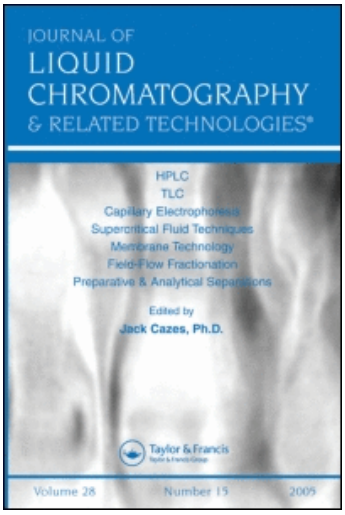

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SCREENING NATURAL PRODUCTS: BIOASSAY-DIRECTED ISOLATION OF ACTIVE COMPONENTS BY DUAL-MODE CCC

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EXTRACTIONS AND PURIFICATIONS

SCREENING NATURAL PRODUCTS: BIOASSAY-DIRECTED ISOLATION OF ACTIVE COMPONENTS BY DUAL-MODE CCC

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ABSTRACT

In the search of new chemical entities, we have developed an integrated biological-physiochemical system for the identification of active compounds in fermentation broths. The system relies upon preliminary fractionation of the microbial crude extract by dual mode high-speed countercurrent (HSCCC, PC Inc.) couple with photodiode array (PDA) detection and bioassay.

INTRODUCTION

Natural products offer a source of molecular diversity in sample sources for screening that can be an essential and productive component in today's modern drug discovery programs. The key to any study of material from natural products is the selection of proper separation methodology for the isolation of compounds. Molecules present in natural product mixtures covers the whole spectrum of polarity. These can very in hydro- and lipophilicity, charge, solubility, stability,

and size. These properties demand mild and versatile separation techniques, especially during initial fraction of the crude extract; because, at this stage, the chemist knows nothing about the properties of the compound of interest. For this reason, countercurrent chromatography (CCC) should be a first choice for initial fractionation of crude extracts, since it offers the chemist a number of very desirable features. A complete chromatography can be achieved in a mater of a few hours. Mild operating conditions prevent decomposition and denaturation of valuable components, and the absent of solid supports rule out catalytic surface effects. An attractive feature of this machine is its ability to perform normal and reversed phase chromatography (dual mode) during the same run. Both polar and non-polar compounds are certain to be fractionated in a single chromatographic run.

Because of the reasons outlined above, at NCE (New Chemical Entities Inc.), in the lead generation program from natural products chemistry, the first fractionation step always relies on fractionation of crude extracts by the dual mode high speed countercurrent chromatography (HSCCC). We have developed a systematic process for isolation and identification of biologically active components from natural products. The system relies upon preliminary fractionation of the microbial crude extract by dual-mode HSCCC coupled with photodiode array (PDA) detection and bioassay. The previously stored and or reported UV-VIS spectra of the biologically active peaks or known compounds, are used for identification. Confirmation of compound identity is by liquid chromatography- mass spectrometry (LC-MS). The approach is an integrated part of our natural products chemistry program, and is being used in both bioassay-guided isolation of bioactive compounds from the crude extract[1] and in the new developed prefractionated library.

EXPERIMENTAL

Countercurrent chromatography (CCC) was performed on a high-speed countercurrent chromatograph (P.C. Inc., Potomac, MD, USA), equipped with a 1.68 mm ID coil (volume 320 mL). Also included were a 10 mL sample loop (with six-way valve) and a valve to permit rapid switching of the solvent between “head” or “tail” ends of the coil. This instrument was connected with a Waters photodiode array spectrophotometric detector model 996 (Millipore Corporation, Marlborough, MA, U.S.A.) and a NEC power mate 486/33I computer. Mass spectra were recorded on a PE Sciex API 150 MCA mass spectrometer interfaced with a Sciex Ion-Spray probe (PE Sciex Perkin Elmer, Toronto, Ontario, Canada). Liquid chromatography was performed with a Hewlett Packard 1100 series binary pump on a C8 (Monitor, 5 μm, 30 × 4.6 mm) column

and a Waters photodiode array (PDA) spectrophotometric detector, model 996 (Millipore Corporation, Marlborough, MA, U.S.A.). The solvent was evaporated from the 96- wheel microtiter plate using a Savant Speedvac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.). NMR spectra were recorded on a Brucker Avance DRX 500 spectrometer (Brucker Instruments Inc., Fremont, CA, U.S.A.).

