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Rapid identification of teleocidins in fermentation broth using HPLC photodiode array and LC/MS methodology

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SUMMARY

During the course of screening for inhibitors of phorbol ester binding to protein kinase C, several actinomycete cultures were discovered that produce active metabolites. HPLC coupled to photodiode array and LC/MS techniques were applied to broth extracts to identify the presence of indolactams belonging to the teleocidin family. Various members of this family were rapidly identified from crude broth extracts using a combination of these spectroanalytical procedures. An analytical HPLC system was developed to optimize separation of teleocidin A and B analogues directly from ethyl acetate extracts of whole broth cultures. This technique allowed us to identify a novel homologue of pendolmycin and demonstrated the utility of photodiode array HPLC coupled with LC/MS as an initial analytical tool in the analyses of these secondary metabolites produced by soil microorganisms.

INTRODUCTION

Protein kinase C (PKC) has been the focus of considerable research activity in recent years to unlock the secrets of its control over cell metabolism. The enzyme exists in various isoforms, and all of these isozymes appear to have a common feature in that they contain a regulatory subunit that is naturally activated by intracellular diacylglycerol [8,9].

The regulatory subunit of the enzyme is also activated by the phorbol esters. These are natural plant diterpenes which have been discovered to be potent skin irritants as well as tumor promoters, and whose toxicity is attributed, at least in part, to their potent PKC agonist activity [1].

We adapted a phorbol ester binding assay to enable screening of natural products and examined microbial culture extracts for the presence of competitive inhibitors of phorbol ester binding. This screen led to the detection of the teleocidin family of indole alkaloids from a number of actinomycete cultures (representatives are shown in Fig. 1). Consequently, a dereplication scheme was developed to ensure rapid identification of these compounds in our screening evaluation process. The purpose of this research was to demonstrate that coupling the use of photodiode array HPLC with mass spectrometry could be a quick method to identify specifically these toxic metabolites of actinomycetes.

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MATERIALS AND METHODS

Microbial cultures

All cultures tested were originally isolated from the soil rhizosphere by Panlabs, Inc. The three teleocidin-producing cultures described in this report were identified as *Streptomyces* sp. (strain SC0035), *Nocardiopsis* sp. (strain SC0037), and *Streptoverticillium* sp. (strain SC0038). All strains were deposited in the culture collection maintained at Sterling Research Group. Growth and production conditions involved inoculation from frozen stock into seed medium containing (% w/v): glucose 2.0; Pharmamedia 1.5 (obtained from Traders Protein, Memphis, TN), $(NH_4)_2SO_4$ 0.3; $ZnSO_4 \cdot 7H_2O$ 0.003; and $CaCO_3$ 0.3. The seed was grown for 2 days at 27 °C and 220 rpm on PsycroTherm shakers (New Brunswick Scientific Co., Edison, NJ), followed by transfer (using a 6%-inoculum) to production medium for an additional 4 days, also at 27 °C and 220 rpm. The principal production medium contained (% w/v): glycerol 2.0; dextrin 2.0; bacto-soytone 1.0; yeast extract 0.3; $(NH_4)_2SO_4$ 0.2; and $CaCO_3$ 0.2, though production of these compounds was observed in a variety of media tested.

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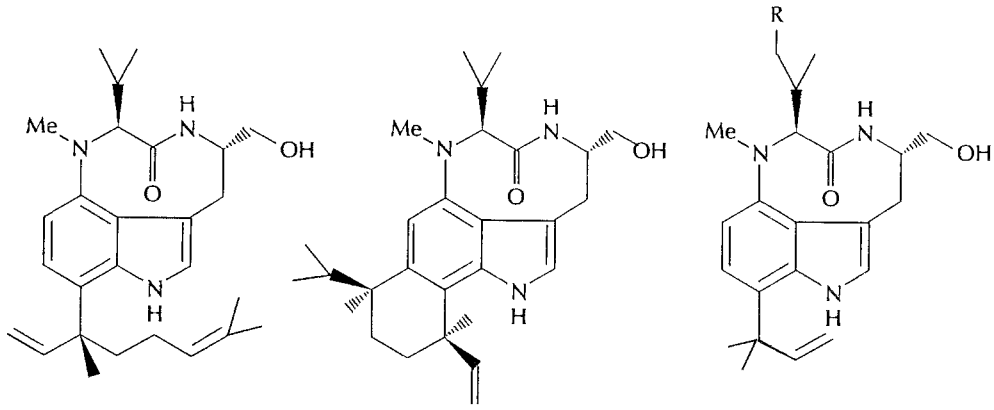

