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ORIGINAL ARTICLE

Pilot-plant cultivation of *Streptomyces griseus* producing homologues of nonactin by precursor-directed biosynthesis and their identification by LC/MS-ESI

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Precursor-directed biogenetic approach was used to produce a range of nonactin homologues in a 50 l fermentor cultivation of strain *Streptomyces griseus* 34/249 obtained by UV mutagenesis. The production medium contained sodium propionate, isobutyrate and isovalerate as individual precursors, and 10 g l^{-1} Diaion HP20 styrene-divinylbenzene resin that maintains suitable precursor concentration by reversibly adsorbing and releasing it. The produced nonactin homologues were separated on two C18 reversed-phase liquid chromatography columns in series and analyzed by MS with ESI source in the positive ion mode. Formation of doubly charged ions was suppressed by an excess of Na^+ ions throughout the process. The production of the homologues increased up to day 5 and then it leveled off. Cultivations with individual precursors yielded a total of 18 nonactin homologues whose spectrum depended on the precursor used. The total production of the homologues was lowered but their spectrum was shifted to higher-molecular-weight compounds.
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Keywords: liquid chromatography/MS-ESI; pilot-plant cultivation; precursor-directed biosynthesisl; *Streptomyces griseus*

INTRODUCTION

The macrotetrolide ionophore antibiotic 'polynactin' family is composed of both enantiomers of nonactic acid (**1**), homononactic acid (**2**) or bishomononactic acid (**3**) and are assembled from four acids connected in a head-to-tail manner into a 32-membered macrocycle. The macrotetrolide antibiotics nonactin (**4**), monactin (**5**), dinactin (**6**), trinactin (**8**), tetranactin (**10**), and their homologues macrotetrolides B (**13**), C (**11**), D (**9**) and G (**7**) (Figure 1) were found in different admixtures and proportions in a variety of *Streptomyces* species.[1,2]

They display a wide range of biological activities, such as antimicrobial, insecticidal, antifungal and immunosuppressive[3] or antitumor.[4] The biological activities of the macrotetrolides are generally traced to their ionophoric properties,[5] and the potencies of these activities appear to parallel the size of the alkyl substituents of the macrotetrolides: tetranactin is often the most potent member of the family whereas nonactin is generally inactive (this gave rise to its name, that is nonactive). Nonactin is also used for the preparation of ion-selective electrodes.[6]

Although there is no information about the biological activities of macrotetrolides B–G,[7] it was reported that the K^+ affinity was found to be highly correlated with the biological activity; for example, the association constant for the K^+ complex of dinactin is sevenfold higher than that of nonactin.[8]

To study the biological activities of compounds containing bishomononactic acid and also higher homologues, that is tetranactin, macrotetrolides B–G (**7**, **9**, **11**, **13**), and nonnatural macrocyclic lactones, these compounds were prepared both by cultivation (see above) and by organic synthesis.[9] Macrotetrolide α was synthesized from two (−)- and two (+)-bishomononactic acids in seven steps[10] but the relevant papers do not mention any biological effects of this compound. Another problem in organic synthesis contrary of the cultivation is the alternation of (−)- and (+)-nonactic acid; that is, two monomers of (−)-nonactic acid and two monomers of (+)-nonactic acid assembled to form (+)-(−)-(+)-(−) 32-membered lactone nonactin. This natural nonactin has about 100-fold stronger antimicrobial activity against *Bacillus anthracis*, *Enterococcus faecalis*, *Staphylococcus aureus* or methicillin-resistant *S. aureus* than the synthetic all(+) or all(−) nonactin.[11]

To prepare sufficient amounts of higher nonactin homologues for further biological studies, we optimized cultivation conditions on a pilot-plant scale including the use of precursor-directed biosynthesis