RESULTS AND DISCUSSION

The examples given below, illustrate the essential role high speed countercurrent chromatography played in accomplishing: i) bioassay-directed initial fractionation, ii) de-replication, iii) identification, iv) isolation of novel antitumoral compounds and v) scale-up isolation of target compound.

Bioassay-Directed Initial Fractionation of Crude Extract

We routinely employ dual-mode HSCCC coupled with PDA detection as a primary tool for the initial assay-directed fractionation of active extracts. A standard HSCCC condition was selected to fractionate all the extracts. A hexane-EtOAc-MeOH-H_2O (1:3:3:3) biphasic solvent system was employed at a flow rate of 3 mL/min. Four hundred milligrams of crude extract were loaded onto the column. The upper phase was employed as the mobile phase and the lower phase as the stationary phase for the first 120 min. of the run. For the remaining 120 min. of operation, the mobile phase and flow direction were reversed. A Waters photodiode array detector was used to analyze all samples.

The fractions were collected in individual tubes using a commercially available fraction collector. Aliquots from each tube were transferred to a 96-well microtiter plate with the help of a robotic system, and tubes with the remaining effluent were stored at –20°C. The solvent was evaporated from the 96-well microtiter plate using a centrifugal vacuum evaporator, and the material in the individual wells was assayed. This procedure provides discrete localization of short segments of the HSCCC effluent stream, allowing an accurate correlation of biological activity with retention time. The PDA detector permits correlation of biological activity, not only with the UV peaks in the chromatogram, but also with the 200-600 nm UV-visible spectrum of the component.

An advantage of this approach is that samples are compatible with all current screening formats. In addition, we can go from an initial detection of activity to a fully characterized component in very rapid fashion because we are fractionating on a preparative scale.

Strategy for Dereplication

The chance of finding novel bioactive compounds from natural products has become more difficult because several thousand metabolites have already been reported in the literature. The rapid characterization of these compounds has become a strategically important area for the natural products chemist involved in a screening program.

Use of an integrated system incorporating dual-mode HSCCC separation, PDA detector, and LC-MS proved to be a valuable tool in the rapid identification of known compounds from microbial extracts.[2] This collection of analytical data has enabled us to make exploratory use of advanced data analysis methods to enhance the identification process. For example, from the UV absorbance maxima and molecular weight for the active compound(s) present in a fraction, a list of potential structural matches from a natural products database (e.g., Berdy Bioactive Natural Products Data Base, Dictionary of Natural Products by Chapman & Hall, etc) can be generated. Subsequently, the identity of metabolite(s) is ascertained by acquiring a proton nuclear magnetic resonance (^{1}H-NMR) spectrum. HSCCC fractions containing known metabolites as determined by de-replication, are eliminated from further study and only fractions with novel structures and high potency are designated "hits" (Fig. 1). It is important to note, that the isolated quantities are sufficient for structure elucidation, as well as, concluding their *in vitro* biological evaluation after a single chromatographic step. As an example, Fig. 2 showed the effectiveness of the procedure in the identification of known compounds within crude extract.

Identification and Isolation of Novel Antitumor Compounds

Fractions passing this critical hurdle are prioritized for structure elucidation and comprehensive biological testing. If necessary, the prioritized fraction is purified further by semi-preparative HPLC. The purified compound is subjected to structure elucidation and detail efficacy and toxicity studies.

For example, in the course of screening for new anti-tumoral compounds from microbial sources, a fungal extract was discovered that exhibited potent activity against the HT29 tumor cell line. The crude extract was fractionated by our standard HSCCC protocol as described above. The activity was concentrated into three chromatographic peaks eluting at 60-69, 108-120, and 219-231 min (Fig. 3). After searching Natural Products Databases with the available UV and molecular weight data, no matches were found. Subsequently, three novel cytochalasans: phomacin A, B, and C were identified from these HSCCC fractions.[3]