TELEOCIDIN A-2
MW = 437

TELEOCIDIN B-1
MW = 451

PENDOLMYCIN (R=H)
MW = 369

METHYLPENDOLMYCIN (R=Me)
MW = 383

Fig. 1. Representative structures of teleocidins.

Sample preparation

All samples were prepared by ethyl acetate (EtOAc) extraction of 4-day-old shake-flask cultures using two extractions of EtOAc with volumes equivalent to the original culture volume. The crude EtOAc extracts were pooled and dried to remove all excess solvent from the sample. Prior to chromatography or bioassay the samples were dissolved in ethanol to prepare a 100-fold concentrate as compared to the original culture volume.

Photodiode array HPLC analysis

A Waters 600E multisolvent delivery system with model 990 photodiode array detector was used to analyze all samples. The mobile phase consisted of a linear gradient starting with 85% solvent A (0.1% trifluoroacetic acid in water), 15% solvent B (acetonitrile) and ending 40 min later with 100% solvent B. The gradient was followed with an additional 10-min rinse with 100% solvent B. The column used was a Waters C_{18} Nova-Pak 8 × 10 cartridge and the flow rate was 2 ml/min.

The photodiode array spectrophotometer was adjusted to collect data every 6 s, for 0–50 min run time. Both accumulation and sensitivity settings on the unit were set to '2'. The unit was set to acquire spectral data from 200 nm to 500 nm with a resolution of 2 nm.

When fractions were tested for bioactivity, 2-ml fractions were collected and dried overnight in a Speed-Vac Concentrator (Savant Instruments, Inc., Farmingdale, NY), then resuspended in ethanol prior to test.

LC/MS analysis

A HP1050 Series Quaternary Pump, Autosampler and Multiple Wavelength Detector were used for the thermospray LC/MS analyses (Hewlett-Packard Company, Palo Alto, CA). The column, the mobile phase and gradient were the same as described above, but the flow rate was 1 ml/min with 0.1% aqueous trifluoroacetic acid added post-column at 0.5 ml/min. These modifications were employed to enhance the thermospray performance. The optical absorbance was monitored at 300 nm, and the injection volume was 5 μl.

The mass spectrometer was a HP5988A with extended mass and positive/negative ion capabilities and equipped with a HP HED detector. The interface between the HPLC and the mass spectrometer was a HP thermospray unit with a post repeller placed opposite to and aligned axially to the sampling orifice. The source temperature was 350 °C and mass spectra were obtained scanning from 150 to 650 amu in 0.8 s. The potential on the repeller was 300 V.

Protein kinase C assay

The method used was based on a procedure originally described by Dunphy et al. [3] modified by the use of rat brain homogenate as a source of receptor. Briefly, the procedure used 80 μg protein per assay and ^{3}H-phorbol dibutyrate (PDBU at 19.1 Ci/mmol; obtained from NEN Research Products, Boston, MA) as the radioligand. The positive reference control, 4-*O*-methylphorbol 12-myristate 13-acetate (K_i = 0.5–0.6 μM), and the (−)-indolactam V standard (K_i = 0.42 μM) were obtained from LC Services Company, Woburn, MA. All samples were originally dissolved in ethanol, then tested at a final concentration of 1% solvent.

