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MALDI-TOF mass spectrometry, an efficient technique for *in situ* detection and characterization of actinomycins

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An extensive study of actinomycins was performed using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS). Actinomycins represent a well-known family of peptidolactone chromopeptides with potent cytostatic and antibiotic properties. Using five well-characterized streptomycete strains, we introduced MALDI-TOF MS as an efficient technique for rapid *in situ* detection of actinomycins in surface extracts of cells picked from agar plates. By this procedure, actinomycin complexes can be investigated with high sensitivity and accuracy in a minimum of time. These studies were complemented by mass spectrometric investigation of actinomycins obtained from culture filtrate extracts and purified by high-performance liquid chromatography to detect yet unknown actinomycin species. By feeding experiments, C-demethyl-actinomycins from *Streptomyces chrysomallus* and *Streptomyces parvulus* as well as *hemi*-actinomycins from *Streptomyces antibioticus* lacking one of the two pentapeptide lactone rings were isolated and characterized as novel variants for structure–activity relationship studies. Structural characterization of the investigated actinomycins was performed by post source decay MALDI-TOF MS. The specific features of the fragmentation patterns of the protonated and cationized forms of selected actinomycins were investigated in detail. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: actinomycins; MALDI-TOF MS; *in situ* detection; structural characterization; PSD-MALDI-TOF MS

Introduction

Actinomycins are a family of closely related chromopeptides with potent anticancer and antibiotic activities of medical and biotechnological interest.[1] An overview on naturally occurring actinomycins was given by Bitzer *et al.*[2] They are produced by a variety of streptomycete strains, each synthesizing a specific set of such compounds. They consist of a 2-amino-4,6-dimethyl-3-ox o-phenoxazine-1,9-dicarboxylic chromophore to which two pentapeptide lactone rings are connected each to one of the carboxylic groups in positions 1 and 9 of the phenoxazinone core. Prominent examples are the actinomycins of the C-type,[3–6] G-type,[7] X-type,[8–10] Y-type[2] and Z-type.[11] The structures of actinomycins and related peptidolactone compounds are shown in Fig. 1.

Variations in the actinomycin structure are restricted to amino acid substitutions in the peptide lactone rings, while the phenoxazinone core is essentially unmodified. In some cases, cyclization is observed between the amino group in position 2 of the chromophore with functional groups of modified threonine residues in the β-ring, such as chlorine or a hydroxyl group at position 4 of the threonine component in the β-ring. In biosynthetic studies, we recently reported *mono*-demethyl and *di*-demethyl derivatives of actinomycin D, which lack one or both methyl groups in 4-position and 6-position of the phenoxazinone core[12] and which are one of the first variations directly at the chromophore. Remarkably, actinomycins function as intercalators with the DNA double helix. Their phenoxazinone core fits between two guanine/cytosine base pairs with the peptidolactone side chains located in the minor grove of the helix. In this way, these compounds can be used as specific inhibitors to study the transcription process.

Several publications have been focused on the structural characterization of actinomycins by mass spectrometric and nuclear magnetic resonance methodologies.[2,7,11–19] In earlier work, a wide spectrum of mass spectrometric techniques has been used for the detection and structural characterization of actinomycins using ionization by electron ionization,[13] plasma desorption,[14] fast atom bombardment[15,16] and more recently by electrospray ionization (ESI).[7,11,19] In this paper, we present a comprehensive and comparative mass spectrometric study of actinomycins using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS). This modern method offers the advantage for rapid *in situ* detection of actinomycins in surface extracts of streptomycete cells picked from agar plates. By this procedure, actinomycin complexes can be investigated with high sensitivity and accuracy in a minimum of time followed by structural characterization using post source decay (PSD)-MALDI-TOF MS. Because of their interesting biological properties and their attractive potential for medical and biotechnological applications, such research is of high interest to make available novel actinomycins for structure–activity relationship studies.

Materials and methods

Chemicals

Actinomycin D was purchased from Applichem (Darmstadt, Germany). The matrix α-cyano-4-hydroxycinnamic acid was from Bruker Daltonics, Bremen, Germany. Etamycin was isolated from cultures of

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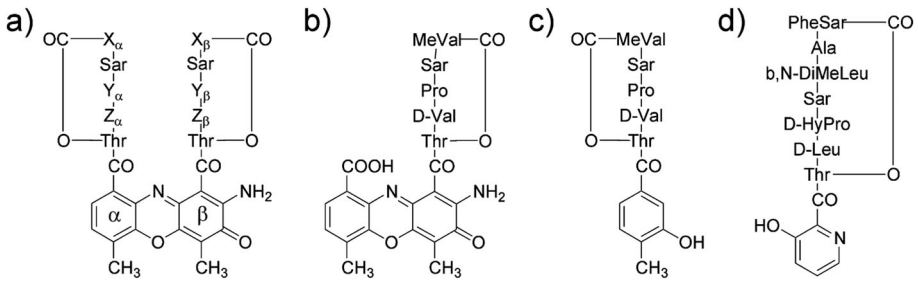

Figure 1. Structures of actinomycin, actinomycin derivatives and the heptapeptidelactone antibiotic etamycin. (a) general structure of actinomycin; (b) *hemi*-actinomycin D; (c) 4-methyl-3-hydroxybenzoic acid-(4-MHB)-pentapeptide lactone (actinomycin half molecule); (d) etamycin. $X_{\alpha,\beta}$, $Y_{\alpha,\beta}$ and $Z_{\alpha,\beta}$ refer to the different amino acid substitutions between the actinomycins. MeVal: L-*N*-methylvaline; Sar: L-*N*-methylglycine; PheSar: phenylsarcosine; β,*N*-DiMeLeu: L-β,*N*-dimethylleucine; D-Hypro: *allo*-D-hydroxyproline.

Streptomyces griseoviridus according to Schlumbohm and Keller.[20] and purified by high-performance liquid chromatography (HPLC). All other chemicals were of the highest purity commercially available.

Strains and cultures

Streptomyces chrysomallus ATCC 11523, *Streptomyces parvulus* ATCC 12434 and *Streptomyces antibioticus* ATCC 14888 (IMRU 3720) were from the American Type Culture Collection. *Streptomyces fradiae* DSM 40711 and *Streptomyces iakyrus* DSM 41873 were from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). All strains were maintained on complete medium (CM) agar[21] or on GYM *Streptomyces* medium (DSMZ). To produce mycelium actively synthesizing actinomycins growth of *S. chrysomallus* and *S. parvulus* in liquid culture was either for up to 2 days in liquid CM[21] or for 3 to 4 days in a glutamate–mineral salts medium.[22] *S. antibioticus*, *S. iakyrus* and *S. fradiae* were grown exclusively in the glutamate–mineral salts medium. All cultures were performed with 1% maltose plus 0.1% glucose as carbon source (both sterilized separately) using 500 ml Erlenmeyer flasks containing 200 ml medium. Incubation was at 28 °C and 220 rpm in a New Brunswick G 25 shaker. The content of actinomycins in liquid cultures was determined by extraction of culture aliquots with ethyl acetate and evaporation to dryness and subsequent spectrophotometric determination at 441 nm using authentic actinomycin D as standard. For preparation of surface extracts, streptomycete cells were picked from agar plates and extracted with 50 µl of 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid. Extracted cells were removed by centrifugation, and the supernatants used for mass spectrometric investigation of actinomycins.

Feeding experiments

Feeding experiments with 3-hydroxyanthranilic acid (3-HA) and 4-methyl-3-hydroxyanthranilic acid (4-MHA) were performed as follows: Mycelium from cultures of a streptomycete actively synthesizing actinomycins was harvested by suction on a Buechner funnel and after washing with tap water resuspended in 40% of the original volume of tap water containing a carbon source (0.5–1% glucose or galactose), 1–2 mM of each L-threonine, L-valine, L-proline, L-glycine and 100 µM L-methionine. Of this suspension, 5–8 ml portions were placed in 100 ml Erlenmeyer flasks, and 3-HA or 4-MHA was added at concentrations of 200 µM. The controls contained no such additive. After shaking at 220 rpm and 30 °C for up to 4 h, each flask was extracted with ethyl acetate, and the extracts were separated by thin layer chromatography (TLC) or HPLC or by a combination of both. For the preparation of 4-MHB pentapeptide lactone, *S. chrysomallus* was grown up to 1 day in liquid CM[21] after which 4-methyl-3-hydroxybenzoic acid (4-MHB) was added in 4 mM concentration. After shaking at 220 rpm and 30 °C for up to 4 days, each flask was extracted with ethyl acetate, and the extracts were separated by HPLC.