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This paper is dedicated to Arnold L Demain, one of the world's leading industrial microbiologists, a pioneer in the research of secondary metabolites, a great human being and for many years my best friend. I wish you all the best in the years to come—JS.
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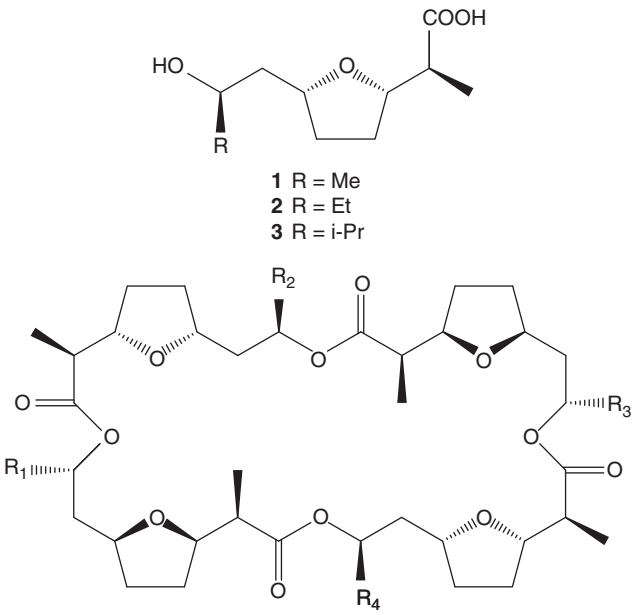


1 R = Me
2 R = Et
3 R = i-Pr

No	R_1	R_2	R_3	R_4
4	Me	Me	Me	Me
5	Me	Me	Me	Et
6	Me	Et	Me	Et
7	Et	Me	i-Pr	Me
8	Me	Et	Et	Et
9	Et	Me	Et	i-Pr
10	Et	Et	Et	Et
11	Et	Me	i-Pr	i-Pr
12	Me	i-Pr	i-Pr	i-Pr
13	Et	i-Pr	Et	i-Pr
14	Me	Et	i-Bu	i-Bu
15	Et	i-Pr	i-Pr	i-Pr
16	i-Pr	i-Pr	i-Pr	i-Pr
17	Et	i-Bu	i-Bu	i-Bu
18	i-Bu	i-Bu	i-Bu	i-Bu

Figure 1 Structures of nonactic acid (including two higher homologues) and natural or synthetic macrotetrolides.

(PDB) and identification of metabolites by liquid chromatography (LC)/MS-ESI.

MATERIALS AND METHODS

Strain and cultivation

A mutant strain *Streptomyces griseus* 34/249 obtained by genetic improvement[12,13] was used. It is deposited in the collection of the Institute of Microbiology (Academy of Sciences of the Czech Republic, Prague). The inoculum was cultivated for 24 h in a complex medium containing glucose 0.5%, soybean meal 1.5% and yeast extract 0.5%; the medium (35 l) in a pilot-plant bioreactor was inoculated with 1.75 l of vegetative inoculum.

For cultivation, the following complex production medium ($g\,l^{-1}$) was used: glucose 20, soybean meal 20, glycerol 5, peptone 2, yeast extract 2, beef extract 2, KH_2PO_4 1, $MgSO_4{\cdot}7H_2O$ 0.05, $CaCO_3$ 1 and resin 10 (pH not adjusted). Diaion HP20 styrene-divinylbenzene resin was purchased from Supelco (Prague, Czech Republic). For activation the resin granules were washed in twice the volume of MeOH and stirred for an hour. Excess MeOH was decanted, and the wetted resin was washed five times with deionized water. The slurry of the activated resin was filtered and the wet resin was added to liquid medium. In mechanically stirred aerobic tank reactor (75L; Bioengineering AG, Wald, Switzerland) the cultivations were performed for 5 days at 28 °C, aeration rate 0.6 VVM and impeller tip speed 1.9 $m\,s^{-1}$. The bioreactor vessel was equipped with four baffles and stirred by two radial six-blade Rushton turbines and one axial or radial impeller above them on the same shaft. The samples were collected after 2 days, then at 1-day intervals for 6 days. The cultivation was terminated after 6 days.

Sodium salt of a short-chain fatty acid (propionate, isobutyrate or isovalerate) was added in an amount of 10 mmol per 1 l of cultivation medium (that is, 33.6 g of sodium propionate (0.35 mol) in 35 l broth in a 50 l fermentor) every 12 h from 0 to 72 h and further in an amount of 5 mmol per 1 l of medium from 84 to 108 h every 12 h. In our study, for instance 85 mmol sodium propionate (8.16 g) per 1 l was added in 10 doses. Jizba *et al.*[13] added 8.33 g sodium propionate per 1 l (500 mg per 60 ml) in a single dose at the beginning of cultivation. The production of the sum of nonactins was 220 $mg\,l^{-1}$ for 1 $g\,l^{-1}$, 260 $mg\,l^{-1}$ for 2.5 $g\,l^{-1}$, 285 $mg\,l^{-1}$ for 5 $g\,l^{-1}$, 310 $mg\,l^{-1}$ for 10 $g\,l^{-1}$, 300 $mg\,l^{-1}$ for 15 $g\,l^{-1}$ and 280 $mg\,l^{-1}$ for 20 $g\,l^{-1}$ resin. Optimum resin concentration was therefore 10 $g\,l^{-1}$ (see above).