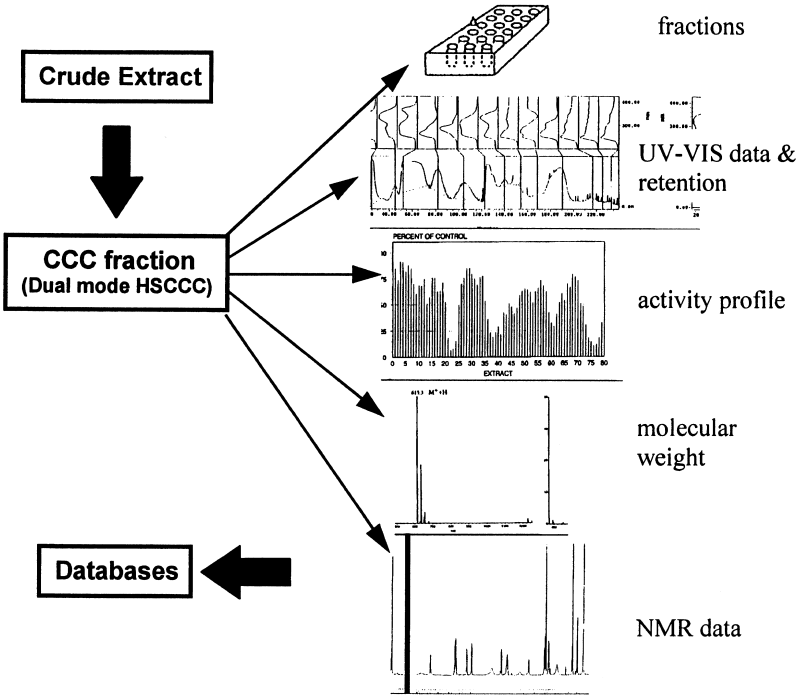

Figure 1. Scheme of the integrated dereplication protocol.

Strategy for Scale-Up Isolation of Target Compound by HSCCC

Most of the time, active compounds obtained from HSCCC fractions are considerable pure, and isolated in reasonable quantities after a single chromatographic run. However, in some instances the active fractions afforded only a few milligrams of a sample that may be enough to perform structure elucidation study, but for comprehensive biological studies larger quantities are necessary. In this situation, more material can be isolated by adapting a strategy based on the information gathered during initial fractionation of the target molecule. For example, an assay-directed HSCCC fractionation afforded only a few milligrams of an active material (SCH 45752, Fig. 4). A large quantity was requested for a detailed biological study. For this purpose, a large scale-up fermentation (40 L) was extracted, which afforded 10.691g of crude extract. From the initial fractionation step, we know that the activity was concentrated into the fractions 17-20, which suggested that compounds having the most favorable distribution towards