HPLC separations

Actinomycin mixtures were fractionated using a Waters HPLC system or a Pharmacia LKB HPLC system (Pump 2248, WWM 2141). Separation was performed on a prepacked EnCaPharm 100-RP18 column (Molnar Institute, Berlin, Germany) isocratically with 60% (by vol.) acetonitrile–water at a flow rate of 1 ml min^{-1}. The detection wavelength was 452 nm.

Mass spectrometric analysis

MALDI-TOF mass spectra were recorded using a Bruker Autoflex instrument equipped with a 337 nm nitrogen laser for desorption and ionization. Of surface extracts or HPLC fractions, 2 µl samples were mixed with the same volume of matrix medium (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid), spotted on the target, air dried and measured. Spectra were recorded by positive ion detection in reflector mode. The acceleration and reflector voltages were 19 and 20 kV, respectively, in pulsed ion extraction mode. Most of the matrix ions were suppressed until 350 Da. The structure of actinomycins was investigated by PSD-MALDI-TOF MS.

Preparation of desaminoactinomycin D and test by TLC

Actinomycin D was incubated with 0.5 ml of 10% HCl for 16 h at 40 °C. After incubation, the product was extracted with 1 ml ethyl acetate, and the extract was evaporated to dryness.[23] The purity of the desaminoactinomycin D (2-hydroxy-actinomycin D) was tested by TLC using ethyl acetate : methanol : water : dimethylformamide (100 : 5 : 5 : 1, by vol.) as the solvent.

Results and discussion

In situ detection of actinomycins in surface extracts of five streptomycete strains by MALDI-TOF mass spectrometry

The actinomycin complexes produced by five representative *Streptomyces* strains, *S. chrysomallus*, *S. parvulus*, *S. antibioticus*, *S. fradiae* and *S. iakyrus*, were investigated by MALDI-TOF MS. For

this purpose, these strains were grown on agar plates using either the CM-medium or the GYM-medium. The best results have been obtained with the CM-medium. After growth for 24 h, cells were picked from the agar plates and extracted with 50% acetonitrile/0.1% trifluoroacetic acid. In this way, surface extracts were prepared, which efficiently can be used for mass spectrometric investigation of products that are attached to the outer surface of the cell wall of the tested *Streptomyces* strains. For most of these organisms, the actinomycins were the dominating metabolites. All these compounds could be detected *in situ* by MALDI-TOF MS with high sensitivity and precision in a minimum of time, frequently without the need for further fractionation and purification. The MALDI-TOF mass spectra of the actinomycins found in surface extracts of the five test strains are shown in Fig. 2. They exhibit peaks for their protonated forms as well as for their sodium and potassium adducts, which dominate in the spectra. Monoisotopic mass numbers were obtained. The resolution of the mass data was in the range of 5000. Deviations from the calculated values were ±0.2 Da. The MALDI-TOF mass spectra of all investigated actinomycins exhibit an unusual pattern of isotope distribution comprising 5 to 6 peaks, which will be discussed later in detail. The mass numbers of the detected $[M+H]^+$ ions and the alkali adducts of actinomycins are listed in Table 1. They essentially correspond to those that have been previously reported for the five test strains.[2,7,11–17] Because it is not possible in our study to discriminate between amino acid substitutions in the α-ring and β-ring and to distinguish optical isomers by MALDI-TOF mass spectrometry, we used the knowledge[1–12] on the structure of actinomycins for assignment of the detected actinomycin product ions.

Detection of C-type and X-type actinomycins

S. chrysomallus forms actinomycins of the C-type comprising C_1–C_3, which differ at the D-Val position of their peptide lactone rings. Actinomycin C_1 (IV, D) that yields a $[M+H]^+$ ion at *m/z* 1255.3 is the well-known reference compound actinomycin D containing D-Val in both rings, while in actinomycins C_2 and C_3 with $[M+H]^+$ ions at *m/z* 1269.3 and 1283.4, one or both D-valine residues are replaced by D-*allo*-Ile, respectively[2,6] (Fig. 2a). *S. chrysomallus* is also a potent producer of nonactins, which in this case dominate in the mass spectrum. Their $[M+H]^+$ ions were found between *m/z* 740 and 840 in Fig. 2b.

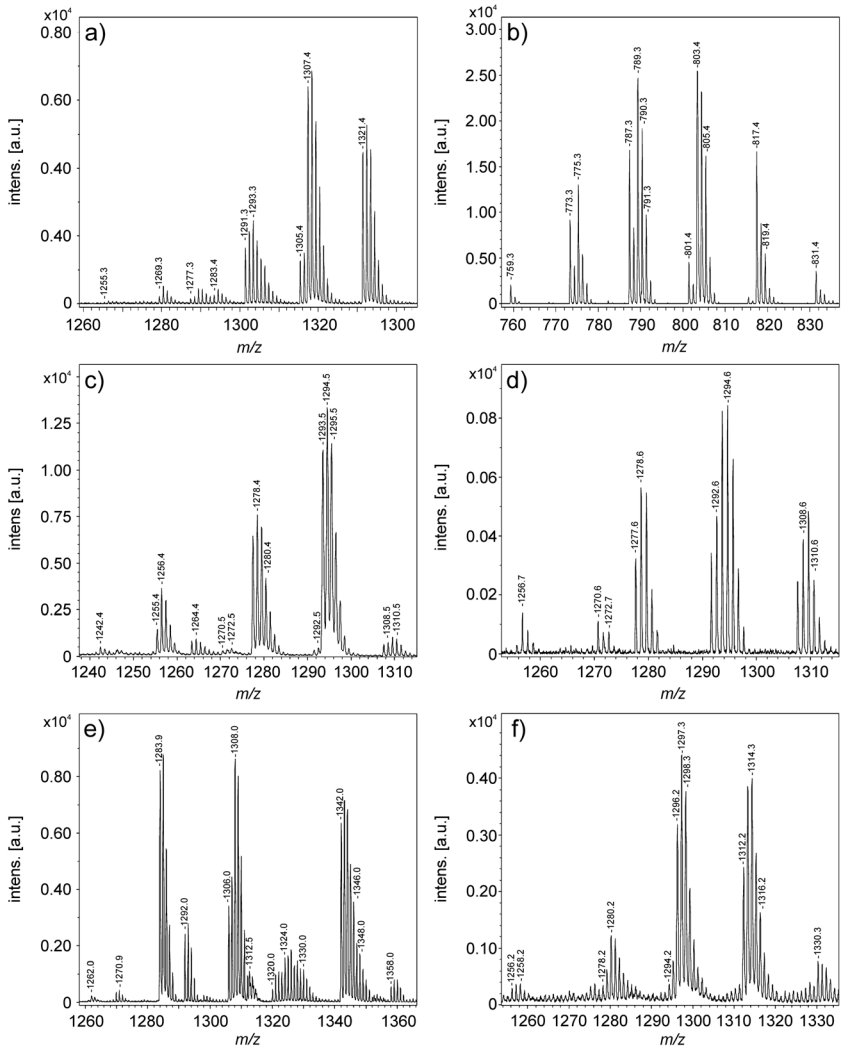

Figure 2. Rapid *in situ* detection of actinomycins in surface extracts of cells taken from agar plates of five streptomycete strains by MALDI-TOF MS. (a and b) *Streptomyces chrysomallus*, (c) *Streptomyces parvulus*, (d) *Streptomyces antibioticus*, (e) *Streptomyces fradiae* and (f) *Streptomyces iakyrus*.

