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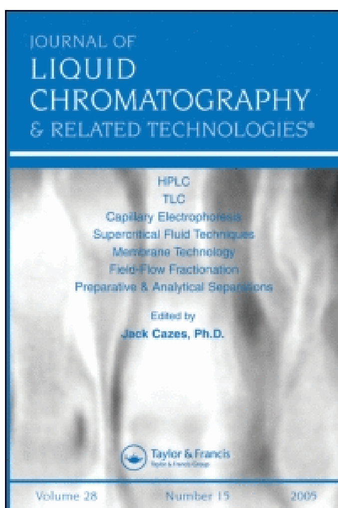

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Use of Coil Planet Centrifuge in the Isolation of Antibiotics

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USE OF COIL PLANET CENTRIFUGE IN THE ISOLATION OF ANTIBIOTICS

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ABSTRACT

In the course of isolating antibiotics from actinomycete fermentations, we have found the Ito Multi-Layer Coil Planet Centrifuge (CPC) can effect separations of such quality on crude fermentation extracts, that frequently, meaningful spectral data may be obtained on the separated bioactive component from a single counter-current chromatography. This paper outlines the use of CPC counter-current distribution in the isolation-separation of siderochelin A, efrotomycin, pentalenolactone, Bu 2313 B, and the closely related congeners, tirandamycins A and B. The discussion includes general methods for solvent system selection and sample preparation. Spectral data on the isolated compounds are presented as evidence for the quality of separations effected. The variety of structural types presented demonstrates the versatility of the coil planet centrifuge as a tool in separation science.

INTRODUCTION

Bioactive natural products of microbial fermentations are often difficult to isolate from crude preparations. From monoclonal, but otherwise unimproved wild strains, a desired bioactive metabolite, in most cases, is produced as less than 0.01% of the

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non-aqueous fermentation components. These components include a wide range of secondary metabolites, as well as unmetabolized media ingredients such as yeast or beef extracts, sugars, starches, and salts. With the exception of ion-exchange or affinity chromatography, most crude preparative methods involve relatively large transfer of some of these unwanted materials to the activity concentrates. An overwhelming number of chemically diverse impurities present in large quantities can mask the chromatographic and other characteristic properties of the desired bioactive metabolite.

Prior to current technology, the initial stages of antibiotic isolation often involved elaborate extraction schemes, followed by numerous liquid-solid and/or liquid-liquid chromatography (LSC, LLC) methods. Antibiotics are usually biosynthesized as mixtures of closely related congeners, the separation of which requires high resolution chromatographic methods. Moreover, as many of these compounds are labile molecules, complex isolation and purification schemes often result in low yields. These facts encourage the tendency to "overload" traditional LSC or LLC systems.

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We have found in the course of isolating antibiotics from actinomycete fermentations, that the Coil Planet Centrifuge (CPC) counter-current system provides a valuable chromatographic tool. Only the minimal requirements of solvent stability and moderate thermal stability are made of the bioactive metabolite, as most separations last no more than eight hours. In addition, the selectivity of this partition system is not easily compromised by a high impurity-to-metabolite ratio. These advantages are well suited to the isolation of antibiotics from microbial fermentations.

MATERIALS AND METHODS

Sample Preparation

The vast majority of known antibiotics which we have isolated have been extracted from the microbial fermentation beers or the

TABLE 1

Sample Preparation Solvent Systems for use with Sephadex® LH-20

Solvent System	Ratios
MeOH 100% (Optional 0.01 N in HCl Content)	
MeOH - CH_2Cl_2	3:2, 2:1, 1:1
MeOH - $CHCl_3$	3:2, 2:1
MeOH - H_2O	3:1, 1:1
MeOH - EtOAc	3:1, 1:1, 1:3
$CHCl_3$ - Heptane - EtOH	10:10:1
$CHCl_3$ - Heptane	1:1

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mycelia using various solvents or adsorbent resins. At this stage the crude extracts normally contain various oils, fatty acids, and other extraneous materials from the fermentation (such as anti-foaming agents) which may adversely affect partition systems. We have found it advantageous at times to chromatograph our crude fermentation extracts on a packing such as lipophilic Sephadex® LH-20 before selecting a CPC solvent system. Depending on the nature of the concentrate we usually elute with one of the solvent systems listed in Table 1. In some instances, such as when a very clean methylene chloride extract of the fermentation is made, this step is omitted.

