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Rapid identification of elaiophylin and geldanamycin in *Streptomyces* fermentation broths using CPC coupled with a photodiode array detector and LC-MS methodologies

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During the course of screening microbial broth extracts in various high through-put bioassays (eg receptor binding or enzyme inhibition), several actinomycete cultures were discovered to produce active metabolites. The natural products elaiophylin and/or geldanamycin are produced by several *Streptomyces violaceusniger* strains, and the bioactivity of the extracts from these cultures was frequently associated with the fractions containing these metabolites. CPC coupled to a photodiode array detector and LC-MS techniques were applied to these broth extracts to ascertain rapidly when these natural products were present. These methodologies allowed us to identify the metabolites quickly in the crude extract, and the application demonstrated further the utility of CPC-photodiode array detection and LC-MS as powerful, initial analytical tools in analyses of the complex metabolite profiles produced by microorganisms.

Keywords: dereplication; CPC; LC-MS; elaiophylin, geldanamycin; actinomycete

Introduction

Several thousand microbial metabolites have been isolated which necessitate an efficient natural product dereplication methodology so that resources are not wasted. Numerous dereplication schemes have been reported that use HPLC and UV-visible absorbance spectral libraries [1,2,5] or HPLC photodiode array or LC/MS methodology [6].

Panlab's Natural Products Chemistry program employs centrifugal partition chromatography coupled to photodiode array detection routinely as a primary tool for the initial bioactive-directed fractionation of active extracts. Centrifugal partition chromatography (CPC) is well suited for performing assay-directed fractionations. The technique does not involve solid phase adsorbents which are inherently more destructive. The coupling of a CPC to a photodiode array detector is a powerful procedure in the dereplication of an active metabolite at an earlier stage of the project. The pattern of metabolites produced by a particular microorganism is reflected in the various separated peaks of the CPC chromatogram. Retention time alone, however, is not a sufficient characteristic for dereplication because of the large number of possible compounds and the possibility of co-eluted metabolites. Therefore, in addition to retention time and UV-visible absorbance profiles, LC-MS data are collected to identify the bioactive component.

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Materials and methods

Microbial cultures

The actinomycete strains used in this study were isolated from terrestrial samples collected around the world. Cells of the isolated actinomycetes were inoculated into seed media containing (% w/v) glucose 20 g, pharmamedia 15 g, $(NH_4)_2SO_4$ 3 g, $ZnSO_4 \cdot 7H_2O$ 0.03 g, $CaCO_3$ 4 g and yeast extract 5 g. The seed culture was grown for 2 days at 28° C at 250 rpm on a shaker (New Brunswick Scientific Co, Edison, NJ, USA), before transfer of a 2-ml aliquot to production medium. The production culture was grown for an additional 4 days, also at 28° C and 250 rpm. The elaiophylin- and geldanamycin-producing cultures were identified as *Streptomyces violaceusniger*.

Sample preparation

Broth extracts were prepared by ethyl-acetate (EtOAc) extraction of 7-day-old shake-flask cultures using three extractions into EtOAc with volumes equivalent to the original culture volume. The crude EtOAc extracts were pooled and concentrated to give a dried sample.

CPC fractionation

Initial fractionations were performed using a commercial high-speed CCC centrifuge (PC Inc, Potomac, MD, USA) that holds an Ito multilayer-coil separation column. A hexane : EtOAc : MeOH : H_2O (1 : 3 : 3 : 3) biphasic solvent system was employed at a flow rate of 3 ml min^{-1}. Four hundred milligrams of extract were loaded onto the column. The upper, less-polar phase was employed as the mobile phase and the lower, more-polar phase as the stationary phase (normal phase chromatography) for the first 120 min of the run. For the remaining 120 min of oper-