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Table 1. A survey of actinomycins of five representative *Streptomyces* strains as detected by *in situ* MALDI-TOF MS

Strain	Actinomycin species	$M + [H,Na,K]^+$ (*m/z*)
a) *In situ* detection in surface extracts:		
S. chrysomallus	Actinomycin C_1 (D, IV)	1255.4/1277.4/1293.4
	C-Demethylactinomycin C_1	1241.4/1263.4/1279.4
	Actinomycin C_2	1269.4/1291.4/1307.4
	Actinomycin C_3	1283.4/1305.4/1321.4
S. parvulus	Actinomycin C_1 (D, IV)	1255.5/1277.5/1293.6
	C-Demethylactinomycin C_1	1241.4/1263.4/1279.5
	Actinomycin V	1269.5/1291.6/1307.5
	Actinomycin I	1271.6/1293.6/1309.6
S. antibioticus	Actinomycin C_1 (D, IV)	1255.7/1277.7/1293.7
	Actinomycin V	1269.7/1291.7/1307.6
	Actinomycin I	1271.7/1293.7/1309.6
S. fradiae	Actinomycin Z_1	1301.2/1323.2/1339.2
	Actinomycin Z_2	1286.0/1308.0/1324.1
		1284.0/1306.0/1322.1*
	Actinomycin Z_3	1320.0/1342.1/1358.2
		1322.3/1344.3/1360.3*
	Actinomycin Z_4	1270.0/1292.0/1306.0
		1268.3/1290.3/1308.3*
	Actinomycin Z_5	1304.2/1326.2/1342.2
		1306.2/1328.2/1344.2*
		1315.3/1337.3/1353.4*
S. iakyrus	Actinomycin G_4	1256.2/1278.3/1294.3
		1258.1/1280.1/1296.2*
	Actinomycin G_3/G_6	1274.3/1296.3/1312.3
		1276.2/1298.2/1314.3*
		1244.3/1266.3/1282.3*
		1314.2/1336.3/1352.3*
b) Detection in culture filtrates from feeding experiments:		
S. chrysomallus	4-MHB-peptidolactone C_1	616.4/638.4/654.4
	4-MHB-peptidolactone C_2	630.5/652.5/668.5
S. antibioticus	C-Demethylactinomycin C_1	1241.7/1263.7/1279.8
	C-*di*-Demethylactinomycin C_1	1227.7/ 1249.7/ 1265.7
	C-Demethylactinomycin V	1255.9/1277.9/1293.9
	C-*di*-Demethylactinomycin V	1241.9/1263.9/1279.9
	C-Demethylactinomycin I	1257.8/1279.8/1295.8
	C-*di*-Demethylactinomycin I	1243.7/1265.7/1281.8
	hemi-Actinomycin C_1 (D, IV)	792.5/814.5/830.6
	C-Demethyl-*hemi*-actinomycin C_1	778.5/800.5/816.5
	C-*di*-Demethyl-*hemi*-actinomycin C_1	764.5/786.5/802.5

The mass data of the protonated form and the alkali adducts of the detected actinomycins were listed. Unidentified species were indicated by an asterisk.

The main actinomycin in the mass spectrum of the surface extract of *S. parvulus* in Fig. 2c is actinomycin D (C_1, IV). In addition, two minor peaks were detected at *m/z* 1269.5 and 1271.6, which could be attributed to the $[M + H]^+$ ions of actinomycins V and I, which hitherto have not been reported in previous characterization of this streptomycete strain. A similar pattern of actinomycins is exhibited by *S. antibioticus* (Fig. 2d).[2–6] It produces a mixture of actinomycin D, actinomycins I and V, which belong to the actinomycin X-complex. They differ from each other in the proline site of their β-ring. While actinomycin D (IV, C_1) is an iso-actinomycin with two identical pentapeptide lactone rings each containing proline, the asymmetric actinomycins I and V (aniso-actinomycins) show a modification at the proline site in the β-ring. Both are minor products. Actinomycins I and V show a 4-hydroxyproline or a 4-oxoproline at this position, respectively.

Detection of G-type and Z-type actinomycins

S. fradiae (Fig. 2e) and *S. iakyrus* (Fig. 2f) produce actinomycins of the Z-type and G-type, respectively.[11,7] In the surface extract of *S. fradiae*, $[M + H]^+$ ions of variants Z_2, Z_3 and Z_4 were detected at *m/z* 1284.0, 1320.0 and 1270.0, while for *S. iakyrus*, $[M + H]^+$ ions of G-type species were found at *m/z* 1256.2 and 1274.3, which could be attributed to G_4 and G_3/G_6. For these two strains, it was difficult to detect all actinomycin variants so far known by *in situ* MALDI-TOF MS and to identify unknown species. Thus,

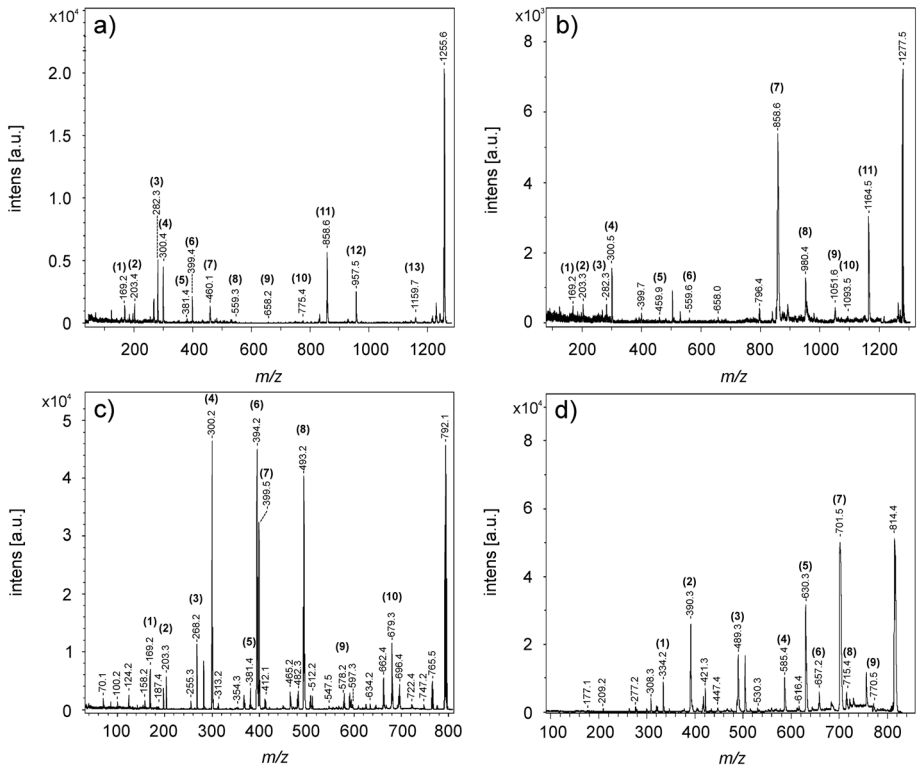

Figure 3. Structure analysis of actinomycin D from *Streptomyces chrysomallus* and *hemi*-actinomycin D produced by feeding of 4-MHA to *Streptomyces antibioticus* by PSD-MALDI-TOF MS. PSD-production spectra of (a) protonated actinomycin D ($[M+H]^+$ *m/z* 1255.6) and (b) its sodium adduct ($[M+Na]^+$ *m/z* 1277.5) and (c) protonated *hemi*-actinomycin D ($[M+H]^+$ *m/z* 792.6) and (d) its sodium adduct ($[M+Na]^+$ *m/z* 814.5). Peptidolactone rings of actinomycin D: X_α, X_β = L-*N*-methylvaline; Y_α, Y_β = L-proline; Z_α, Z_β = D-valine.

efficient fractionation and further purification of the actinomycin mixture were needed. Therefore, we separated both the surface extracts and the ethyl acetate extracts of the culture supernatants of *S. fradiae* and *S. iakyrus* by HPLC (data not shown). By combination of MALDI-TOF mass spectrometric analysis with fractionation by HPLC, we succeeded in identifying most of the Z-type and G-type actinomycins so far described in the literature.[11,7] For example, in this way also, the $[M+H]^+$ ions of Z_1 and Z_3 were detected at *m/z* 1301.2 and 1303.1. Z_1 and Z_3 appeared as minor products in the ethyl acetate extracts. In addition, some unidentified actinomycins of the G-type and Z-type were found whose structures still remain to be elucidated. They are marked in Table 1 by an asterisk. The characterization of these unknown actinomycins needs further intensive separation and purification.