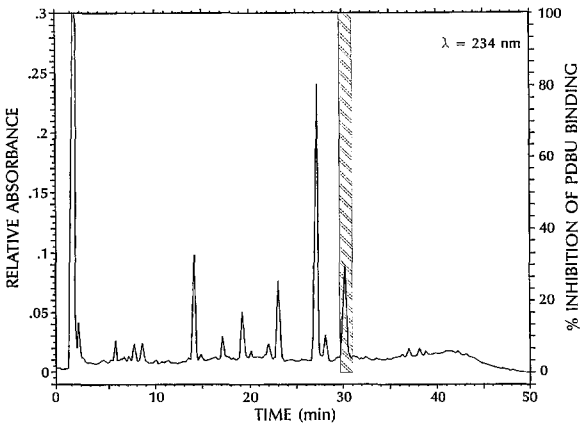

Fig. 2. Bioactivity of SC0035 extract.

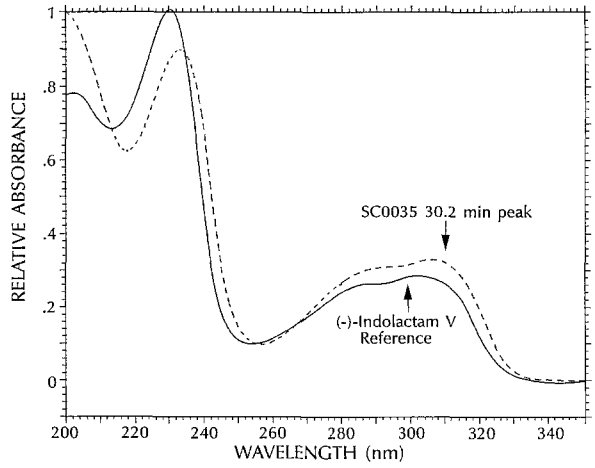

Fig. 3. UV spectra of SC0035 bioactive component and indolactam reference compound.

RESULTS

Initial screening of EtOAc extracts from actinomycetes showed that three cultures, designated as SC0035, SC0037 and SC0038, possessed potent inhibitors of PDBU binding. The active components were found to be stable with no change in activity observed when the extracts were exposed to acid (pH = 2.5), alkali (pH = 9.0), or elevated temperature (90 °C). Additional studies comparing quantities extracted from cells versus supernatant fluid revealed that the active components were approximately 90% cell associated.

We directed our efforts at this early stage of characterization to develop a quick identification protocol for these compounds. Use of HPLC/photodiode array methods enabled us to separate the active components in the EtOAc extract and to detect the active components exhibiting similar UV chromophores. The UV spectra of these meta-