Selection of a Solvent System

The selection of a CPC solvent system is based primarily on the partition ratio of antibiotic activity in a given test system. These systems (usually less than a total of 5 ml) are made up with one solvent containing a sample aliquot of the crude antibiotic mixture. The systems are shaken, gently at first, and phase separation should occur within 30 seconds (1). (A second vigorous shaking, such as one on a vortex mixer, may be employed to rate each system in terms of its tendency to form emulsions, as well as settling time). Serial dilutions (i.e., 1, 1/2, 1/4, 1/8, etc.)

TABLE 2

Partition Bioassay Example

Dilution	1	1/2	1/4	1/8
% Concentration	100	50	25	12.5
Zone of Inhibition (mm) Upper	20	18	16	14
Zone of Inhibition (mm) Lower	18	16	14	12

Concentration Ratio $\frac{U}{L} = \frac{100}{50} = \frac{2}{1}$

A Serial Dilution Bioassay of Upper and Lower Phases of System U:L in Which an Antibiotic Has Been Partitioned

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are then made from the upper and lower phases and assayed by the agar disc diffusion method with a bacterial strain sensitive to the antibiotic. The antibiotic activity slope, that is, the relation between the change in the diameter of the zone-of-growth-inhibition and the antibiotic concentration, is the same for both upper and lower phases. It is, therefore, possible to measure antibiotic concentration partition ratios indirectly with reasonable accuracy by using an empirical extrapolation. An example of this is given in Table 2.

The range of ratios with which we normally work is from 1:1 to 3:1 in favor of the stationary phase. With a high impurity-to-metabolite ratio and relatively large sample loads (50-500 mg), we find that even a slight advantage to the mobile phase tends to elute the desired metabolite too near the solvent front.

Apparatus Used

All isolations were performed using a flow-through coil planet centrifuge (2). The CPC was equipped with a #14 Ito multilayer coil that had an approximate retention volume of 325 ml. Added to the system were the following: 4-way rotary variable

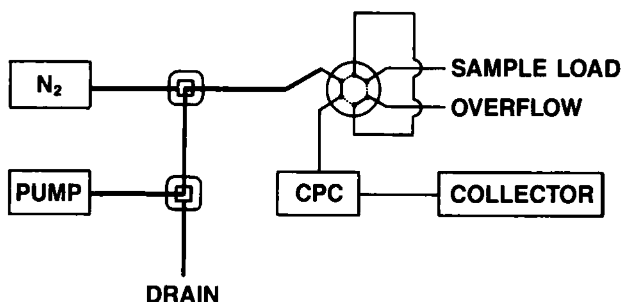

Figure 1. Isolation CPC system employing variable loop injector and 3-way slide values.

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loop injection valve (Rheodyne), two 3-way slider valves (Altex) (see Figure 1), Milton Roy Minipump® (46-460 ml/hr), small bore restrictor tubing at CPC outlet (to produce back pressure), and Gilson FC-220 race track fraction collector.

Solvent systems were made up to total approximately 4 liters and were vigorously shaken and allowed to settle, before use. The CPC was filled with stationary phase, at which point the load sample was injected and pushed onto the column by mobile phase. (The load samples were partitioned between a 5-10 ml combination of mobile and stationary phase, the latter entering the column first). The CPC was connected in the tail-inlet mode and in all cases, except as indicated, the motor was run forward at 800 RPM. Flow rates were 5 ml/min except again as indicated. Fractions eluted were monitored by disc bioassay on bacterial agar plates.

Several milligrams (2-10) of material from the most active fractions were submitted for 300 or 360 MHz ^{1}H NMR. All ^{1}H NMR spectra were produced using either a Nicolet NMC-360 wide bore or Nicolet QE-300 spectrophotometer. The actual spectra are presented as evidence of the purity of samples obtained by this simple isolation procedure. The antibiotics were further characterized by ^{13}C NMR, mass spectrum, IR, and UV to complete identifi-

cation. The separations in each case are presented as percent of antibiotic activity of that in the most active fraction (% a) versus fraction number as per assay on bacterial agar plates. Point S on these elution profiles marks the point at which the stationary phase begins discharge (nitrogen pressure blow-out).

EXPERIMENTAL

I. Siderochelin A (3)

A 20 liters harvest was made of a microbial fermentation, active against *Bacteroides fragilis*. The fermentation was filtered through Dicalite® and the filtrate (pH 7.0) was extracted with a total of 20 liters of methylene chloride. The methylene chloride extract was dried over sodium sulfate and concentrated to 400 mg of an oil-like residue.