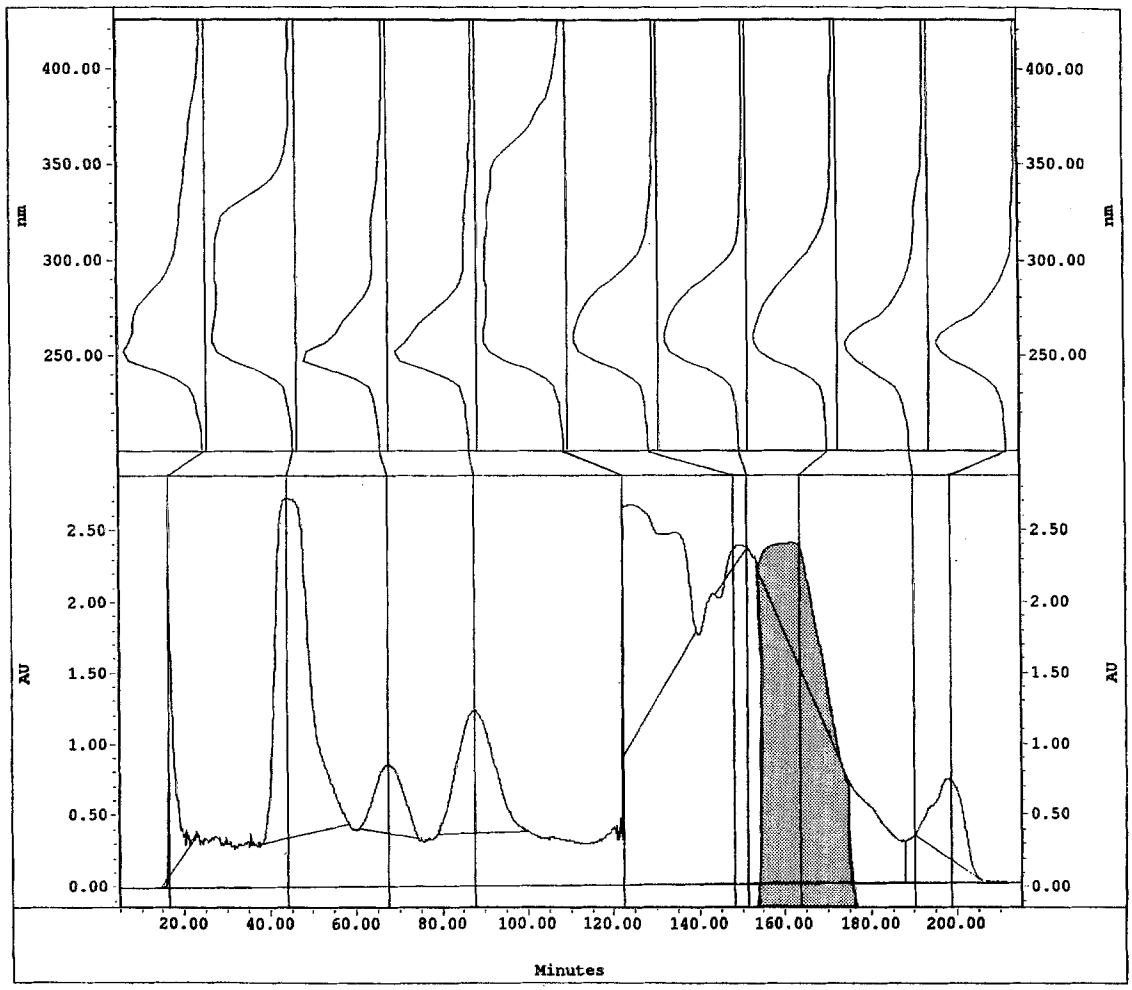


Figure 1 CPC separation of an EtOAc extract of actinomycetes strain 9999AE. (Shaded area represents the elution of elaiophylin)