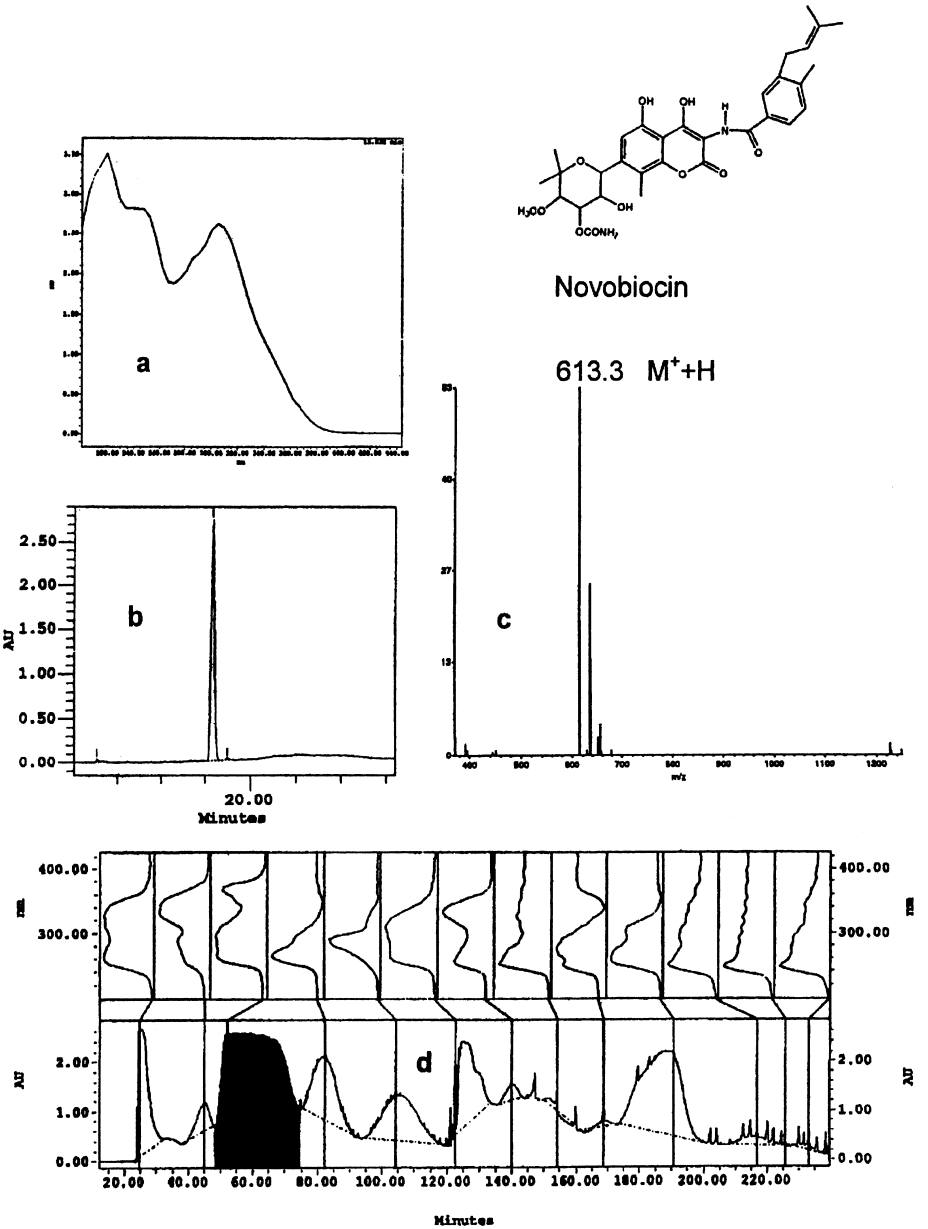

Figure 2. (a) UV spectrum of the HPLC peak eluting at 16 minutes; (b) analytical HPLC chromatogram of the pooled active CCC fraction eluting in tubes 51 to 75; (c) ion-spray mass spectrum (positive ion) of the HPLC peak eluting at 16 minutes in b; (d) HSCCC separation of the EtOAc extract of actinomycete strain #21,000 AE (shaded area corresponds to active fractions).

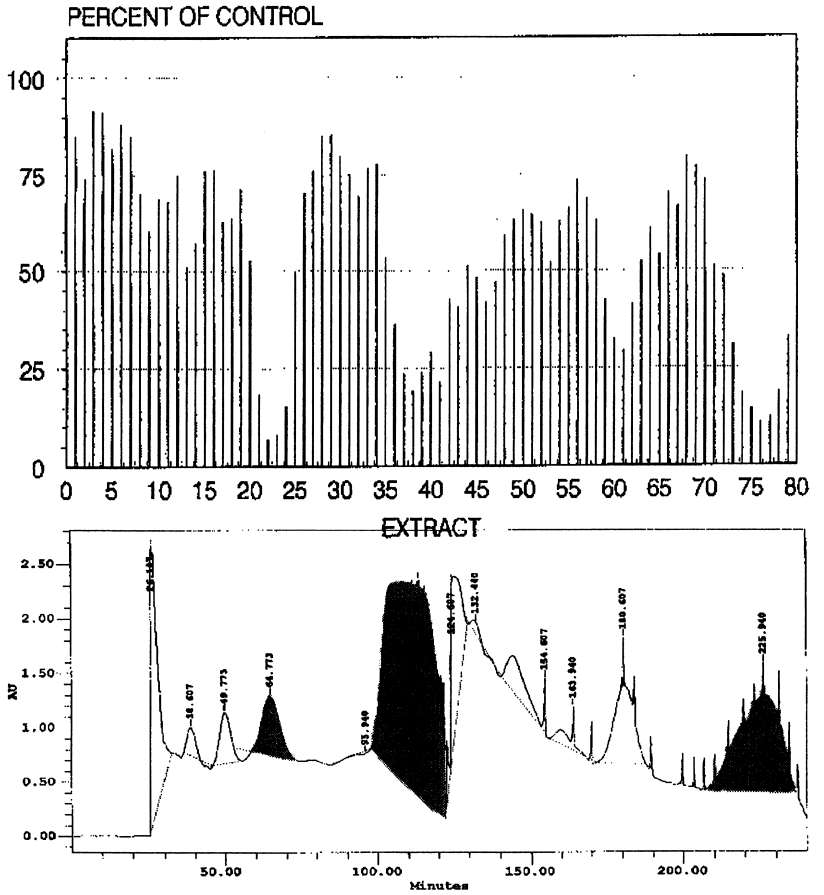

Figure 3. Activity profile and HSCCC chromatogram of an EtOAc extract of a fungus strain 14078FH. (Shaded area represents the elution of active fractions. Please note that the collection time for each fraction was 3 minutes.)

the upper phase of the solvent system was used during HSCCC chromatography. Therefore, the entire extract was partitioned first between upper and lower phase of the hexane-EtOAc-MeOH-H_2O (1:3:3:3). As expected, the upper phase containing over 99% of the active component on evaporation gave 2.980 g of a residue. At this stage the amount became easy to handle and was fractionated by five HSCCC chromatographic runs, which yielded 457 mg.