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II. Efrotomycin (4)

A 15 liters harvest of a microbial fermentation, active against *Bacteroides fragilis*, was filtered through Dicalite®, and the filtrate was freeze-dried at neutral pH. The beer solids were extracted with a total of 4.5 liters of methanol in three portions. The active methanolic concentrate (17.5 g) was chromatographed on a Sephadex® LH-20 column (5 cm diameter x 80 cm) in 50% (aq) methanol. The concentrated major active band (5.7 g) was flash chromatographed on a Baker C_{18} capped silica column (2.5 cm diameter x 30 cm), eluting with 300 ml of H_2O, and then 300 ml of methanol. The active methanol eluates were concentrated to 670 mg of a solid residue.

III. Pentalenolactone (5)

The pH 4.0 adjusted filtrate of a 20 liters harvest of a microbial fermentation, active against *Bacteroides fragilis*, was extracted with a total of 20 liters of methylene chloride. The active organic extract was dried over sodium sulfate and concentrated to 900 mg of a semi-solid residue.

IV. Bu 2313 B (6)

A 10 liters harvest of a microbial fermentation, active against *Bacteroides fragilis*, was filtered through Dicalite® and the pH 7.0 adjusted filtrate was extracted with a total of 10 liters of methylene chloride. The active organic extract was dried over sodium sulfate and concentrated to 530 mg of an oil-like residue.

V. A 201 E (7)

The pH 7.0 adjusted filtrate of a 10 liters harvest of a microbial fermentation active against *Staphylococcus aureus* was extracted with a total of 10 liters of ethyl acetate. The active organic extract was dried over sodium sulfate and concentrated to 350 mg of an oil-like residue.

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VI. Tirandamycin A and B (8, 9)

A 20 liters harvest of a microbial fermentation, active against *Bacteroides fragilis*, was filtered over Dicalite® and the pH 3.0 adjusted filtrate was extracted with a total of 12 liters of ethyl acetate. The organic extract was dried over sodium sulfate and concentrated. The active ethyl acetate concentrate (1.3 g) was chromatographed on a Sephadex® LH-20 column (5 cm diameter x 90 cm) in methanol. The major active band was concentrated and the methanolic concentate (900 mg) was again chromatographed on another Sephadex® LH-20 column (5 cm diameter x 90 cm) in a system composed of n-heptane-$CHCl_3$-EtOH (10:10:1). The major active band eluted was concentrated to 134 mg of a semi-solid residue.

Effective purification and separations of tirandamycin A and B have also been accomplished employing systems composed of CCl_4-$CHCl_3$-MeOH-H_2O (1:1:1:1) and CCl_4-$CHCl_3$-MeOH-H_2O (5:5:6:4). The lower phase was used as the stationary and the order of elution was reversed from that in the example above.

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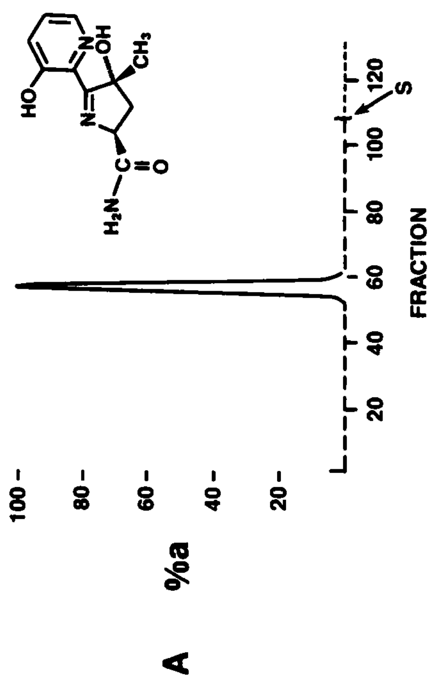

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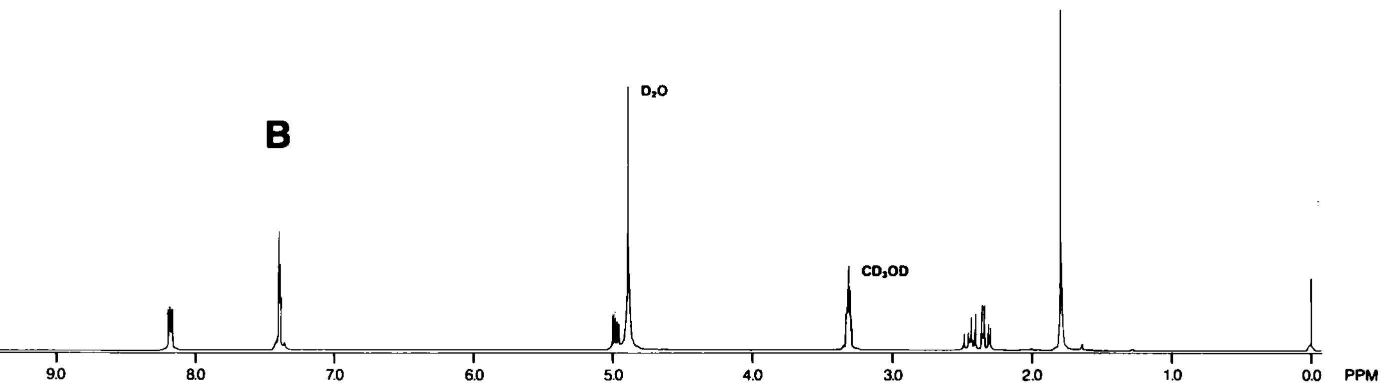