Characterization of novel actinomycins obtained from feeding experiments

Novel actinomycin species such as C-demethylactinomycins and *hemi*-actinomycins were obtained by feeding actively growing mycelia of streptomycete strains with precursors of actinomycin biosynthesis. *S. chrysomallus* and *S. parvulus* were fed with 3-h ydroxy-anthranilic acid (3-HA), which is incorporated into pentapeptide lactone precursors in competition with the regular precursor 4-MHA. In this way, two novel actinomycins were identified as the *mono*-C-demethyl and *di*-C-demethyl derivatives of actinomycin D lacking either one or both methyl groups in positions 4 and 6 of the phenoxazinone core. In *S. parvulus* these were obtained in a yield of at most 10%. In this way, we generated biosynthetically actinomycin species with modifications at the chromophore for the first time.[12] These compounds were also detected in traces by MALDI-TOF MS under natural conditions in surface and culture filtrate extracts of unsupplemented *Streptomyces* cultures. Using this technique for *S. antibioticus*, the *mono*-C-demethyl and *di*-C-demethyl derivatives of all its actinomycin products, actinomycins D, I and V, were found (Table 1).

Other novel actinomycins were obtained by feeding *S. antibioticus* with excess of 4-MHA and 3-HA. The phenoxazinone moiety of actinomycins is formed in the last step of their biosynthesis by oxidative condensation of two 4-MHA-pentapeptide lactone precursors. 4-MHA shows a reactive *o*-aminophenol structure, which is able to directly condense with 4-MHA-pentapeptide lactone units resulting in a phenoxazinone moiety with only one instead of two pentapeptide lactone rings as in actinomycin. In this way, *hemi*-actinomycins were created as new compounds, which were characterized by MALDI-TOF MS (Table 1). External addition of 3-HA to mycelium of *S. antibioticus* instead of 4-MHA led to the formation of C-demethyl-*hemi*-actinomycins lacking one or both methyl groups in positions 4 and 6 of the phenoxazinone core. Such *hemi*-actinomycins could be detected in minute quantities by MALDI-TOF MS also under natural conditions in long-term cultures of *S. antibioticus*. In this way, novel actinomycin species were generated that can be used for structure–activity relationship studies towards medical and biotechnological applications.

In this paper, we give an overview on the yet identified species of these new actinomycins. The pathways for the production of the C-demethyl derivatives and their biochemical characterization are described in the study of Crnovčić *et al.*[12] For the *hemi*-actinomycins such data are presented in another recent paper.[24]

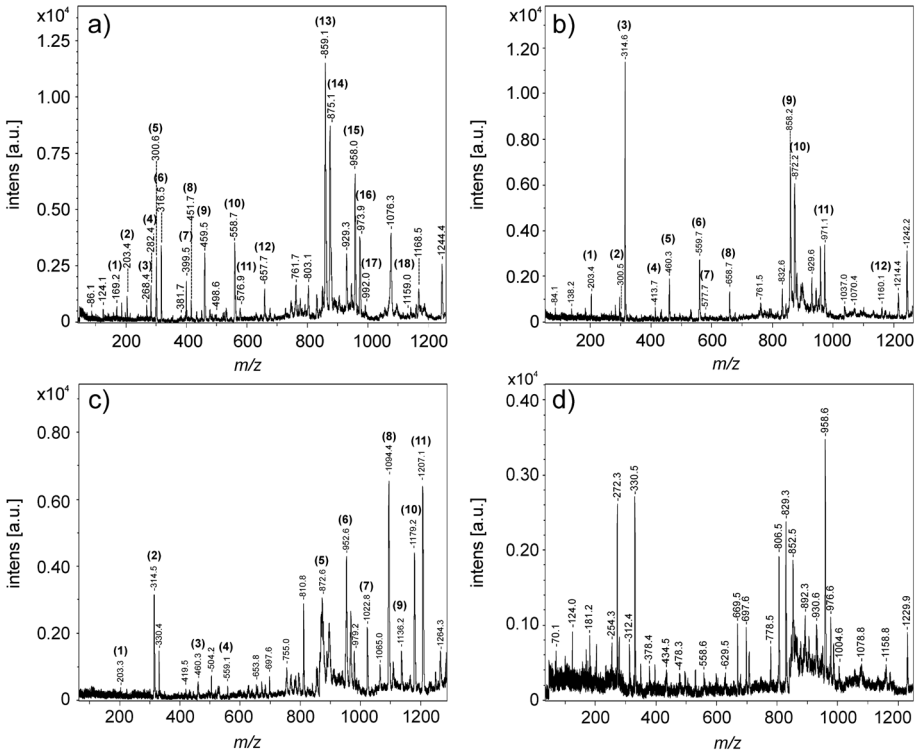

Figure 4. Structure analysis of a X-type, Z-type and G-type actinomycin produced by *Streptomyces antibioticus, Streptomyces fradiae* and S*treptomyces iakyrus*, respectively, by PSD-MALDI-TOF MS. PSD-fragment spectra of (a) protonated actinomycin I ($[M+H]^+$ *m/z* 1272.0), (b) protonated actinomycin Z_4 ($[M+H]^+$ *m/z* 1270.1), (c) the sodium adduct of actinomycin Z_4 ($[M+Na]^+$ *m/z* 1292.2) and (d) protonated actinomycin G_4 ($[M+H]^+$ *m/z* 1257.8). In all PSD-MALDI-TOF spectra, the dominant precursor ion is cut off for a better presentation of the fragment peaks. Peptidolactone rings of (a) actinomycin I: X_α, X_β = L-*N*-methylvaline; Y_α = L-proline; Y_β = trans-4-hydroxyproline (Hyp); Z_α, Z_β = D-valine. (b and c) actinomycin Z4: X_α = L-*N*-methylvaline; X_β = L-*N*-methylalanine; Y_α = *cis*-5-methylproline; Y_β = *cis*-5-methyl-4-oxoproline; Z_α, Z_β = D-valine. (d) actinomycin G4: X_α = L-*N*-methylvaline; X_β = L-*N*-methylalanine; Y_α = *trans*-3-hydroxy-*cis*-5-methylproline; Y_β = L-proline; Z_α, Z_β = D-valine.

Structural characterization of actinomycins by PSD-MALDI-TOF MS

Structural characterization of some of the investigated actinomycins and related compounds was performed by fragment analysis using PSD-MALDI-TOF MS. Examples of the investigated C-type, G-type, X-type and Z-type actinomycins are presented in Figs. 3 and 4. The obtained product ions are summarized in Tables 2–5. Each table is divided into two parts: A and B. Part A shows the product ions of the peptidolactone moieties, while in part B, we have listed product ions that were formed from losses of amino acids and peptide constituents of the actinomycin precursor ions, still containing the phenoxazinone core.

In the PSD-MALDI-TOF product ions spectra of all investigated actinomycins, the isotope distribution of the product ions was not well resolved representing average mass data. Generally, the product ions peaks of the peptide lactone rings are sharp and symmetric. Here, the experimental *m/z* data were found between 0.1 and 0.5 Da higher than the calculated monoisotopic values (see parts A in Figs. 2–4). In contrast, the product ions originating from losses of amino acid and peptide residues still containing the phenoxazinone core are broad. A part of them shows still some fine structure. Here, the minor first peak visible for these product ions was found near the calculated average mass values, but frequently the peaks show only a broad envelope. In this case, their maxima were found approximately 1 Da higher than the calculated average values because of the high intensity of the second peak seen in the unusual isotope pattern of the MALDI-TOF mass spectra of actinomycins discussed later.

In the most stable form of the $[M+H]^+$ ions of actinomycins, the ionizing proton presumably is mainly located at the amine substituent in position 2 of the phenoxazinone core. To rationalize the fragmentation pathways, it was assumed that this proton is transferred to the less basic lactone bond. Roboz *et al.*[16] proposed an initial McLafferty rearrangement at the lactone linkage between the C-terminal amino acid (methylvaline or methylalanine) and the threonine in position 1. Thomas *et al.*[18] discussed a charge-site initiated mechanism for opening of the protonated lactone rings of actinomycins, which results in open-chain pentapeptides including a dehydrothreonine residue with the ionizing proton residing at the C-terminal carboxyl group from which it can readily be shifted to amide linkages in the same pentapeptide chain. It was postulated that fragmentations start from this arrangement or from related structures with both rings opened to form linear peptides attached to the phenoxazinone ring system through dehydrothreonine residues.

