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Identification of alkaloids and polyketides in an *Actinomycete* by high-performance liquid chromatography with mass spectrometric and UV–Visible detection

Marc Stadler[a,*], Thomas Henkel[a], Hartwig Müller[a], Karlheinz Weber[a], Harald Schlecker[b]

[a]*Bayer AG, Pharma Research Center Wuppertal, Natural Products Research, P.O.B. 10 17 09, D-42096 Wuppertal, Germany*
[b]*Bayer AG, Pharma Research Center Wuppertal, Dept. Of Structural Research, P.O.B. 10 17 09, D-42096 Wuppertal, Germany*

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Abstract

Analytical HPLC with UV–Vis diode array detection and positive electrospray HPLC–MS were employed to characterise the secondary metabolites of *Actinomycetes* spec. strain BA 909 in crude mycelial extracts and preparative HPLC fractions. It was shown that the strain produced two families of antibiotics previously reported in the literature, namely the polyketides leptomycin A (**1**) and B (**2**), kazusamycins A (**3**) and B (**4**) and the alkaloids nybomycin (**5**) and deoxynybomycin (**6**), respectively. Compounds **1**–**4** were identified by comparison of their HPLC–UV and HPLC–MS data with authentic materials obtained from the original producer strain, whereas the identity of the nybomycins **5** and **6** was established by comparison of structural data with those of reference compounds. From HPLC–UV–Vis analyses it was established that both groups of metabolites are produced as complex mixtures of congeners. The employment of a single HPLC–UV–Vis method, aided by HPLC–MS of selected fractions, allowed for simultaneous detection of the main metabolites in the crude extracts during fermentation. Known metabolites could be distinguished from tentatively new congeners. For example, the data available from HPLC–UV–Vis and HPLC–MS analyses point towards the presence of an anguinomycin A (**7**) isomer, two new isomers of kazusamycins A and B (**8** and **9**), and a new nybomycin derivative (**10**) in addition to the known compounds **1**–**6**. The co-occurrence of both leptomycin and nybomycin type metabolites in the same *Actinomycete* strain was observed for the first time.

Keywords: *Actinomycetes*; Alkaloids; Polyketides; Leptomycin; Nybomycin; Antibiotics

1. Introduction

Analytical HPLC monitoring nowadays constitutes a well-established standard evaluation method in natural products research. Besides the established HPLC–UV–Vis techniques, high-performance liquid chromatography–mass spectrometry (HPLC–MS) was introduced about 5 years ago as a powerful additional analytical method for early-stage investigation of bioactive natural products. Several studies on the behaviour of pure natural products, e.g. of microbial [1–3] and plant [4–8] origin, have already been published. Upon investigation of several pure natural products of different chemical types and origins, electrospray ionisation (ESI) was more suitable for detection than atmospheric pressure chemical ionisation (APCI) [9], but it was noted that several compounds gave no or only weak signals in

*Corresponding author.

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either the positive or negative ESI mode, according to their structural type and the chromatographic conditions employed. Thus it cannot always be anticipated that metabolites of different biogenetic origin in heterogeneous crude extracts will be detectable using a standard protocol.

In the course of a screening of natural products extracts, samples from the *Actinomycete* strain BA 809 showed strong antifungal activities, and preliminary HPLC analyses pointed towards the occurrence of two distinct groups of secondary metabolites. Strain BA 809 was chosen as a model organism to evaluate the suitability of both above mentioned analytical methods for identification of its secondary metabolites

We report here a standard method for the simultaneous detection and identification of polyketides and alkaloids in the mycelial extracts of this *Actinomycete* by analytical HPLC methodology (Fig. 1).