HPLC and MS instrumentation

A 1100 Series LC-mass-selective detector (VL model; Agilent Technologies, Santa Clara, CA, USA) equipped with APCI source and combined with Agilent's 1100 Series autosampler (model G1329A), and thermostatted column compartment (model G1316A) was used for reversed-phase LC/MS separations. The instrument was also equipped with four-solvent (model G1312A with additional two-way low-pressure solvent switching valve) and two-solvent (model G1312A) gradient pumps. Both pumps were connected to the flow path using a T-connector.

Two Hichrom HIRPB-250AM 250×2.1 mm ID, 5-μm phase particle columns were connected in series to obtain a high-efficiency column of approximately 53 000 plates per 50 cm. The samples were separated using a gradient solvent program: initial from 80% H_2O-20% THF with 0.1 M AcONa to 20% H_2O-80% THF with 0.1 M AcONa within 90 min; the composition was returned to the initial conditions over 8 min. A peak threshold of 0.3% intensity was applied to the mass spectra. The eluent flow rate was 0.25 $ml\,min^{-1}$, the column temperature was 28 °C and the volume of injected sample was 2 μl.

The column eluent was introduced into the ESI source and the MS was operated in the positive ion mode (ESI) by applying a voltage of 3000 V to the capillary and the following set of conditions: nebulizer gas 50 psi, drying gas 9 $l\,min^{-1}$, ESI desolvation temperature was 325 °C, fragmentation voltage 1.0 V, peak width 0.2 min and monitoring mass range 100–1000 Da. Agilent 1100 Series LC/MSD Trap software Version 4.2 was used.

RESULTS AND DISCUSSION

Precursor-directed biosynthesis

In its simplest form, PDB consists in feeding a precursor analogue to wild-type strains of a producing organism and building this unnatural precursor into a new product. This approach thus generates novel structurally diverse microbial secondary metabolites by combining chemical and biological methods.[14,15] It is effective in generating new analogues of natural products but requires basic knowledge of the cultivation of the producer organism and the target natural product's biosynthetic process. A correct precursor should be used; with a wrong precursor, for example 1 mM nonactic acid having a furan ring, the production of nonactin was inhibited by more than 90%, no effect on biomass production being found up to the 10 mM concentration of added precursor.[16]

Another strategy, mutasynthesis, uses mutant microorganisms deficient in a key aspect of the biosynthetic pathway; substitution of the natural precursor with a precursor analogue can again lead to the production of a new natural product. As documented, for example, by Bode *et al.*[17] in their study of myxalamid production by myxobacterial mutant strains so manipulated, these strains may eventually exhibit the same or a weaker incorporation of suitable precursors into the

desirable product than the wild-type strains. Another reason for using PDB is that it can be used without time-consuming genetic manipulations using methods of modern molecular biology.

Because the concentration of a precursor during the fermentation varies in dependence on its dosage intervals, we added into the fermentor adsorbent resin, which keeps the precursor concentration relatively constant by reversibly adsorbing and releasing it.[18,19] The optimum resin concentration in our experiments was $10\,g\,l^{-1}$.

Cultivation

UV mutagenesis of the parent strain of *S. griseus* yielded the strain 34/249 that exhibited predominantly cycloheximide-blocked synthesis, was resistant to glucose repression of morphogenesis, produced lower amounts of peptide antibiotics and increased levels of nonactic acid and its homologues including nonactin and its homologues.[20]