Product ion spectra of protonated actinomycin D and hemi-actinomycin D

Figure 3 shows the PSD-MALDI-TOF product ion spectra of the protonated form of the reference compound actinomycin D $[M+H]^+$ at *m/z* 1255.6 (Fig. 3a) and of its sodium adduct $[M+Na]^+$ at *m/z* 1277.5 (Fig. 3b). Actinomycin D is an example of an iso-actinomycin carrying two identical depsipeptide rings showing only one set of product ions of the peptide lactone moieties (Table 2A). For the protonated form of actinomycin D, all dipeptide, tripeptide and tetrapeptide product ions of the peptidolactone rings were found, among them the Y-ions that originate from successive decomposition of the linearized

Table 2. Product ions of the $[M+H]^+$ and $[M+Na]^+$ ions of actinomycin D obtained by PSD-MALDI-TOF MS

	a) $[M+H]^+$ *m/z* 1255.6		b) $[M+Na]^+$ *m/z* 1277.5	
	m/z			
	Calc.	Found	Found	Found
A. Product ions of the peptide lactone moieties:				
	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+Na]^+$
VP	197.2	197.3 (l)	197.4 (l)	
PMeG	169.1	169.2 (m) (1)	169.2 (l) (1)	
MeGMeV	185.1	185.3 (l)	185.2 (l)	
H-MeGMeV-OH	203.1	203.4 (m) (2)	203.3 (l) (2)	
VPMeG	268.2	268.3 (m)	268.3 (l)	
PMeGMeV	282.2	282.3 (h) (3)	282.3 (l) (3)	
H-PMeGMeV-OH	300.2	300.4 (h) (4)	300.5 (h) (4)	
VPMeGMeV	381.3	381.4 (l) (5)	381.4 (l)	
H-VPMeGMeV-OH	399.3	399.4 (h) (6)	399.7 (l)	
TVPMeGMeV	482.3	482.4 (l)	482.4 (l)	
B. Product ions generated by elimination of parts of the peptide lactone moieties from actinomycin D precursor ions:				
Loss of:				
V	1157.3	1157.6 (l)	—	—
P	1159.3	1159.7 (l) (13)	—	—
MeG	1185.4	1185.6 (l)	—	—
MeV	1143.3	1143.7 (l)	—	1164.5 (h) (11)
2 MeV	1030.2	—	—	1051.6 (m) (9)
MeVMeG	1072.3	—	1072.6 (l)	1093.5 (l) (10)
MeV + MeVMeG	959.2	959.5 (l)	—	980.4 (l) (8)
2 MeVMeG	888.2	—	—	909.6 (l)
MeGP	1088.3	1088.7 (l)	—	—
PV	1060.2	1059.5 (l)	—	—
PMeGMeV	975.2	975.5 (l)	—	—
H-PMeGMeV-OH	957.2	957.5 (h) (12)	—	980.4 (l)
H-VPMeGMeV-OH	858.1	858.6 (h) (11)	858.6 (h) (7)	879.7 (l)
TVPMeGMeV	775.1	775.4 (l) (10)	775.7 (l)	796.4 (m)
H-PMeGMeV-OH from both rings	658.0	658.2 (m) (9)	658.0 (l)	—
H-PMeGMeV-OH from one ring and H-VPMeGMeV-OH from the other	558.9	559.3 (l) (8)	559.6 (l) (6)	—
H-VPMeGMeV-OH from both rings	459.8	460.1 (m) (7)	459.9 (l) (5)	—

The numbers set in parentheses behind masses of actinomycin D product ions corresponds to the numbers indicating prominent mass peaks in the PSD-MALDI-TOF fragment spectra of the precursor ions of actinomycin D in Fig. 3a and b. l, m and h mean low, medium and high intensity. The calculated reference data in part A and part B are monoisotopic and average, respectively.

peptide lactone moieties from their C-terminus. In addition, the hydrated species of Y_2–Y_4 (*m/z* 203.2, 300.3 and 399.4) were visible, which are the dominant peaks in the lower part of the PSD spectrum. In the upper part, above *m/z* 400 product ions were detected that are generated by elimination of amino acid and peptide residues from the $[M+H]^+$ ion of actinomycin D still containing the phenoxazinone core (Table 2B). Complementary to the product ion pattern of the peptide lactone moieties in part A in the range above *m/z* 450, those ions that originate from the loss of hydrated tripeptide and tetrapeptide portions from one or both rings dominate (Table 2B).

Figure 3c and d shows the MALDI-TOF product ion spectra of the protonated form and the sodium adduct of *hemi*-actinomycin D found at *m/z* 792.6 and 814.5, respectively, produced by *S. antibioticus*, which is induced by feeding cells in the growth medium with 4-MHA. *Hemi*-actinomycins are lacking one of the peptide lactone rings. A list of product ions of *hemi*-actinomycin D is shown in Table 3. Part A shows a complete set of dipeptide, tripeptide and tetrapeptide product ions of the single peptidolactone ring, which is identical with that of actinomycin D (Table 2A).

Product ion spectra of protonated actinomycin I, Z_4 and G_4

Figure 4 exhibits product ion spectra of the $[M+H]^+$ ion of actinomycin I at *m/z* 1272.0 produced by *S. antibioticus* as an example of a X-type actinomycin (Fig. 4a), of the protonated form ($[M+H]^+$at *m/z* 1270.1, Fig. 4b) and of the sodium adduct ($[M+Na]^+$ at *m/z* 1292.2; Fig. 4c) of actinomycin Z_4 from *S. fradiae* as well as of the $[M+H]^+$ ion of actinomycin G_4 at *m/z* 1257.8 (Fig. 4d) from *S. iakyrus*. All these actinomycins belong to the group of aniso-actinomycins exhibiting different α-rings and β-rings with specific amino acid substitutions in each ring (Fig. 1). These variants

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Table 3. Product ions of the $[M+H]^+$ and $[M+Na]^+$ ions of *hemi*-actinomycin D obtained by PSD-MALDI-TOF MS

	$[M+H]^+$ *m/z* 792.6		$[M+Na]^+$ *m/z* 814.5
	m/z		
	Calc.	Found	Found
A. Product ions of the peptide lactone moieties			
	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$
VP	197.2	197.3 (l)	—
PMeG	169.1	169.2 (m) (1)	169.2 (l)
MeGMeV	185.1	185.4 (l)	185.2 (l)
H-MeGMeV-OH	203.1	203.3 (m) (2)	203.2 (l)
VPMeG	268.2	268.2 (m) (3)	—
PMeGMeV	282.2	282.3 (m)	282.2 (l)
H-PMeGMeV-OH	300.2	300.2 (h) (4)	300.2 (l)
VPMeGMeV	381.3	381.4 (l) (5)	—
H-VPMeGMeV-OH	399.3	399.5 (h) (7)	—
TVPMeGMeV	482.3	482.3 (l)	—
B. Product ions generated by elimination of parts of the peptide lactone moiety from *hemi*-actinomycin D precursor ions			
		m/z 792.6	*m/z* 814.5	*m/z* 770.5
	$[M+H]^+$	$[M+H]^+$	$[M+Na]^+$	$[M+Na-CO_2]^+$
Loss of:	Calc.	Found	Found	Found
V	693.3	693.5 (l)	715.6 (m) (8)	—
P	695.3	695.4 (l)	—	—
MeG	721.4	—	—	—
MeV	679.3	679.3 (m) (10)	701.6 (h) (7)	657.2 (m) (6)
MeGMeV	608.3	608.3 (l)	630.4 (h) (5)	586.5 (m) (4)
H-MeGMeV-OH	590.3	590.3 (l)	612.3 (l)	—
PMeGMeV	511.2	511.3 (l)	533.1 (l)	489.3 (h) (3)
H-PMeGMeV-OH	493.2	493.2 (h) (8)	515.2 (l)	—
VP	596.2	596.3 (l)	—	—
H-VP-OH	578.2	578.2 (l) (9)	—	—
VPMeG	525.2	525.3 (l)	—	—
H-VPMeG-OH	507.2	507.2 (l)	530.0 (l)	—
VPMeGMeV	412.1	412.2 (l)	434.2 (l)	390.4 (h) (2)
H-VPMeGMeV-OH	394.1	394.2 (h) (6)	416.1 (l)	—
TVPMeGMeV	311.1	311.1 (l)	334.2 (m) (1)	—

The numbers set in parentheses behind masses of *hemi*-actinomycin D product ions corresponds to the numbers indicating prominent mass peaks in the PSD-MALDI-TOF fragment spectra of the precursor ions of *hemi*-actinomycin D in Fig. 3c and d. l, m and h mean low, medium and high intensity.

were distinguished by two sets of peptide lactone product ions according to the composition of both rings (see parts A in Tables 4 and 5). For all tested aniso-actinomycins, almost all peptide lactone product ions were detected. The general features of the product ion spectra of these G-type, X-type and Z-type actinomycins were similar to those of the reference compound actinomycin D. In each case, the hydrated tripeptide and tetrapeptide production ions dominate in parts A of Tables 4 and 5, and complementarily, the product ions originating by the loss of these species from the $[M+H]^+$ precursor ions show the highest intensities in parts B.