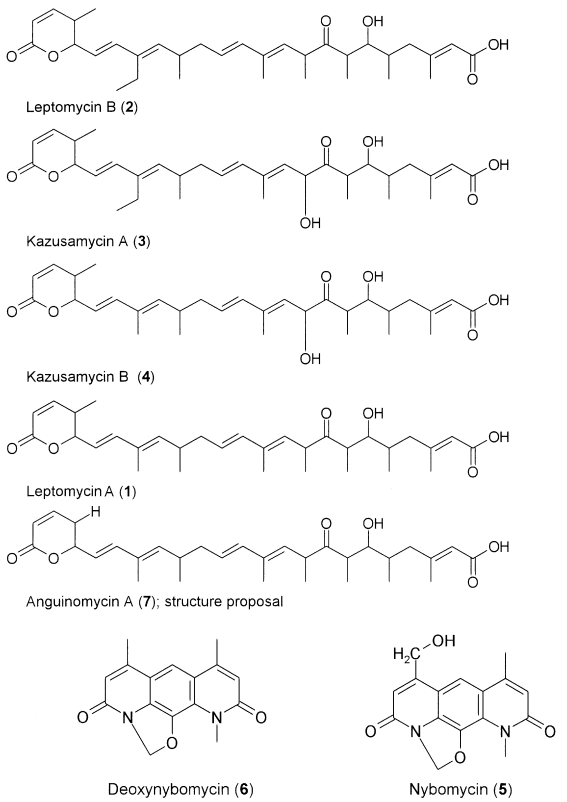

Fig. 1. Structures of metabolites from *Actinomycetes* sp. BA 809 and ATCC 39366.

2. Experimental

2.1. Chemicals

All solvents (gradient grade) and chemicals (analytical grade) were obtained from Merck (Darmstadt, Germany) if not stated otherwise.

2.2. Culture media

The Q6 medium consisted of: glucose 0.4%, glycerine 2%, cottonseed meal 1%; tap water, pH 7.2 sterilised at 121°C for 60 min.

2.3. Organisms and fermentation conditions

Actinomycetes sp. strain BA 809 was isolated from soil and deposited in liquid nitrogen at the Bayer culture collection, Wuppertal, Germany. Strain *Streptomyces* spec. ATCC 39366 was kindly provided by American Type Culture Collection (Rockville, MD, USA). For production of secondary metabolites, BA 809 was propagated in a Biostat E (Braun Melsungen, Germany) stirring fermenter containing 10 l sterile Q6 medium for 96 h at 28°C with a stirring speed of 350 rpm at an aeration rate of 3.5 l sterile air/min. This culture was used as inoculum for a 200-l stirring fermenter (Biologische Verfahrenstechnik, Stuttgart, Germany). The fermentation was carried out in Q6 medium at 28°C with agitation (350 rpm) and aeration (3.5 l of sterile air per min). Daily samples (100 ml) were taken, and the mycelia were obtained from the culture broth by centrifugation (15 min at 1000 *g*) and extraction with acetone. After 120 h the culture was harvested. The combined mycelia were extracted and treated as described below. For cultivation of the reference strain ATCC 39366, 1-l Erlenmeyer flasks containing 300 ml Q6 medium were inoculated with 2 ml of a glycerine culture. The fermentation was performed at an incubation temperature of 28°C on a rotary shaker (240 rpm) for 120 h. Samples were extracted according to the protocol used for BA 809.

2.4. Preparation of mycelial extracts

The mycelia (1200 g dry mass from 200-l culture) were separated from the culture broth (which was discarded), freeze-dried and extracted three times with 10 l each of acetone. The extracts were combined, and the solvents were evaporated in vacuo (40°C) to yield ~120 g of an oily crude product. Aliquots of this product were applied to preparative HPLC columns.

For HPLC analyses of crude extracts during fermentations, the freeze-dried mycelia from 100 ml of the culture broth were extracted twice with 100 ml each acetone for 30 min in an ultrasonic bath. The combined acetone extracts were evaporated in vacuo (40°C), and the oily residues were redissolved in 2 ml of methanol. Aliquots (1–5 μl, depending on the concentrations of leptomycins and nybomycins) of these solutions were used for HPLC analyses.

2.5. Analytical HPLC methods

HPLC–UV–Vis analyses were carried out using a HP1090 (Hewlett-Packard, Waldbronn, Germany) automated liquid chromatograph comprising an integrated autoinjector, and a photodiode array detection system. Reversed-phase separations were carried out at 40°C using a Knauer (Berlin, Germany) Eurospher 100 C_{18} (5 μm) column (100×2 mm I.D.). The column was eluted at a flow-rate of 0.75 ml/min in the gradient mode, using 0.005% H_3PO_4–CH_3CN as mobile phase (gradient: 0–100% CH_3CN 15 min; 100–100% CH_3CN 5 min; 100–0% CH_3CN 5 min). UV spectra were recorded in the range 210–400 nm, and HPLC–UV traces for analyses were plotted at 220 nm. At this wavelength, the limit of detection was 1–2 ng for the standard compounds, leptomycin B (**2**) and nybomycin (**5**).