Strain 34/249 was cultivated in a 50 l fermentor and production of nonactin homologues was studied by LC/MS. As seen in Figure 1 and Tables 1 and 2, addition of different precursors resulted in the production of different nonactin homologues. As reported previously,[12] cultivation without precursors provided nonactin as a major product, the proportions of its higher homologues in culture medium decreasing with increasing molecular weight (Figure 2a). Figure 3 shows the results of LC/MS analysis of the broth on cultivation day 6. The production of the homologues increased up to the fifth day and then it leveled off. A similar situation was found after addition of sodium propionate, isobutyrate and isovalerate (Figures 2b–d). The addition of the precursors resulted on the one hand in a lowering of both total production and the production of the homologues, on the other hand, it brought about a change in their spectrum (Figures 2a–d). The highest drop in production, observed in our cultivation with sodium isovalerate (20% of control), though sizable, was much less than the 96% drop in production relative to the wild-type observed by Walczak *et al.*[20] with the mutant strain *S. griseus* ΔnonKJ.

Identification of macrolide mixture by RP-HPLC/MS-ESI

The methods currently in use for separation and identification of nonactin and its homologues are HPLC or MS;[1] for example, C18 RP-HPLC, using THF–water gradients with sodium salts solution as chelating agent.[21] On the basis of RP-HPLC analysis of acetone

Table 1 LC and MS data of macrotetrolides, that is (ionophore+H)$^+$

Name	*No*	R^1	R^2	R^3	R^4	*RT (min)*	*(1/4+H)$^+$*	*(2/4+H)$^+$*	*(3/4+H)$^+$*	*(M+H)$^+$*
Nonactin	**4**	Me	Me	Me	Me	17.48	185	369	553	737
Monactin	**5**	Me	Me	Me	Et	19.58	185, 199	369, 383	553, 567	751
Dinactin	**6**	Me	Et	Me	Et	22.72	185, 199	369, 383	567, 581	765
Macrotetrolide G	**7**	Et	Me	*i*-Pr	Me	25.30	185, 199, 213	383, 397	595, 581, 567	779
Trinactin	**8**	Me	Et	Et	Et	25.87	185, 199	383, 397	581, 595	779
Macrotetrolide D	**9**	Et	Me	Et	*i*-Pr	29.76	185, 199, 213	383, 411	581, 595, 609	793
Tetranactin	**10**	Et	Et	Et	Et	30.46	199	397	595	793
Macrotetrolide C	**11**	Et	Me	*i*-Pr	*i*-Pr	35.05	185, 199, 213	383, 397, 411, 425	581, 595, 623	807
Macrotetrolide	**12**	Me	*i*-Pr	*i*-Pr	*i*-Pr	38.76	185, 213	397, 425	609, 637	821
Macrotetrolide B	**13**	Et	*i*-Pr	Et	*i*-Pr	39.33	199, 213	411	609, 623	821
Isobutyl nonactin analog	**14**	Me	Et	*i*-Bu	*i*-Bu	44.75	185, 199, 227	383, 411, 425, 453	609, 637, 651	835
Macrotetrolide	**15**	Et	*i*-Pr	*i*-Pr	*i*-Pr	45.32	199, 213	411, 425	623, 637	835
Macrotetrolide α	**16**	*i*-Pr	*i*-Pr	*i*-Pr	*i*-Pr	52.44	213	425	637	849
Isobutyl nonactin analog	**17**	Et	*i*-Bu	*i*-Bu	*i*-Bu	70.80	199, 227	425, 453	651, 679	891
Isobutyl nonactin analog	**18**	*i*-Bu	*i*-Bu	*i*-Bu	*i*-Bu	83.26	227	453	679	905

Table 2 LC and MS data of macrotetrolides, that is (ionophore+Na)$^+$

Name	*No*	*(1/4+Na)$^+$*	*(2/4+Na)$^+$*	*(3/4+Na)$^+$*	*(M+Na)$^+$*
Nonactin	**4**	207	391	575,	759
Monactin	**5**	207, 221	391, 405	575, 589	773
Dinactin	**6**	207, 221	391, 405	589, 603	787
Macrotetrolide G	**7**	207, 221, 235	405, 419	617, 603, 589	801
Trinactin	**8**	207, 221	405, 419	603, 617	801
Macrotetrolide D	**9**	207, 221, 235	405, 433	603, 617, 631	815
Tetranactin	**10**	221	419	617	815
Macrotetrolide C	**11**	207, 221, 235	405, 419, 433, 447	603, 617, 645	829
Macrotetrolide	**12**	207, 235	419, 447	631, 659	843
Macrotetrolide B	**13**	221, 235	433	631, 645	843
Isobutyl nonactin analog	**14**	207, 221, 249	405, 433, 447, 475	631, 659, 673	857
Macrotetrolide	**15**	221, 235	433, 447	645, 659	857
Macrotetrolide α	**16**	235	447	659	871
Isobutyl nonactin analog	**17**	221, 249	447, 475	673, 701	913
Isobutyl nonactin analog	**18**	249	475	701	927