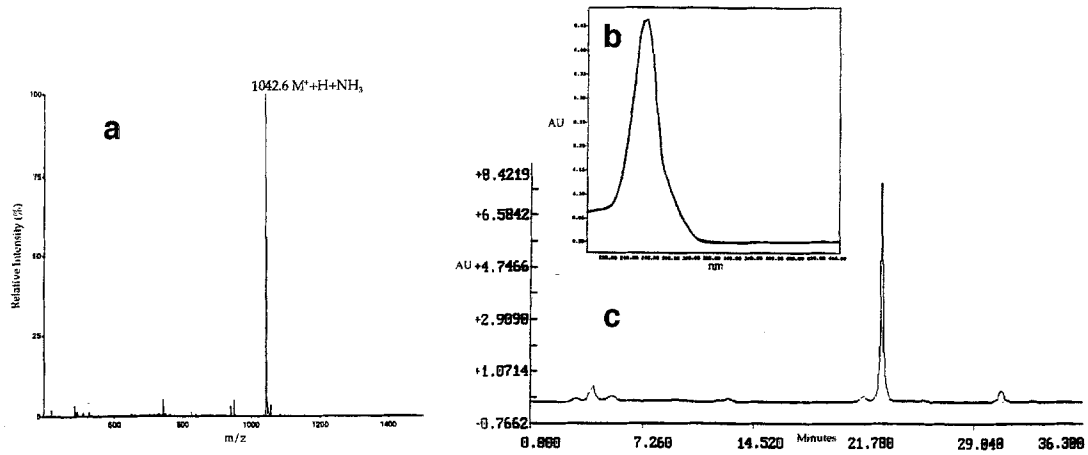


Figure 2 Characterization of the active compound contained in the CPC fractions eluting at 151–171 min: (**a**) Ion-spray (positive ion) mass spectrum of the HPLC peak eluting at 22 min; (**b**) UV spectrum of HPLC peak eluting at 22 min; (**c**) Analytical RP-HPLC of the combined CPC fractions eluting at 151–171 min

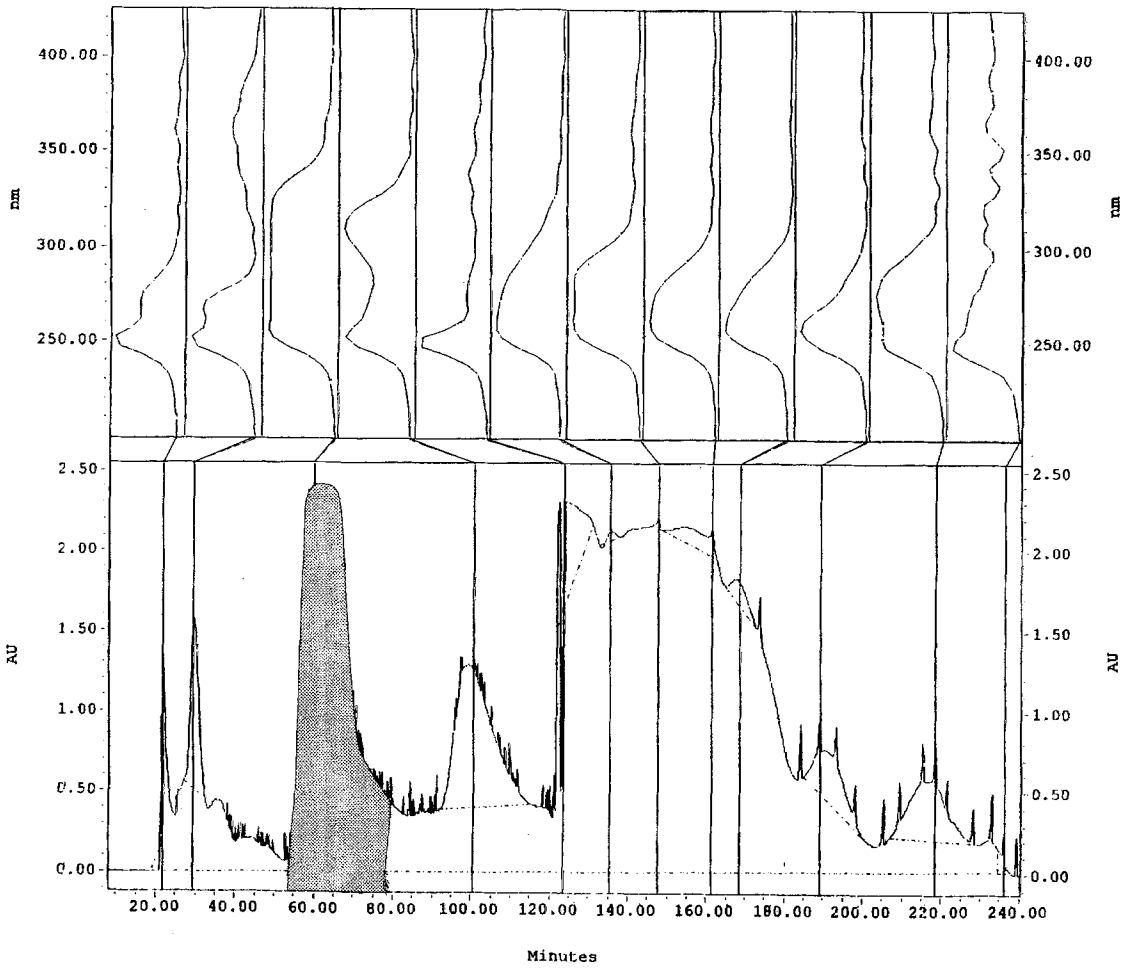


Figure 3 CPC separation of an EtOAc extract of actinomycetes strain 10000AA. (Shaded area represents the elution of geldanamycin)