HPLC–MS analyses were accomplished on a TSP (Darmstadt, Germany) liquid chromatograph directly coupled with a Finnigan (Bremen, Germany) MAT 900 mass spectrometer. Column: Waters (Milford, MA, USA) Symmetry C_{18} (5 μm, 150×2.1 mm); mobile phase: 0.01 *M* HCl–CH_3CN; flow 0.5 ml/min; temperature 20°C. Gradient: 90% 0.01 *M* HCl 1 min; 90% 10% 0.01 *M* HCl 8 min; 10% 0.01 *M* HCl 5 min; 10–90% 0.01 *M* HCl 0.5 min; 90% 0.01 *M* HCl 2.5 min; flow-rate: 0.5 ml/min. The mass spectrometer was equipped with an ESI interface. ESI was carried out in the positive mode to produce protonated molecular ions, using the following parameters: scan speed: 1.5 s/decade against scan; scan range 150–1200; resolution: 2000; capillary voltage: 4.750 V; identity of nebuliser gas: N_2 99.999%; nebuliser gas pressure: 5 bar; heated capillary temperature: 220°C. Negative ESI was attempted with the crude extract using neutral conditions (same HPLC system as described above without addition of acid to aqueous mobile phase), but the crude extract did not separate fairly without the addition of acid to the mobile phase. No calibration with standard compounds was carried out as this method was only used for qualitative purposes (identification of congeners).

2.6. Standard compounds

Compounds **1**–**4** were obtained by preparative chromatography from both BA 809 and ATCC 39366, identified by comparison of their spectroscopic data (proton NMR, mass spectra) with those reported in Literature and subsequently employed as both external and internal standards. The nybomycins (**5** and **6**) were also isolated to purity and identified by comparison of their spectroscopic data with those reported in the literature and with authentic materials available in the Bayer repository of pure natural products. The retention times given in Table 1 resulted from several experiments using the same HPLC columns.

2.7. Preparation of the starting material for HPLC separations

Prior to preparative HPLC the crude mycelial acetone extract was dissolved in methanol (100 mg ml^{-1}) and applied in portions of each 200 mg to Baker BondElut 6 ml SiOH columns. The columns were washed with 6 ml each of methanol. The combined methanolic desorbates were evaporated in vacuo, redissolved in 300 μl each of methanol and applied onto Baker BondElut C_{18} columns (~100 mg crude extract per column). The C_{18} columns were washed with 6 ml each of MeOH. The combined methanolic desorbates from 200-l culture broth yielded approximately 20 g of an oily intermediate

Table 1
Retention times (t_R) of compounds **1**–**10** in the gradient system used for HPLC–UV–Vis (Figs. 2 and 3)

Compound		t_R (min)
1	Leptomycin A	11.574
2	Leptomycin B	11.988
3	Kazusamycin A	10.666
4	Kazusamycin B	10.251
5	Nybomycin	7.612
6	Deoxynybomycin	5.971
7	Anguinomycin A or isomer[a]	11.424
8	New kazusamycin B isomer[a]	10.071
9	New kazusamycin A isomer[a]	10.478
10	New nybomycin derivative[a]	5.741

[a] These compounds were only identified tentatively from LC–MS and LC–UV data.

product **1** after evaporation in vacuo. Using a similar procedure, 250 mg of crude materials were obtained from the mycelia of 900 ml culture broth of ATCC 39366.

2.8. Preparative HPLC

For preparative separations a Gilson Abimed (Langenfeld, Germany) 305/306 HPLC unit equipped with a 811C dynamic mixer, a 119 UV–Vis detector, a 202 fraction collector, a 806 manometric module and a manual injection loop, were employed. Approximately 70 mg of intermediate product **1** (obtained after cartridge fractionation of the crude extract as described above) was divided into portions of ~100 mg, dissolved in 1 ml of methanol and applied by injection onto a Merck Hibar LiChrosorb C_{18} column (particle size 7 μm; column size 250× 20 mm; mobile phase: 0.1% trifluoroacetic acid (TFA)–methanol gradient; flow: 9 ml/min; gradient from 20 to 100% methanol in 30 min, thereafter elution at 100% methanol for 30 min; thereafter regeneration of the column). The main fractions were pooled according to UV absorbance of the peaks at 280 nm (UV maximum of leptomycins). Pure compounds leptomycin B (**2**; 9 mg from 100 mg crude extract; obtained at t_R=37–39 min), leptomycin A (**1**; 0.5 mg from 100 mg crude extract; t_R=36 min), kazusamycin A (**4**; 0.5 mg from 100 mg crude extract; t_R=29–30 min) and kazusamycin B (**3**; 3 mg from 100 mg crude extract; t_R=33–34 min) were separated under the chosen conditions. During chromatography it was established that most of the starting materials consisted of lipophilic UV-inactive materials, and therefore the yields of leptomycins were rather low.