Product ion spectra of cationized actinomycins

The PSD-MALDI-TOF product ions spectra of cationized actinomycins are simpler than those of the protonated forms. Those for the sodium adducts of actinomycins D and Z_4 are shown in Figs 3–5. In Tables 2, 4 and 5, the obtained product ions of actinomycins D, I and Z_4 are listed and compared with those from their protonated forms. Similar results were obtained for the potassium adducts. Generally, the following features have been observed for the fragmentation of the cationized actinomycins. In their PSD-MALDI-TOF product ion spectra, the most abundant ions are those that are formed by elimination of the C-terminal amino acid residues MeV and MeA. These product ions usually appear with the highest intensities followed by those that originate from subsequent elimination of the Sar residues following in the sequence starting from the C-terminus. Here, it cannot be decided from our measurements, whether Sar is eliminated in a coordinated manner successively after the end-standing residue has been split off or as the MeV/MeA–MeG dipeptides. In contrast, in the mass spectra of the protonated forms, such peaks were not found or are of minor intensity, like the peak indicating the elimination of one MeV residue.

A schematic illustration of these fragmentation steps is shown in Fig. 5 for the sodium adducts of the reference compound actinomycin D and of actinomycin Z_4. For actinomycin D as an iso-form fragments indicating the loss of L-MeV from one and both rings found at *m/z* 1164.6 and 1051.6, respectively, dominate followed by those originating by further elimination

Table 4. Product ions of the $[M+H]^+$ and $[M+Na]^+$ ions of actinomycin I obtained by PSD-MALDI-TOF MS

	a) $[M+H]^+$ *m/z* 1272.0		b) $[M+Na]^+$ *m/z* 1294.0
	m/z		
	Calc.	Found	Found
A. Product ions of the peptide lactone moieties: branch 1			
	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$
VP	197.2	197.3 (m)	197.5 (l)
PMeG	169.1	169.2 (m) (1)	169.4 (1)
MeGMeV	185.1	185.4 (h)	185.4 (l)
H-MeGMeV-OH	203.1	203.4 (h) (2)	203.4 (l)
VPMeG	268.2	268.4 (h) (3)	268.6 (l)
PMeGMeV	282.2	282.4 (h) (4)	282.4 (l)
H-PMeGMeV-OH	300.2	300.6 (h) (5)	300.3 (m)
VPMeGMeV	381.3	381.7 (l)	381.7 (l)
H-VPMeGMeV-OH	399.3	399.5 (l) (7)	399.7 (l)
TVPMeGMeV	482.3	482.8 (l)	482.4 (l)
A. Product ions of the peptide lactone moieties: branch 2			
VHyp	213.2	213.3 (m)	213.3 (l)
HypMeG	185.1	185.4 (h)	185.5 (l)
MeGMeV	185.1	185.4 (h)	185.5 (l)
H-MeGMeV-OH	203.1	203.4 (h) (2)	203.4 (l)
VHypMeG	284.2	284.4 (m)	284.1 (l)
HypMeGMeV	298.2	298.5 (l)	—
H-HypMeGMeV-OH	316.2	316.5 (h) (6)	316.8 (l)
VHypMeGMeV	397.3	397.6 (l)	—
H-VHypMeGMeV-OH	415.3	415.7 (m) (8)	415.6 (l)
TVHypMeGMeV	498.3	498.6 (l)	498.8 (l)
B. Product ions generated by elimination of parts of the peptide lactone moieties from actinomycin I precursor ions:			
		m/z 1272.0	*m/z* 1294.0
	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+Na]^+$
Loss of:	Calc.	Found	Found	Found
V	1173.3	1174.1 (l)	—	—
P	1175.3	1175.9 (l)	—	—
Hyp	1159.3	1160.0 (l)	—	—
MeG	1201.4	—	—	—
MeV	1159.3	1159.0 (l) (18)	—	1181.1 (h)
2 MeV	1046.2	..	—	1067.9 (m)
MeVMeG	1088.3	..	—	1109.8 (l)
2 MeV + MeG	975.2	..	—	996.9 (l)
2 MeVMeG	904.2	..	—	927.5 (l)
PMeG	1104.3	..	—	—
H-PMeG-OH	1086.3	..	—	—
HypMeG	1088.3	1089.1 (l)	1088.7 (l)	1109.8 (l)
PMeGMeV	991.2	992.0 (l) (17)	991.2 (l)	—
HypMeGMeV	975.2	975.2 (l)	—	996.8 (l)
H-PMeGMeV-OH	973.2	973.9 (m) (16)	973.4 (l)	994.8 (l)
H-HypMeGMeV-OH	957.2	957.5 (h) (15)	—	—
VPMeGMeV	892.1	892.9 (l)	892.4 (l)	—
VHypMeGMeV	876.1	876.2 (m)	876.0 (l)	—
H-VPMeGMeV-OH	874.1	875.1 (h) (14)	874.6 (l)	—
H-VHypMeGMeV-OH	858.1	859.1 (h) (13)	—	879.4 (l)
TVPMeGMeV	791.1	791.7 (l)	791.1 (l)	—
TVHypMeGMeV	775.1	775.6 (l)	—	796.7 (m)
H-PMeGMeV-OH + H-HypMeGMeV-OH	657.8	657.7 (m) (12)	658.1 (l)	—
PMeGMeV + H-HypMeGMeV-OH	675.8	675.5 (l)	—	—
H-PMeGMeV-OH + H-VHypMeGMeV-OH	558.9	558.7 (m) (10)	559.1 (l)	580.6 (l)
H-HypMeGMeV-OH + H-VPMeGMeV-OH	558.9	558.7 (m)	559.1 (l)	—
HypMeGMeV + H-VPMeGMeV-OH	576.9	576.9 (l) (11)	577.4 (l)	—
H-VHypMeGMeV-OH + H-VPMeGMeV-OH	459.8	459.5 (m) (9)	460.2 (l)	—

The numbers set in parentheses behind masses of actinomycin I product ions corresponds to the numbers indicating prominent mass peaks in the PSD-MALDI-TOF fragment spectrum of the precursor ions of actinomycin I in Fig. 4a. l, m and h mean low, medium and high intensity.
Hyp, 4-hydroxyproline.