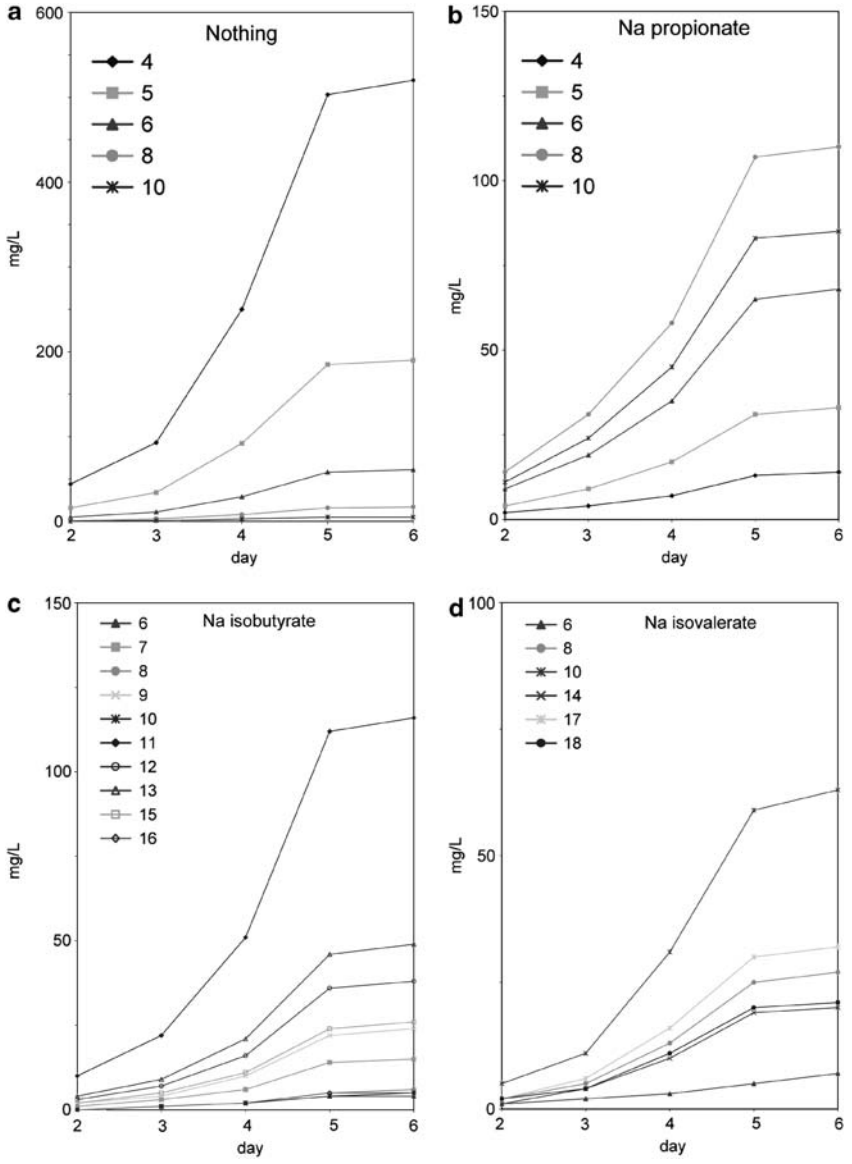
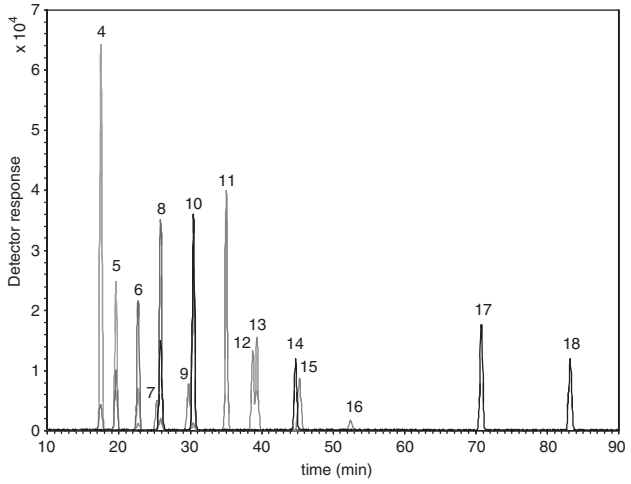