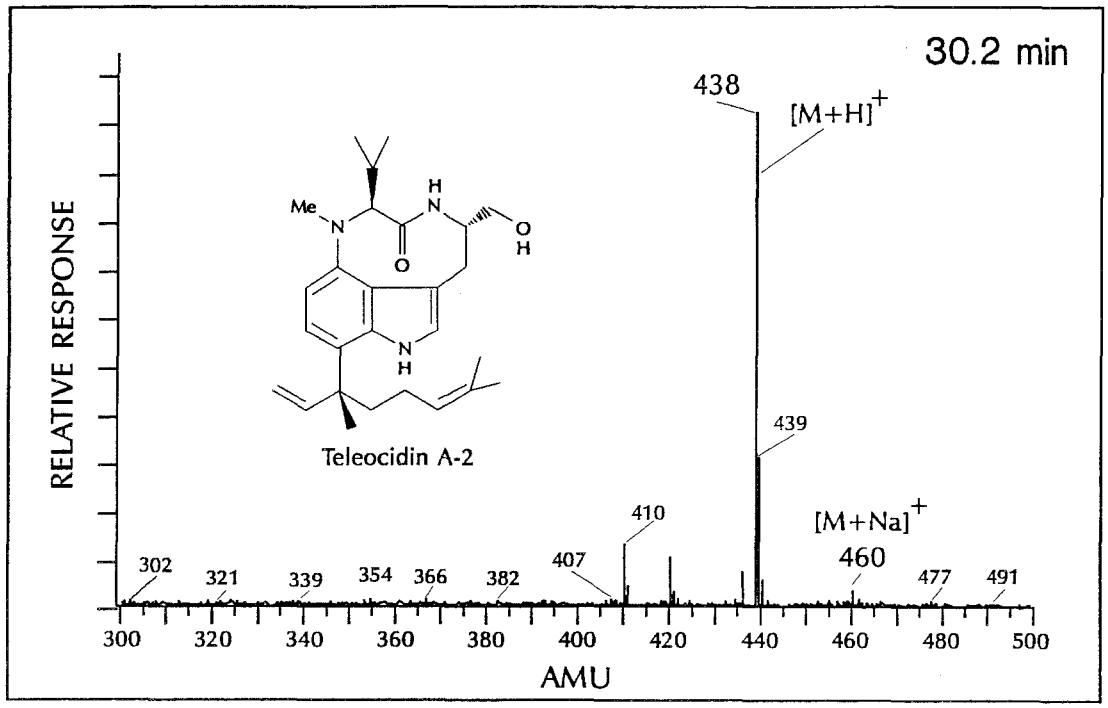

Fig. 4. Mass spectrum of SC0035 bioactive peak.

bolites were typical of compounds that possess an indolactam ring, such as the teleocidins and the olivoretins [10] produced by soil actinomycetes. In order to confirm the presence of the teleocidins in the extracts we further developed an HPLC method to separate several members of this class and coupled this method with on-line MS data to identify each member.

HPLC of the extract from culture SC0035 resolved a single active peak which eluted at 30 min (Fig. 2) using the described conditions. The UV chromophore of 234, 284 and 304 nm, indicated an indolactam (Fig. 3). Upon LC/MS analysis, with addition of 0.1% aqueous trifluoroacetic acid added post-column and modification of the flow rate, this component exhibited M_r = 437, (Fig. 4) which is the same as those of two known isomeric indolactam alkaloids, lyngbyatoxin A (teleocidin A-1) [2,11] and teleocidin A-2 [11]. In order to confirm its identify, isolation of the active compound was achieved by vacuum