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Table 5. Product ions of the $[M+H]^+$ and $[M+Na]^+$ ions of actinomycin Z_4 from *Streptomyces fradiae* obtained by PSD-MALDI-TOF MS

	a) $[M+H]^+$ *m/z* 1270.1		b) $[M+Na]^+$ *m/z* 1292.2
	m/z		
	Calc.	Found	Found
A. Product ions of the peptide lactone moieties: branch 1			
	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$
VMeP	211.2	211.5 (l)	—
MePMeG	183.1	183.4 (l)	183.3 (l)
MeGMeV	185.1	185.4 (l)	185.3 (l)
H-MeGMeV-OH	203.1	203.4 (m) (1)	203.3 (m) (1)
VMePMeG	282.2	282.4 (m)	282.2 (l)
MePMeGMeV	296.2	296.7 (m)	296.1 (l)
H-MePMeGMeV-OH	314.2	314.6 (hh) (3)	314.5 (h) (2)
VMePMeGMeV	395.3	395.5 (l)	395.2 (l)
H-VPMeGMeV-OH	413.3	413.7 (m) (4)	413.5 (l)
TVMePMeGMeV	496.3	496.2 (l)	—
A. Product ions of the peptide lactone moieties: branch 2			
VOMeP	225.2	—	—
OMePMeG	197.1	197.2 (l)	197.4 (l)
MeGMeA	157.1	157.7 (l)	157.7 (l)
H-MeGMeA-OH	175.1	175.2 (l)	—
VOMePMeG	296.2	296.7 (m)	296.1 (l)
OMePMeGMeA	282.2	282.4 (m)	282.2 (l)
H-OMePMeGMeA-OH	300.2	300.5 (l) (2)	300.6 (l)
VOMePMeGMeA	381.3	381.3 (l)	381.7 (l)
H-VOMePMeGMeA-OH	399.3	399.6 (l)	399.5 (l)
TVOMePMeGMeA	482.3	482.4 (l)	—
B. Product ions generated by elimination of parts of the peptide lactone moieties from actinomycin Z_4 precursor ions			
		m/z 1270.1	*m/z* 1292.2
	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+Na]^+$
Loss of:	Calc.	Found	Found	Found
V	1171.3	1171.2 (l)	—	—
MeP	1159.3	1160.1 (l) (12)	—	—
OMeP	1145.3	1145.2 (l)	—	—
MeG	1199.4	—	—	—
MeV	1157.3	—	—	1179.2 (h) (10)
MeV + MeG	1086.3	—	—	1108.9 (l)
MeA	1185.4	1185.9 (l)	—	1207.1(h) (11)
MeA + MeG	1114.4	—	—	1136.2 (m) (9)
MeV + MeA	1072.3	—	—	1094.4 (h) (8)
MeV + MeA + MeG	1001.3	—	—	1022.8 (m) (7)
MeV + MeA + 2MeG	930.3	930.1 (m)	—	952.6 (m) (6)
MePMeG	1088.3	1088.7 (l)	—	1110.9 (l)
H-MePMeG-OH	1070.3	1070.4 (l)	—	—
OmePMeG	1074.3	—	—	—
MePMeGMeV	975.2	975.9 (l)	—	—
OmePMeGMeA	989.3	989.9 (l)	—	—
H-MePMeGMeV-OH	957.2	957.2 (h)	—	979.2 (l)
H-OMePMeGMeA-OH	971.3	971.1 (h) (11)	—	—
VMePMeGMeV	876.1	—	876.2 (l)	898.2 (m)
VOMePMeGMeA	890.2	—	—	—
H-VMePMeGMeV-OH	858.1	858.2 (h) (9)	857.8 (l)	—
H-VOMePMeGMeA-OH	872.1	872.2 (h) (10)	872.6 (m) (5)	894.2 (l)
TVMePMeGMeV	775.1	—	—	—
TVOMePMeGMeA	789.2	789.6 (l)	—	—
H-MePMeGMeV-OH + H-OMePMeGMeA-OH	658.1	658.7 (m) (8)	658.3 (l)	—
MePMeGMeV + H-OMePMeGMeA-OH	676.1	676.3 (l)	—	697.6 (l)
H-MePMeGMeV-OH + H-VOMePMeGMeA-OH	559.0	559.7 (h) (6)	559.1 (l) (4)	—
H-OMePMeGMeA-OH + H-VMePMeGMeV-OH	559.0	559.7 (h)	559.1 (l)	—
OMePMeGMeA + H-VMePMeGMeV-OH	577.0	577.7 (l) (7)	—	—
H-VOMePMeGMeA-OH + H-VMePMeGMeV-OH	459.9	460.3 (h) (5)	460.3 (l) (3)	—

The numbers set in parentheses behind the masses of actinomycin Z_4 product ions corresponds to the numbers indicating prominent mass peaks in the PSD-MALDI-TOF fragment spectra of the precursor ions of actinomycin Z_4 in Fig. 4b and c. l, m and h mean low, medium and high intensity.

MeP, *cis*-5-methylproline; OMeP, *cis*-5-methyl-4-oxoproline.

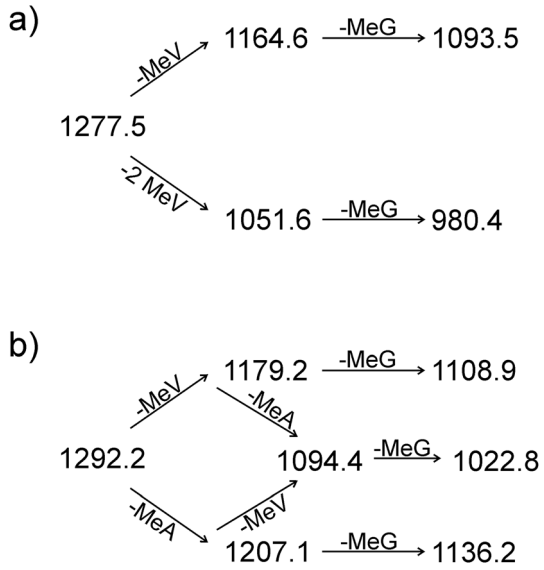

Figure 5. Fragmentation scheme of the sodium adducts of (a) actinomycin D ($[M+Na]^+$ *m/z* 1277.5) and (b) actinomycin Z_4 ($[M+Na]^+$ *m/z* 1292.2). The dominant product ions in the PSD-MALDI-TOF mass spectra can be attributed to the loss of the MeV and MeA residues involved in peptide lactone formation.

of the L-Sar-residues to form the medium intensity peaks at *m/z* 1093.5 and 980.4 (Fig. 3b). Similar features were observed also for the *hemi*-actinomycins. For example, the dominating peaks in the product spectrum of the sodium adduct of *hemi*-actinomycin D $[M+Na]^+$ at *m/z* 814.5 in Fig. 3d are those that originate from the loss of MeV at *m/z* 701.6 and of MeV/MeG at *m/z* 630.4. A specific feature of the sodium adduct of *hemi*-actinomycin D is the decarboxylation of the free carboxyl group at the phenoxazinone core leading to the loss of CO_2. Interestingly, the decarboxylated species showed a successive elimination of MeV, MeG, P and V. The corresponding product ions appeared in high intensity (Fig. 3d and Table 3).

In the case of actinomycin Z_4 as an example for an aniso-form with different α-rings and β-rings, the product ions pattern is more complex. Here, the product ions resulting from the loss of L-MeV from the α-ring (at *m/z* 1179.2) and L-MeA from the β-ring (at *m/z* 1207.1) as well as that obtained by the simultaneous expulsion of both L-MeV and L-MeA from either ring *m/z* 1094.4 represent the prominent peaks in the PSD spectrum of the sodium adduct of actinomycin Z_4. Thereafter, the L-Sar residues were successively eliminated from both rings. Single elimination of the other amino acid residues of the peptidolactone rings either was missing or occurs only in minor extent. Generally, also for the alkali adducts, many of the product ions that had been observed for the protonated forms were found at minor intensities. In particular, the peptide product ions obtained for the peptidolactone rings showed much lower intensities or even were completely missing.

Our results are consistent with previous mass spectrometric studies on the degradation of cationized peptides.[25–27] Although the degradation process is complex, frequently the dominating pathway is the elimination of the C-terminal amino acid. The alkali cations bind primarily to the C-terminus promoting the loss of the C-terminal residue. This process involves migration of an oxygen atom inducing the loss of the terminal amino acid by expulsion of CO and an imine. This mechanism is observed in general for both metastably and collisionally activated ions and is the basis of a method for identification of the C-terminal amino acid.[26]

MALDI-TOF mass spectra exhibit an unusual isotope pattern of the actinomycin mass peaks.