Figure 2 Production of macrotetrolide homologues of nonactin: (**a**) without addition of sodium salts of short-chain fatty acids, (**b**) with addition of sodium propionate, (**c**) with addition of sodium isobutyrate and (**d**) with addition of sodium isovalerate. Numerals at curves correspond to the designation in Tables 1 and 2.



extracts of *S. griseus* DSM40695 mycelia, which revealed a mixture of nonactin-tetranactin,[22] we also used C18 reversed phase with an additional gradient elution with THF–water and with addition of sodium ions. This method separated not only the nonactin homologues differing in a methylene group (see Figure 3) but also the so-called critical pairs, that is macrotetrolide G (**7**)-trinactin (**8**), macrotetrolide D (**9**)-tetranactin (**10**), macrotetrolide (**12**)-macrotetrolide B (**13**) and isobutyl nonactin analogue (**14**)-macrotetrolide (**15**) that have the same molecular weight and differ only in R_1-R_4, see Figure 1 and Tables 1 and 2.

Figure 3 Separation of macrotetrolide homologues of nonactin from mutant strain *Streptomyces griseus* 34/249 by means of RP-HPLC/MS-ESI. For peak assignment, see Table 1 (green, without sodium salts of short-chain fatty acids; blue, sodium propionate was added; red, sodium isobutyrate was added; black, sodium isovalerate was added). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Another possibility of identifying the metabolites is direct flow injection by APCI or ESI-MS. Secondary metabolites such as nonactin were examined with ESI and APCI MS in both positive and negative ion mode. In contrast to positive mode of ESI and APCI,[23] negative ionization mode did not provide satisfactory results.

Jani *et al*.[24] described simultaneous analysis of nonactin and related macrotetrolide homologues using direct infusion LC/MS with ESI. The authors added K^+ acetate into the medium because, when analyzing mixtures of NH_4^+ adducts, other ions present in the fermentation fluid interfered, resulting in very complex spectra.

Because the broth contains an excess of calcium, though in the form of insoluble carbonate, the doubly charged [ionophore+Ca]$^{2+}$ complex may present a problem. For example, the ion at *m/z* 408 corresponds to loss of 2×184 Da from [nonactin+Ca]$^{2+}$.[25] We succeeded in suppressing the formation of doubly charged ions by using an excess of Na^+ ions throughout the process.

Na^+ ions were used as a compensating agent for several reasons. First, sodium is monoisotopic in contrast to potassium (93% of ^{39}K and 7% of ^{41}K); this feature of potassium makes ESI spectra much more complex.[24] Second, Na^+ ions are in all circumstances in a large excess during the cultivation because short-chain acids are present as sodium salts.

In agreement with the paper by Shen and Brodbelt,[25] and in contrast to earlier studies,[2] we also did not observe any dehydration of nonactin complexes, presumably reflecting the lack of hydroxyl groups. As shown in Figure 1, all ionophores have a cyclic structure

