pathways could be proposed after analysing a set of null mutants as in the case of bialaphos (Murakami *et al.*, 1986). However, no cofeeding was observed in the case of the isolated *S. ghanaensis* null mutants. This may be due to minute amounts of cosynthesis products, which are insufficient for the total inhibition of *Staph. aureus* and the subsequent production of clear inhibition zones. Another more likely possibility may be the lack of, or difficulty in, intermediate uptake between the mutants.

In order to elucidate the biosynthetic pathway, further analyses of the mutant products using biochemical methods were necessary. None of the analysed mutants produced the moenomycins A_{12}, C_1, $A+C_3$ and C_4. The mutants 3, 32 and 1146 synthesized more hydrophilic compounds with shorter retention times than those of the moenomycin components, but longer than that of the moenomycin A degradation product MC (Table III). The amount of the compounds secreted by the mutants was very small so that it was not possible to isolate enough material to carry out a HPLC-MS analysis.

The wild-type *S. ghanaensis* (ATCC 14672) releases moenomycin with the completely intact lipid chain. This can then bind to the transglycosidase of *Staph. aureus* p209 (van Heijenoort *et al.*, 1992), resulting in the formation of clear zones of inhibition. The mutants 3 and 32 produce moenomycin intermediates which are more hydrophilic than the moenomycin components, owing to the shorter retention times in the HPLC column. One may speculate that these mutants have turbid

zones of inhibition because the shorter lipid chains of their intermediates cannot retain the transglycosidase; this probably means that the binding efficiency has been reduced and only a partial inhibition of the test organism is possible. The mutant 1146 synthesizes an intermediate, which is rather hydrophilic, but more hydrophobic than MC; which contains only the UV chromophore and the sugar portion of moenomycin A. This mutant product perhaps has a too short lipid chain to be able to bind properly to the transglycosidase and function as a competitive inhibitor. It shows no inhibition zones in the bio-assay. The C_{25} lipid chain (moenocinol) is composed of 3 isoprene units, and the central C_{10} part (C-5 through C-22) does not obey the isoprene rule (Böttger and Welzel, 1983). It is most probable that the mutants 3 and 32 are involved much earlier in the biosynthesis of the lipid chain than the mutant 1146. Since all these intermediates still possess the UV chromophore, which is bound to the last sugar moiety, the sugar portion with perhaps the exception of the branched glucose (unit D, Table I) may be still present and unchanged. These data suggest that the length of the synthesized lipid portion is extremely important for the antibiotic activity of the mutant intermediates. The data obtained after mutant analyses, did not establish whether moenocinol is attached as a complete unit to the moenomycin oligosaccharide, or less likely whether the lipid part is subsequently synthesized on the attached sugar part.

The essential, functionally important parts of the moenomycin molecule are the units E-F-G-H-I (Fehlhaber *et al.*, 1990) presented in Table I. Removal or oxidation of the lipid unit of moenomycin A lead to a total loss of antibiotic activity (Marzian *et al.*, 1994), whereas saturation of all the double bonds in the lipid part of moenomycin A (Welzel *et al.*, 1983) and the deletion of the sugar portion (Welzel *et al.*, 1987, Fehlhaber *et al.*, 1990) did not affect its antibiotic activity. The lipid chain has to perhaps insert itself in the membrane of the damaged bacterium, in order to compete with the C_{55}-lipid carrier. Differences in the *in vivo* activity against *Staph. aureus* and the *in vitro* inhibition of the transglycosylase were noticed. Whereas the hexahydroxy compound obtained after the oxidation of moenomycin A was inactive both *in vivo* and *in vitro*, the tetrahydroxy-moenomycin A de-

rivative was inactive *in vivo* but showed high activity in the *in vitro* test systems. This observation seems to indicate that a non-polar lipid part is necessary for the attachment of the antibiotic to the cytoplasmic membrane of the test organisms. In the case of the *in vitro* systems, the enzyme active site may be more directly accessible. Furthermore, a degradation product of moenomycin A obtained after the removal of both the lipid and the chromophore part exhibited distinct activity in an *in vitro* test system but it was inactive *in vivo* (Marzian *et al.*, 1994). Based on these data it seemed likely that the major intermediates from the mutants 3 and 32 have incomplete lipid parts, are therefore more polar and cannot attach strongly to the cytoplasmic membrane of *Staph. aureus*. This would lead to only a partial inhibition of the test organism. The intermediate from the mutant 1146,

which probably has a very short lipid chain, was very polar. This could account for its failure to attach to the cytoplasmic membrane and thus produced no inhibition zones. Based on the biological activity and the analytical data, we assume that a modification and in particular a shorter lipid portion, drastically influences the inhibitory activity of moenomycin.

Acknowledgments

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