Intermediate product **2** (12 mg from 100 mg crude extract) contained a heterogeneous mixture of compounds with nybomycin-like UV spectra and was obtained from preparative HPLC of intermediate product **1** on RP18 at t_R=22–26 min. This nybomycin mixture was further processed using the same hardware and injection procedure as described above by preparative reversed-phase HPLC on a Merck (Darmstadt, Germany) HiBar column (LiChrosorb CN; particle size 7 μm; column size 250× 25 mm; mobile phase: 0.1% TFA–methanol gradient; flow-rate: 7 ml/min; continuous gradient: 25 min at 25% acetonitrile, thereafter from 25 to 100% acetonitrile in 30 min). Under these conditions, compounds **5** (3 mg from 12 mg intermediate product **2**; t_R=17–21 min) and **6** (1.2 mg from 12 mg intermediate product **2**; t_R=23–26 min) were isolated to purity.

3. Results and discussion

3.1. Production, isolation and identification of main metabolites

BA 809 was grown for 120 h in a 200-l stirring fermenter. Details of fermentation conditions and preparation of mycelial extracts are described in Section 2. Two different groups of main metabolites, each exhibiting very characteristic chromophores, were detected by HPLC–UV analyses (see UV spectra in Fig. 2a).

The more polar group of compounds was separated from the second metabolite complex by preparative HPLC on RP18. Subsequent HPLC on CN materials afforded pure compounds **5** and **6**. Two of these were identified as the known *Actinomycete* metabolites nybomycin (**5**) and deoxynybomycin (**6**), respectively [10]. Besides these known compounds, several minor metabolites with similar UV spectra were detected (Fig. 3).

The second, less hydrophilic group of metabolites in the crude extracts exhibited a typical chromophore with UV maxima in the range of 280 nm (Fig. 2a),