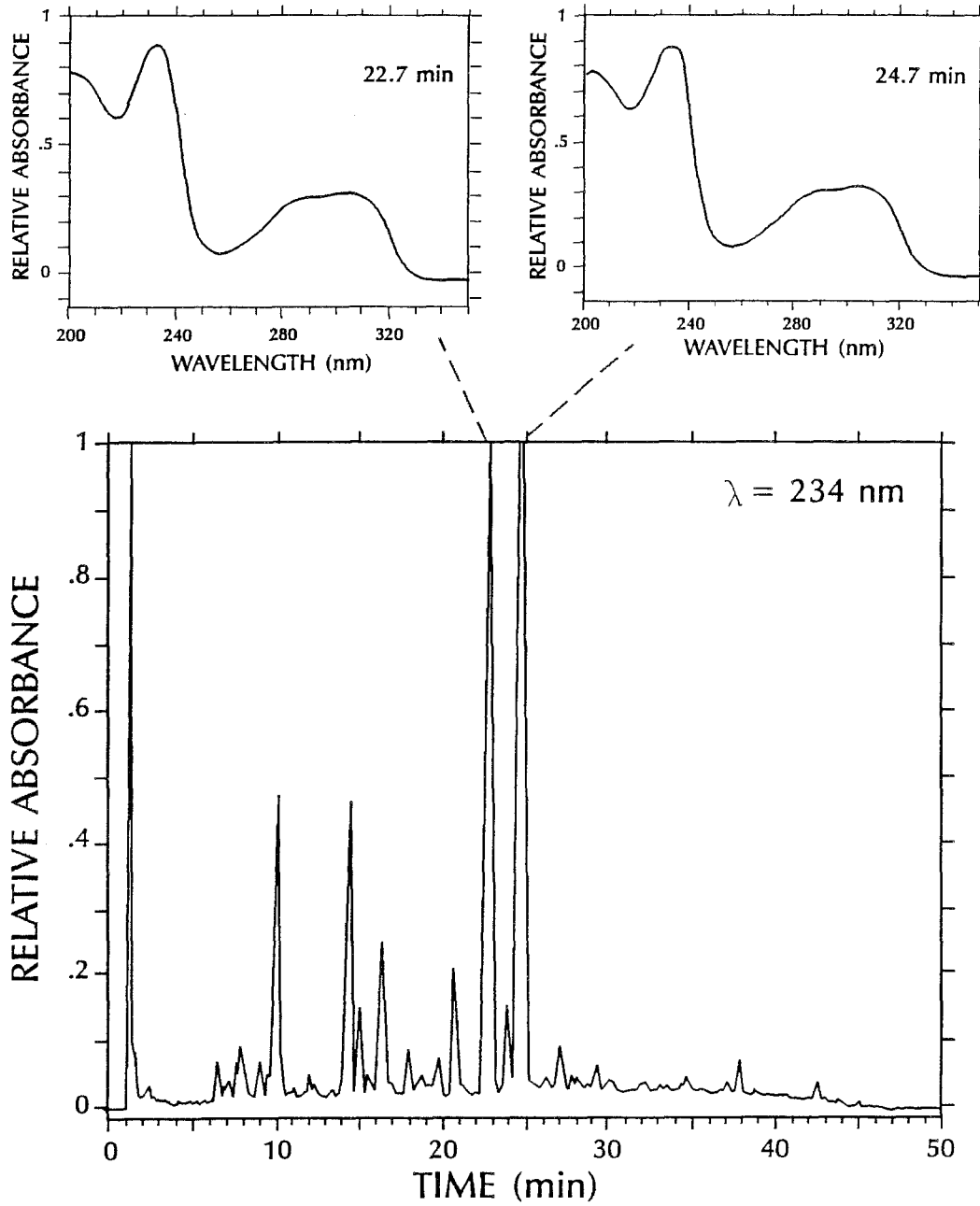

Fig. 5. UV spectra of bioactive HPLC peaks from SC0038 culture extract.

flash silica gel chromatography and HPLC. Analysis of ^{1}H and ^{13}C NMR spectral data and measurement of the optical rotation (− 188°, C = 0.2, MeOH) indicated that this component was in fact teleocidin A-2.

In the case of the extract from culture SC0037, application of the HPLC method coupled with mass spectroscopy revealed production of two indolactam alkaloids. The UV spectra of two components eluting at 23 and 25 min were virtually superimposable (Fig. 5). The thermospray LC/MS analysis indicated that the molecular weight of the early eluting component was 369 (Fig. 6), which matched with that of a known compound, pendolmycin [15]. The mass of the 25-min component was 383 (Fig. 6). The difference of 14 Da between these two components and the presence of the commonly observed mass fragments of $[MH-H_2O]^+$ and $[MH-CO]^+$ for teleocidins in both compounds suggested that the 25-min component was a methylated homologue of pendolmycin.

To confirm the structures, both compounds were isolated by flash chromatography and HPLC. The spectral data of the 23-min component including MS, ^{1}H and ^{13}C NMR, optical rotation, IR and CD are in agreement with the reported values of pendolmycin [15]. The 25-min component was found to be a novel homologue of pendolmycin which we have termed 16-methylpendolmycin. Its structure was established by high resolution mass spectrometry, CD measurement, and ^{1}H and ^{13}C NMR spectral data with the aid of extensive 2-D NMR experiments [13].

Utilization of photodiode array HPLC and LC/MS techniques on a third culture, SC0038, demonstrated the presence of two related indolactam alkaloids eluting at 30 min and 32 min (Figs. 7 and 8). The MS spectral data (M_r = 437) and UV absorbance profile (234, 284 and 304 nm) of the 30-min component were identical with those of teleocidin A-2 from SC0035, suggesting a teleocidin A structure [2,11]. The component eluting at 32 min was noted to have a slightly different UV chromophore with absorbance peaks only at 234 nm and 288 nm indicative of a different structure from that of the 30-min component. LC/MS of the 32-min component yielded a molecular weight of 451, which matched that of a teleocidin B analogue [5].