The MALDI-TOF mass spectra of all investigated actinomycins exhibit an unusual pattern of their isotope distribution (Fig. 6a and b). Normally, the first peak indicating a molecule containing only 12C-atoms shows the highest intensity followed by peaks of species including one or two 13C-atoms of decreasing intensities. For example, nonactins produced by *S. chrysomallus* (Fig. 3b) and peptidolactone compounds, such as the 4-MHB-pentapepti dolactone derivative of actinomycin[28] (Fig. 6e) as well as the heptapeptide lactone etamycin[29] (Fig. 6f), are distinguished by such a normal pattern. In contrast for the MALDI-TOF mass spectra of actinomycins, peak ensembles have been obtained, which exhibit five to six peaks, the second of which always shows the highest intensity. This is shown in Fig. 6a and b for the MALDI-TOF mass spectra of actinomycin D and the corresponding *hemi*-actinomycin in a surface extract of *S. antibioticus*. These effects are specific for MALDI-TOF MS. Using ESI-MS, normal isotope patterns were observed for actinomycins (Fig. 6c and d). The unusual patterns observed in MALDI-TOF mass spectra are specifically related to the phenoxazinone core of actinomycins. Peptidolactone compounds, like the 4-MHB-peptidolactone and etamycin, which contain a benzoic or a 3-hydroxypicolinic core, do not show such an effect. In a similar way, in the PSD-MALDI-TOF product ion spectra of actinomycins, only those ions still containing the phenoxazinone chromophore revealed such an anomalous pattern, while for the peptide product ions of the peptidolactone rings, only three isotope peaks were observed. Resuming these results, we conclude that the described anomalous behavior occurs as a result of the use of MALDI-TOF MS and depends specifically on the presence of the phenoxazinone core of actinomycins.

The reason for this anomaly may be the overlap of the mass spectra of two actinomycin species, which differ in molecular mass by 1 Da. For example, such a situation could occur when the amino group in position 2 of the phenoxazinone core is converted into a hydroxyl group, because it is known that such a replacement can be achieved by mild acidic hydrolysis.[23] Therefore, we tested whether actinomycins would be modified in this manner during sample preparation for MALDI-TOF MS, because the matrix α-cyano-4-hydroxy-cinnamic acid used in our study is a carboxylic acid and the matrix medium contained 0.1% trifluoroacetic acid.

In this case, we expected a normal isotope distribution in the MALDI-TOF mass spectrum of pure 2-hydroxy-actinomycin D (desaminoactinomycin D). To prove this assumption, we prepared this species as the reference compound[23] and tested by TLC, if this compound was formed in our samples for MALDI-TOF MS. Using ethyl acetate : methanol : water : dimethyl formamide (100 : 5 : 5 : 1, by vol.) as the solvent, the 2-hydroxy derivative showed a quite different R_f-value of 0.09 as the original actinomycin D ($R_f = 0.61$), but a spot at this position was not found in our samples for mass spectrometry. Obviously, the putative conversion of 2-amino-actinomycin D to 2-hydroxy-actinomycin D did not occur in our sample preparation. In addition, the MALDI-TOF mass spectrum of 2-hydroxy-actinomycin D showed a similar anomalous isotope pattern of the mass peaks as the mass spectra of the other actinomycin compounds. As the consequence, other reasons must be responsible for the

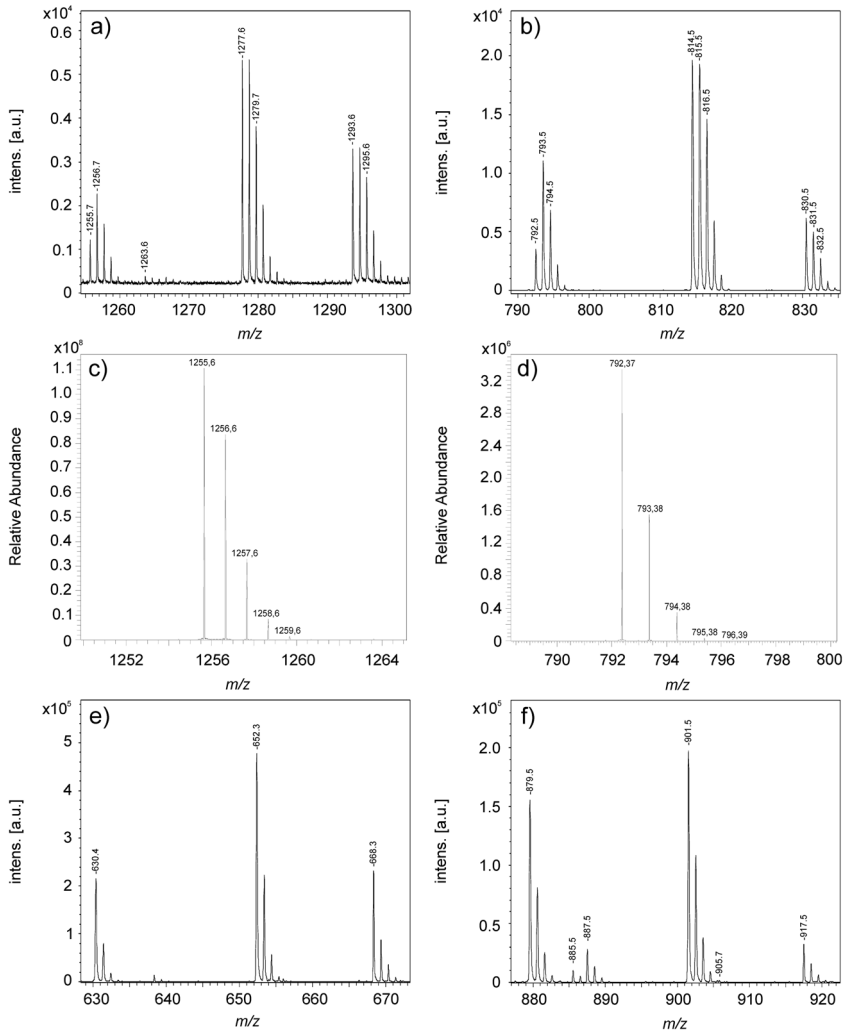

Figure 6. Isotope patterns of the mass peaks of MALDI-TOF mass spectra for actinomycin D (a); *hemi*-actinomycin D (b); 4-methyl-3-hydroxybenzoic acid-pentapeptide lactone (e) and etamycin (f); and the ESI-MS mass spectra for actinomycin D (c) and *hemi*-actinomycin D (d).

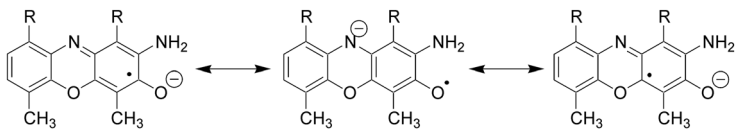

Figure 7. Mesomeric forms of the semiquinone radical of the phenoxazinone core.

observed anomalous behavior. Because the phenoxazinone core of actinomycins shows a quinoid structure, we infer the following mechanism to explain this anomaly. During the mass spectrometric measurements by laser excitation of actinomycins, phenoxazinone radicals are induced. Their anionic form (Fig. 7) is able to accept a proton leading to an increase of its molecular mass by 1 Da. Consequently, the anomaly in the MALDI-TOF mass spectra would originate from a superposition of the mass spectra of the non-modified actinomycin molecule and its radical anion.

Conclusions

MALDI-TOF MS of surface extracts of five well-characterized *Streptomyces* strains was demonstrated as a rapid, efficient technique for the detection and structural characterization of actinomycins *in situ*. By feeding experiments with the precursors 3-HA and 4-MHA, C-de methyl derivatives and *hemi*-actinomycins were obtained as novel actinomycin variants. Fragmentation of the protonated and cationized forms of selected actinomycins was performed by PSD-MALDI-TOF MS. The specific features of the fragmentation patterns of these species were investigated in detail. The most intense peaks in the PSD spectra of the protonated forms were attributed to the hydrated tripeptide and tetrapeptide ions of the pentapeptide lactone moieties and those that originate from the loss of these portions from the $[M+H]^+$ ions of actinomycins. In contrast, in the PSD spectra of the cationized actinomycins, those product ions that arose by elimination of the two C-terminal amino acid residues of the linearized peptide lactone side chains dominate. MALDI-TOF mass spectra of all investigated actinomycins exhibit an unusual pattern of isotope distribution comprising five

to six mass peaks, which can be interpreted by the formation of phenoxazinone anion radicals in the course of laser excitation of the actinomycin samples. Our results represent an extensive study of actinomycins by MALDI-TOF MS, and we consider that they are a valuable contribution for the understanding of actinomycins on the molecular level.

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