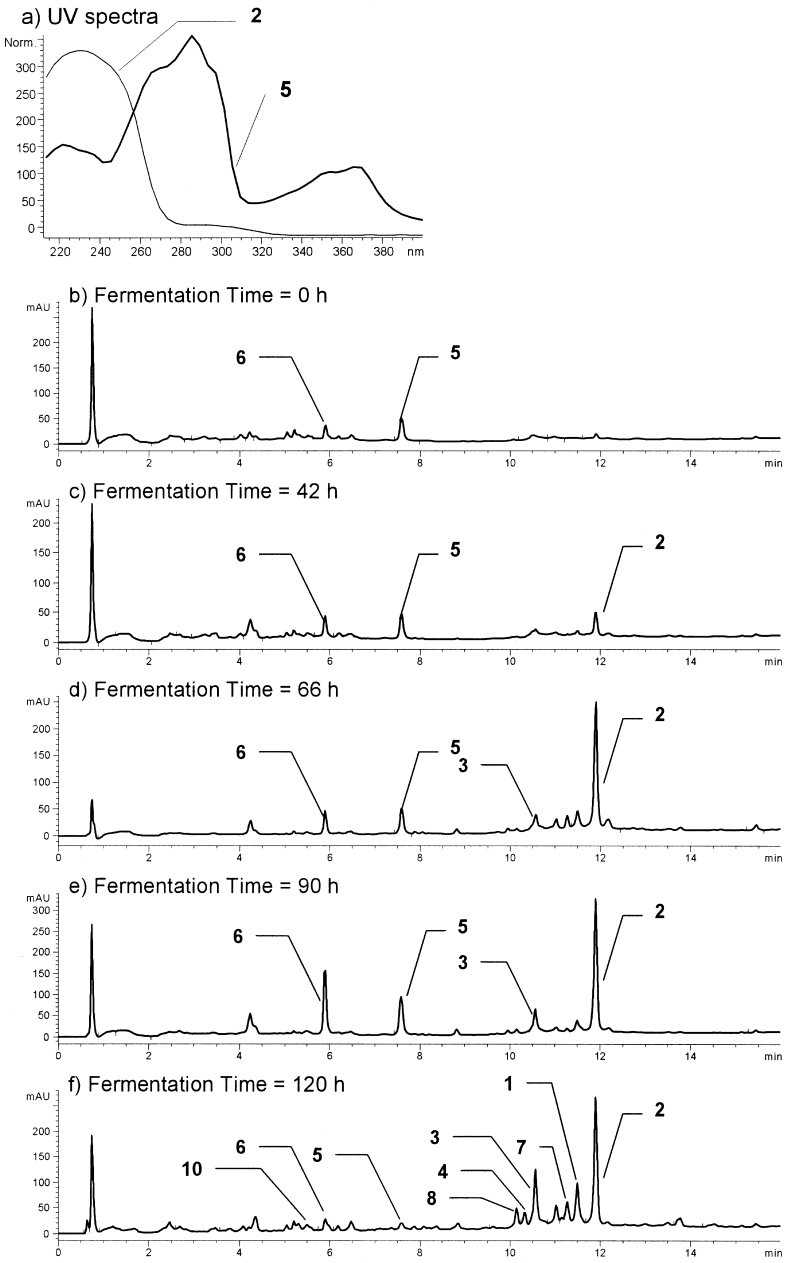

Fig. 2. (a) UV spectra of leptomycin B (**2**) and nybomycin (**5**) during HPLC analysis; (b)–(f): Comparison of HPLC–UV chromatograms (220 nm) of mycelial acetone extracts from samples taken during fermentation of BA 809 at different fermentation times (Knauer Eurospher 100 C_{18}; mobile phase: 0.005% H_3PO_4–ACN); for compound names and retention times see Table 1.

which was identical for all congeners. The main products were isolated by preparative HPLC as described in Section 2.

The identity of compounds **1**–**4** with leptomycins A and B [11] and kazusamycins A and B [12], respectively, was established by mass spectrometry, ^{1}H-NMR spectroscopy and comparison of data with those reported in literature.

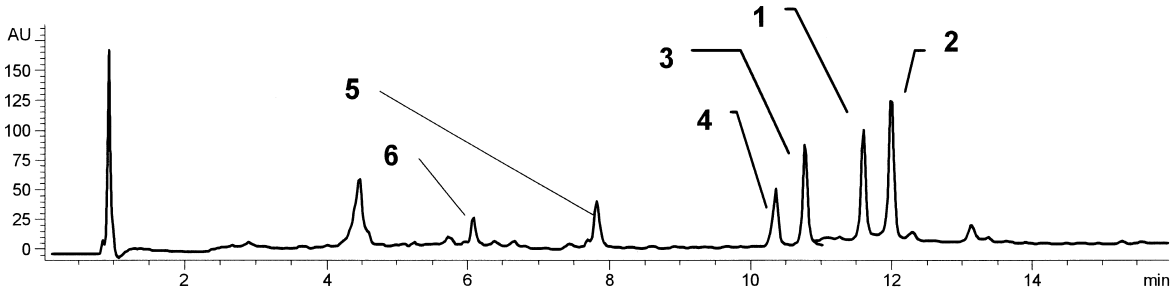

Fig. 3. HPLC–UV chromatogram (220 nm) of mycelial extract from reference strain ATCC 39366 (Knauer Eurospher 100 C_{18}; mobile phase: 0.005% H_3PO_4–ACN); for compound names and retention times see Table 1.

Interestingly the time course of production of both types of metabolites (Fig. 2b to f) suggests that at first, nybomycin (**5**), desoxynybomycin (**6**) and leptomycin B (**2**) are the main products and may be isolated in good yields if the fermentation is terminated after 90 h. At a later stage of fermentation, several peaks exhibiting the typical UV spectra of the nybomycins as well as the leptomycin family) became detectable, whereas the amount of **2** and **5** decreased. Thus, several congeneric metabolites were obtained if the fermentation was prolonged to 114 h or more.