DISCUSSION

Since our purpose in screening for PDBU binding inhibitors was to identify PKC antagonists, we here interested in rapidly identifying cultures which produced teleocidins, which are known PKC agonists [4,6], and

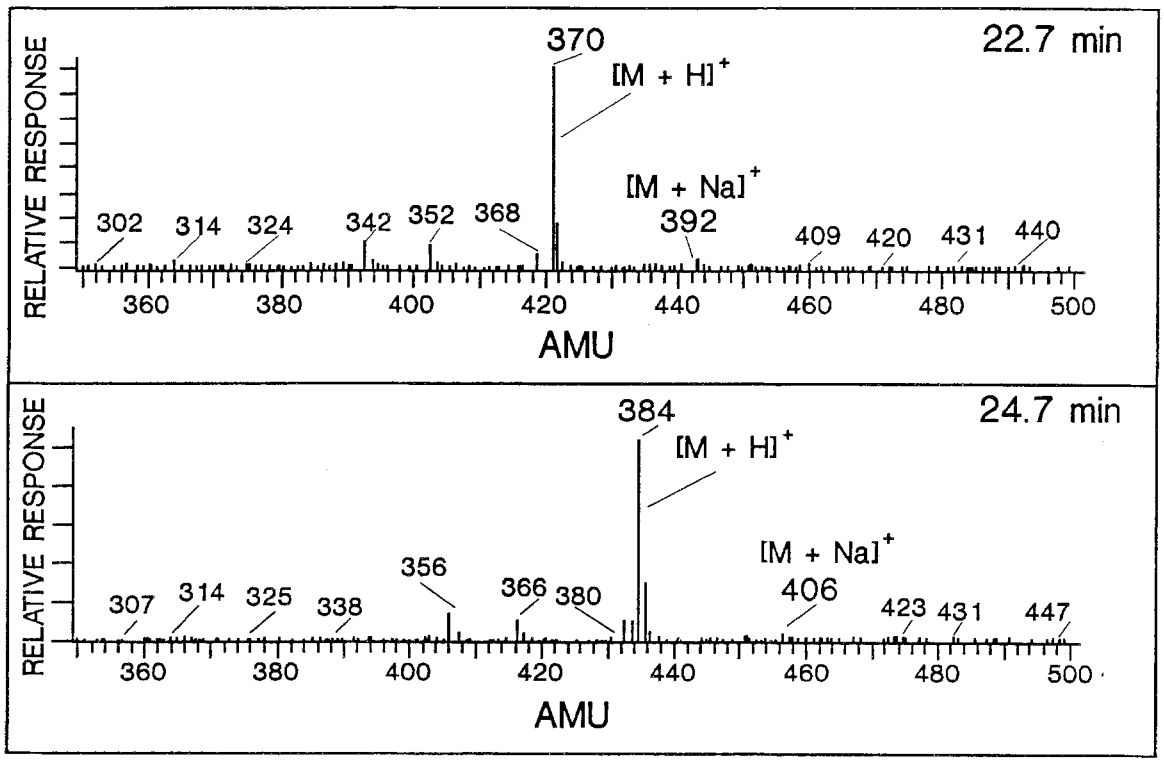

Fig. 6. Mass spectra of bioactive HPLC peaks from SC0037 culture extract.