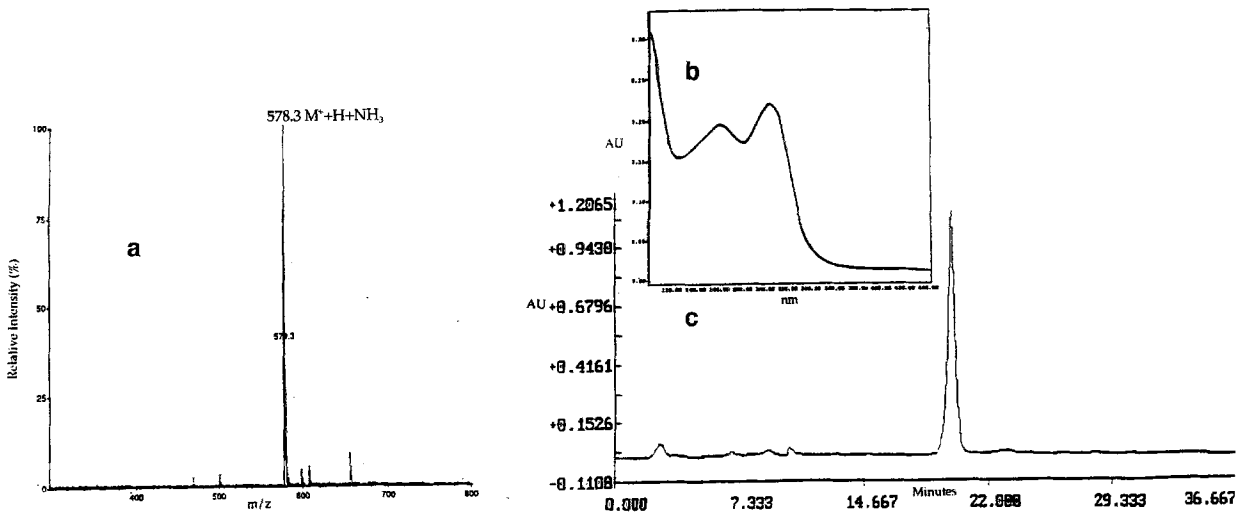
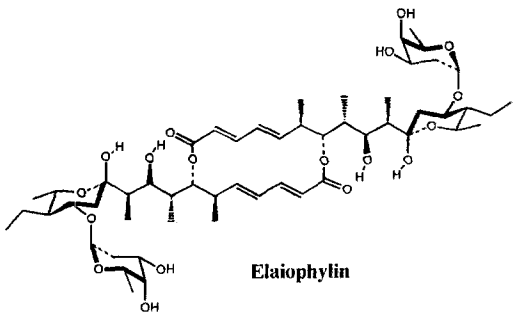


Figure 4 Characterization of the active compound contained in the CPC fractions eluting at 57–78 min: (**a**) Ion-spray (positive ion) mass spectrum of the HPLC peak eluting at 20 min; (**b**) UV spectrum of HPLC peak eluting at 20 min; (**c**) Analytical RP-HPLC of the combined CPC fractions eluting at 57–78 min



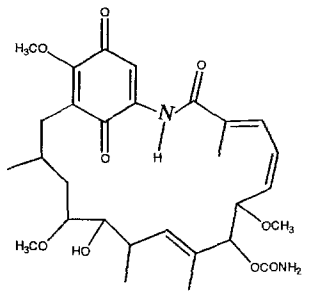


Figure 5 Structures of elaiophylin and geldanamycin

ation, the mobile phase and flow direction (reverse phase chromatography) were reversed. A Waters model 911 photodiode array detector (Millipore Corporation, Marlborough, MA, USA) was used to analyze all samples. A total of 80 9-ml fractions were collected. Aliquots (0.9 ml) from each fraction were transferred to a microtiter plate and concentrated. The dried aliquots were resuspended in DMSO prior to testing them.