As *Streptomyces* sp. ATCC 39366 had previously been reported to produce various leptomycin derivatives [13], this strain was purchased from ATCC as a reference organism and cultivated using similar conditions as with BA 809. The pure compounds **1**–**4** derived from ATCC 39366 were identical to those from BA 809. Besides these leptomycins **1**–**4**, the nybomycins (**5** and **6**) were also isolated and identified in the extracts from ATCC 39366 (see Fig. 3). Unlike in BA 809, no considerable amounts of congeneric metabolites were detected at prolonged fermentation times in ATCC 39366. This observation may point towards 'late biosynthesis' enzyme activities in BA 809, which are not present in the ATCC strain. Table 1 shows that it was possible to distinguish between known and new compounds of each type by comparison of the HPLC characteristics (UV spectra, retention times) of crude extracts.

The co-occurrence of leptomycins and nybomycins in the same organism was never observed before and illustrates the creativity of *Actinomycetes* as producers of chemically diverse bioactive natural products. In addition to the compounds mentioned here, several additional minor peaks were detected in the crude extracts. However, only those exhibiting the typical HPLC–UV signals of leptomycins and nybomycins were analysed more thoroughly.

3.2. HPLC–MS characteristics of leptomycins

Upon positive ESI HPLC–MS analyses, the characteristic UV peaks of leptomycin B (**2**) and kazusamycin A (**3**) gave corresponding molecular ions at m/z 541 $[(M+H)^+]$ and 557 $[(M+H)^+]$, respectively (see Fig. 4). The mass spectra also show signals at m/z 523 (leptomycin B; **2**) and m/z 539 (kazusamycin A; **3**), respectively, which might be due to loss of H_2O $[M+H-H_2O]^+$. The additional minor ionisation peaks detected in the ESI spectra cannot be assigned to specific fragments as their intensity in comparison to the molecular ion varied during several experiments, and they may therefore be due to instrumental artefacts or impurities still remaining in the samples after chromatographic separations. However, the molecular ions of each compound were the strongest LC–MS signals observed with each compound of this type.

Two further leptomycin type congeners (compounds **1** and **4**) [13,14] were isolated and identified. They proved identical to kazusamycin B (**4**) and leptomycin A (**1**), which had both previously been reported as metabolites of ATCC 39366. Their HPLC–UV and HPLC–MS data matched with those of two compounds obtained from the mycelial extracts of strain ATCC 39366, using similar fermentation and isolation procedures. While compound **4** showed ESI signals at m/z 543 $[M+H]^+$ and 525

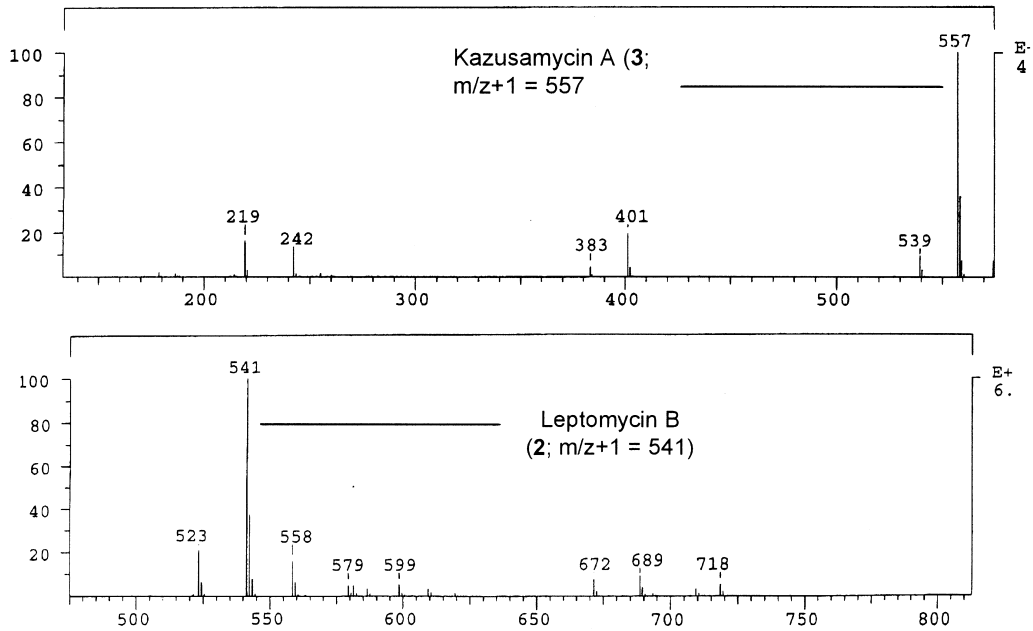

Fig. 4. Positive ESI HPLC–MS spectra of leptomycin B (**1**) and kazusamycin A (**2**). Samples from preparative HPLC.