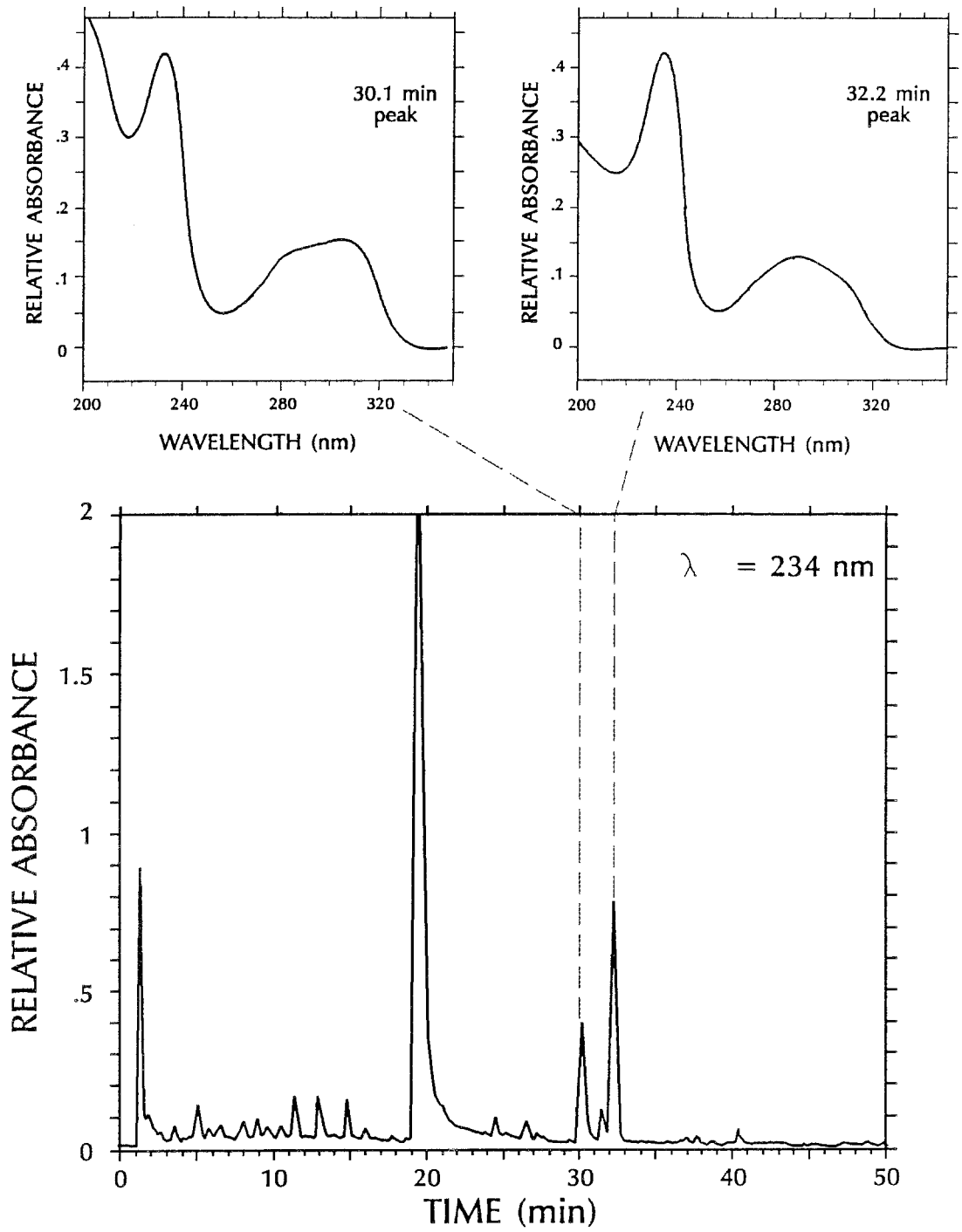

Fig. 7. UV spectra of bioactive HPLC peaks from SC0038 culture extract.

separating these organisms from the total group to be pursued. We thus developed a rapid dereplication scheme for crude culture extracts by coupling photodiode array spectroscopy with HPLC methods [7].