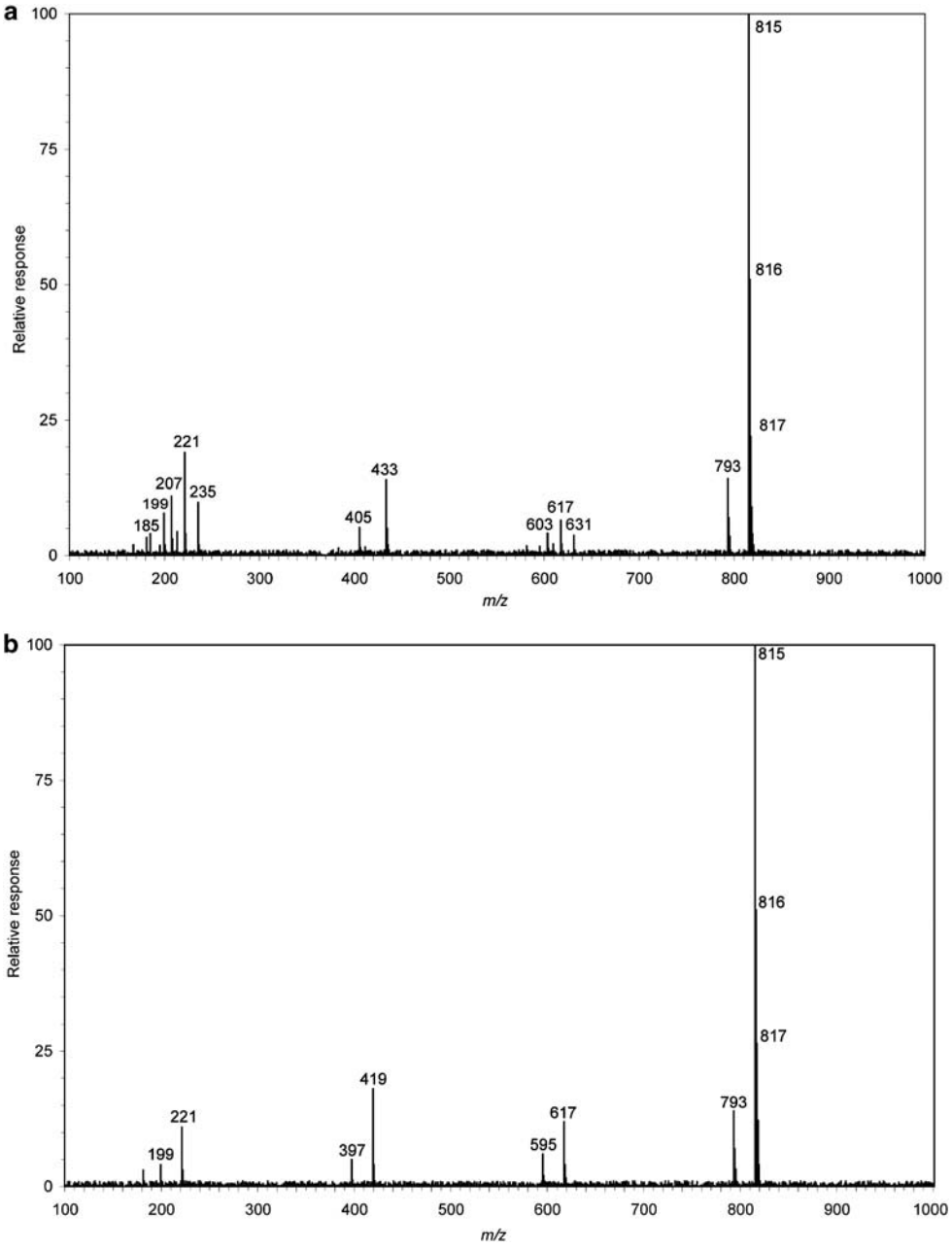


Figure 4 (**a**) ESI spectrum of macrotetrolide D in peak 9 and its major fragmentation pathway. (**b**) ESI spectrum of tetranactin in peak 10 and its major fragmentation pathway.

and therefore multiple covalent bonds must be broken to create observable fragment ions. The nonactin–alkali metal complexes dissociate by a series of losses of nonactic acid (184 Da). For example, the major fragments observed for $[nonactin+Na]^+$ are *m/z* 575, 391 and 207 (that is, loss of 184 or 2×184 or 3×184 Da). One possible proposed pathway is given in literature,[25] but it is not known whether the products are cyclized or not. Similar pathways likely account for the formation of each of the fragment ions; their numerical values are given in Tables 1 and 2. Figures 4a and b also show ESI mass spectra of two compounds from one possible critical pair; that is, compounds **9** and **10** that have the same summary formula and differ only in the substituents (Me, Et, *i*-Pr, see Figure 1). These values can then serve for identification of individual nonactin homologues and determination of their proportions in the mixture.

CONCLUSIONS

Pilot-plant scale cultivation in a 50l fermentor using PDB and the mutant strain *S. griseus* strain 34/249 yielded mixtures selectively enriched in several nonactin homologues according to the precursor used. Though the presence of different precursors usually lowers the overall production, the method can be used for producing novel nonactin homologues.

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