$[M+H-H_2O]^+$, *m/z* 527 $[M+H]^+$ and 509 $[M+H-H_2O]^+$ were observed for compound **1**.

Whereas not all minor congeners could be detected as single peaks at the stage of the crude extract, HPLC–MS analyses of the enriched preparative HPLC fractions gave better resolution, and several congeners were characterised by their respective ESI mass spectra. Compound **7** (peak in Fig. 2f) exhibited the leptomycin chromophore, and ESI-MS spectra showed the molecular ion at *m/z* 513 $[(M+H)^+]$. The only known leptomycin derivative with this molecular mass would be anguinomycin A (**7**) [15], which is lacking the methyl group in the γ-pyrone ring system. However, there would be many possible isomeric structures, and the detected compound might well constitute a novel anguinomycin A isomer. Another peak (**8** in Fig. 2f) exhibited mass and UV spectra identical to kazusamycin A (**2**) but appeared at a shorter retention time, suggesting the presence of a new kazusamycin A (**3**) isomer. For compound **9** (not detectable in crude extracts but only in fractions from preparative HPLC as shown in Fig. 5), ESI LC–MS spectra revealed the molecular ion at *m/z* 543 $[(M+H)^+]$, suggesting that it constitutes a novel isomer of kazusamycin B (**4**, also present in Fig. 5). No leptomycins with the respective masses other than kazusamycins A and B were hitherto reported, suggesting that the detected compounds **8** and **9** constitute new isomers.

3.3. HPLC–MS characteristics of nybomycins

The available data points towards the presence of new members of the nybomycin family in the crude extract of BA 809 as well. Whereas nybomycin (**5**) and deoxynybomycin (**6**) gave molecular ions at *m/z* 299 and *m/z* 283, respectively, another compound **10** showed the molecular ion at *m/z* 269 and might thus be lacking a methyl group as compared to deoxynybomycin (**6**). Such a compound has not yet been reported in the literature. However, the isolation and structure elucidation of compounds **7**–**10** is ongoing and will be reported separately. Further nybomycin type congeners were also present in ATCC 39366, according to preliminary experiments.

The biological activities of both groups of metabolites (especially the leptomycin family) are well investigated. The highly cytotoxic and strong antifungal leptomycins were shown to interact within the eukaryotic cell cycle [11,12,16,17], and the

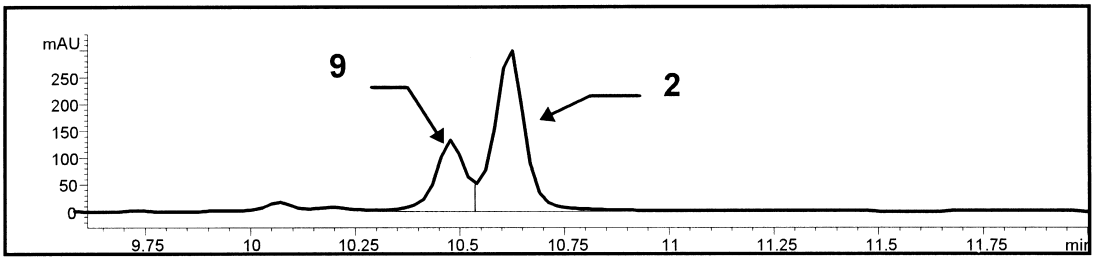

Fig. 5. Detection of kazusamycin A (**2**) and its isomer (**9**) in a preparative HPLC fraction of the mycelial extract from strain BA 809: Zoomed section of a HPLC–UV chromatogram (220 nm).

nybomycins also exhibited weak antifungal effects [10].

4. Conclusion

The presented results clearly demonstrate the progress in natural products extract screening methodology. The simultaneous detection and identification of alkaloids and polyketides from the same crude extracts was achieved by a time-saving combination of chromatographic and spectrometric methods. HPLC–UV and HPLC–MS libraries of known secondary metabolites (either prepurified or obtained from crude extracts of reference organisms) provide additional information to distinguish between new and known compounds in congeneric mixtures. Less than 50 μg of partially purified materials were necessary for safe identification of known metabolites (**1**–**6**). However, preparative chromatography, NMR spectroscopy and 'conventional' MS methodology will still remain necessary for the structure elucidation of minor metabolites and new compounds.

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