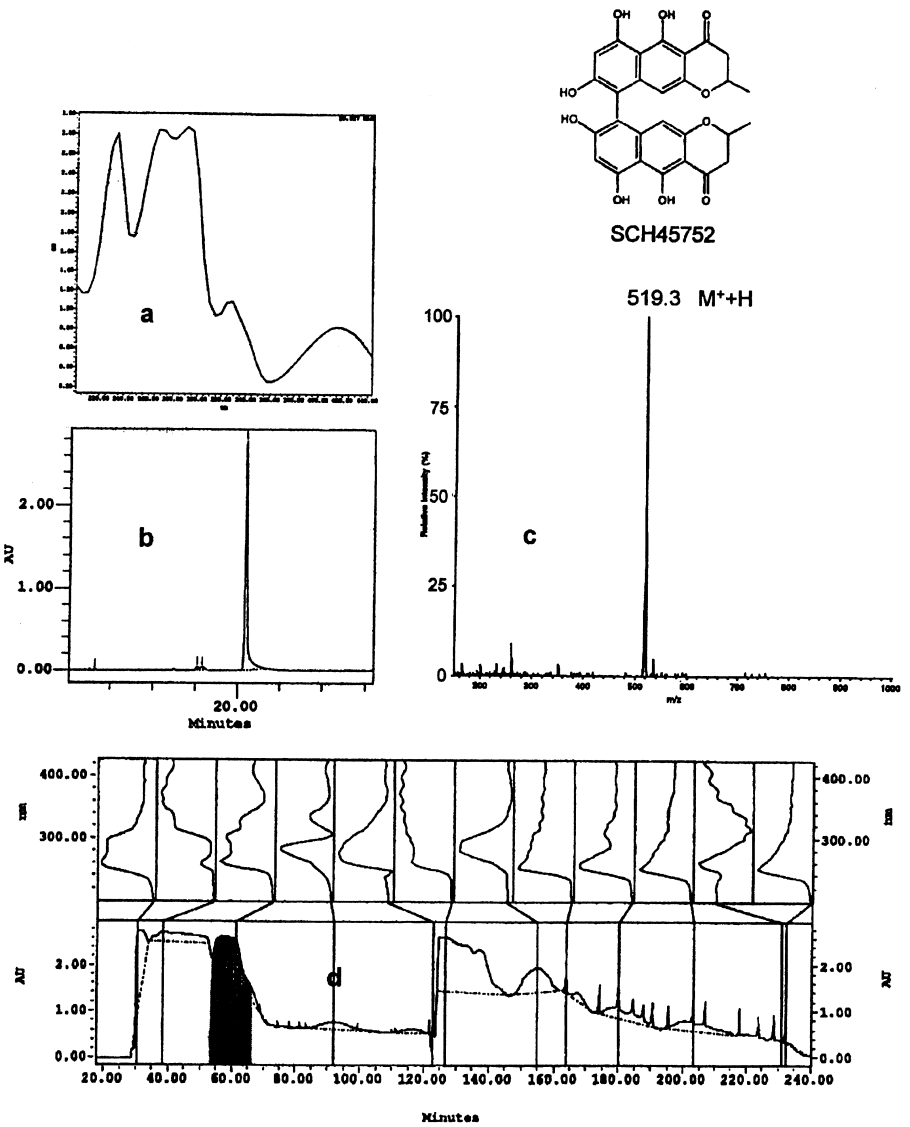

Figure 4. (a) UV spectrum of the HPLC peak eluting at 21 minutes; (b) analytical HPLC chromatogram of the pooled active CCC fraction eluting between 54 and 63 minutes; (c) ion-spray mass spectrum (positive ion) of the HPLC peak eluting at 21 minutes in b; (d) HSCCC separation of the EtOAc extract of fungal strain #21,000 FE. (Shaded area corresponds to active fractions.)

CONCLUSION

Because the nature of the active compound present in natural product extracts is unknown, the dual mode HSCCC is well suited for performing assay-directed fractionations, since it does not involve solid phase adsorbents and, thus, is inherently less destructive. Its versatility is furthermore underlined by the speed of operation, straightforward scaling up, and applicability toward the entire range of polarity of natural products. An additional merit of this technique is that the isolated quantities are sufficient for structure determination, as well as, concluding their *in vitro* biological evaluation after a single chromatographic step. Use of an integrated system incorporating HSCCC separation, photodiode array detector, and LC-MS, rapidly provided profiles and structural information extremely useful for metabolite identification, and therefore, should find widespread application.

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