LC/MS analysis

Mass spectra were recorded on a PE Sciex API III triple-quadrupole mass spectrometer interfaced with a Sciex Ion-Spray probe. Liquid chromatography was performed with a Perkin Elmer Binary Pump 250 and an LC480 Auto Scan Diode Array Detector. Separations were achieved using a linear gradient (80% 2 mM ammonium acetate solution pH 5.3, with 20% CH_3CN to 100% CH_3CN over 30 min) on a C_{18} Waters Novapak 8 × 100 mm cartridge at the flow rate of 1 ml min^{-1}. The post-column split to the mass spectrometer inlet was 0.2 ml min^{-1}.

Results

Several times during the course of screening actinomycetes broth extracts in various high through-put bioassays, the bioactivity of the extracts (eg receptor binding or enzyme inhibition) was associated with the CPC fractions eluting at 151–171 min and/or 57–78 min. We directed our efforts at this early stage to the development of a quick identification protocol for these two metabolites.

The CPC of the extract from culture 9999AE resolved an active peak that eluted from 151–171 min with a UV absorption profile at λ_{max} 250 nm (Figure 1). An aliquot of the pooled active CPC fractions was injected onto the LC-MS to obtain characteristic mass data on the peak. Analysis of the ion-spray MS spectra indicated that the molecular weight of the HPLC peak eluting at 22 min was m/z 1042 ($M^+ + H + NH_3$, Figure 2). The CPC, HPLC and MS data matched those of an authentic sample of elaiophylin, which was further supported by analysis of the ^{1}H-NMR and ^{13}C-NMR spectral data [3] of the CPC fractions. The pooled CPC fractions were purified further by semi-preparative HPLC. The purified elaiophylin fraction was evaluated again in the assay, confirming that elaiophylin was indeed responsible for the bioactivity of this extract.

In the case of extract from cultures of *S. violaceusniger* strain 10000AA, application of the CPC method coupled with LC-MS revealed production of geldanamycin. The UV absorbance profile (λ_{max} 260 and 305 nm) of the active component eluting at 57–78 min, was consistent with a quinone moiety (Figure 3). Upon LC-MS analysis, the HPLC peak eluting at 20 min gave an ($M^+ + H + NH_3$) ion at m/z 578, which matched the molecular weight of geldanamycin [4] (Figure 4). The CPC fractions afforded substantially purified material and the bioassay results showed that geldanamycin was responsible, at least in part, for the activity of the extract. The structure (Figure 5) was further confirmed by ^{1}H-NMR and ^{13}C-NMR data [4]. Note that *S. violaceusniger* strain 10000AA also produced elaiophylin. The presence of elaiophylin was recognized in the CPC chromatogram prepared from an extract of *S. violaceusniger* strain 10000AA by its characteristic retention time and UV absorption profile (Figure 3).

Discussion

Considerable effort is required to eludicate the structure of the active compound when a crude extract is found to be active in a screening assay. Hence it is important that screening hits which are previously discovered structures be identified quickly. For this purpose, we have developed an efficient dereplication scheme designed to identify compounds or metabolites rapidly that are known to be active in a particular assay. We have replaced traditional Kupchan solvent–solvent partitions and initial HPLC fractionation techniques by a more efficient CPC (Centrifugal Partition Chromatograph) coupled to a photodiode array detector followed by LC-MS. These combined methodologies allow us to identify a known metabolite quickly in a crude extract. For example, after obtaining the absorbance maxima and molecular weight from LC-MS spectral data for the active compound and/or components present in an active fraction, this information is used to generate a list of potential structural matches from a natural products data base (eg Berdy Bioactive Natural Products Data Base, Medimpex Hungarian Trading Co for Pharmaceutical Products, Budapest, Hungary). The application of CPC coupled to photodiode array detection and LC-MS techniques is among the most efficient methods for this purpose. This methodology allowed us to identify quickly elaiophylin and geldanamycin in several extracts derived from *S. violaceusniger* and it enabled us to prioritize CPC fractions obtained from extracts at an earlier stage.

In conclusion, the use of CPC coupled with photodiode array detection and LC-MS proved to be a valuable technique for rapid identification of elaiophylin and geldanamycin in broth from actinomycetes cultures. This approach should find widespread application where dereplication schemes are needed for known compounds that possess a characteristic chromophore.

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