The original description of the teleocidins by Takashima and Sakai [15] contained an analysis of the chromophore indicating a slight red shift of the indole group's λ_{max} from 280 nm to $\approx$300 nm. This characteristic was observed when we separated and analyzed components in our culture extracts by photodiode array

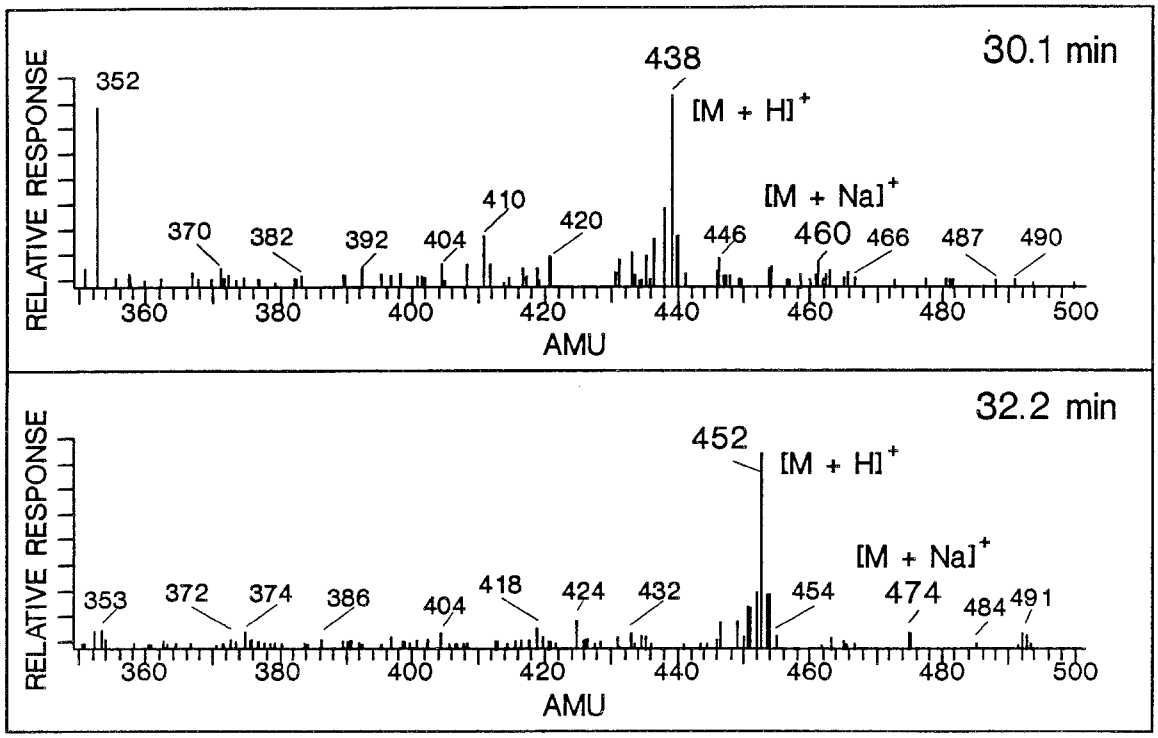

Fig. 8. Mass spectra of bioactive HPLC peaks from SC0038 culture extract.

HPLC. All extracts containing this indole chromophore exhibited potent PDBU inhibitory activity. Testing HPLC eluates from the extracts in the PDBU binding assay confirmed that these indole alkaloids were indeed responsible for the bioactivity of the extracts.

We also developed a LC/MS procedure to characterize structurally these active components compared to known teleocidins. To our knowledge this is the first reported example of applying LC/MS methodology to identify teleocidins in culture extracts. The use of this analytical tool and comparison of the resulting mass spectral data with those of known teleocidins [5,10–12,14,15] allowed us to characterize successfully different analogues of the teleocidin A, teleocidin B and pendolmycin classes. Additionally, this method gave us rapid detection of a novel teleocidin compound, 16-methylpendolmycin, present in culture SC0037. This compound contains an *N*-methylisoleucine moiety rather than the *N*-methylvaline moiety more commonly found in pendolmycin and other teleocidins.

In conclusion, the use of photodiode array spectroscopy coupled with HPLC separation techniques proved to be a valuable tool in the rapid identification of the indolactam alkaloids from microbial broths. When coupled with mass spectroscopy, we were able to identify specific classes of teleocidins. These analytical tools should be generally useful where dereplication schemes are needed for known compounds which possess a characteristic chromophore, especially when these compounds are produced as families of components.

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