Figure 2. Siderochelin A

A. CPC separation elution profile. Solvent system, $CHCl_3$-MeOH-H_2O (7:13:8); stationary, lower phase; load sample, 400 mg/5 ml; stationary phase retained, 84%; fraction volume 15 ml. %a is the percent of antibiotic activity of that in the most active fraction. Point S is the beginning of the stationary blow-off (N_2).

B. ^1H NMR of material from fractions 57-59.

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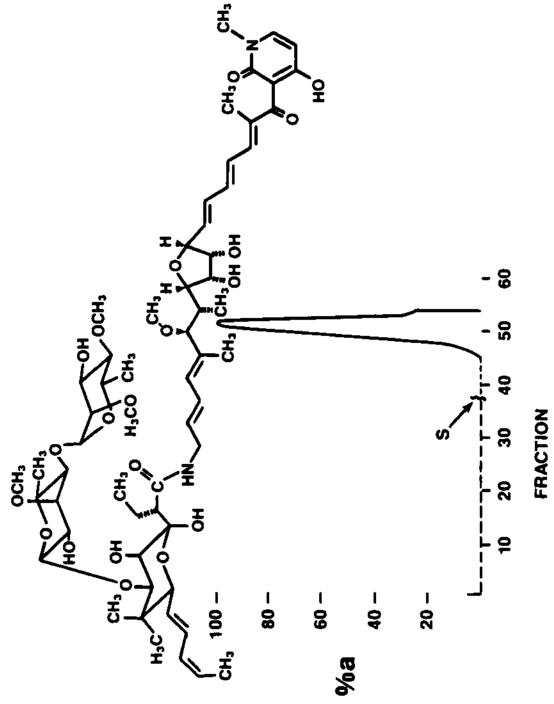

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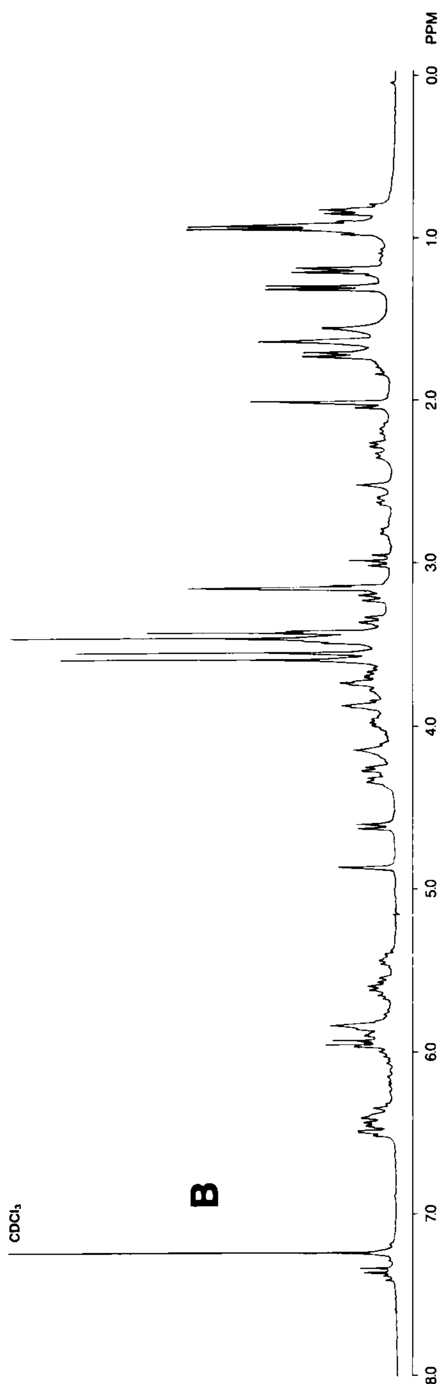

Figure 3. Efrotomycin

A. CPC separation elution profile. Solvent system, CCl_4-$CHCl_3$-MeOH-H_2O (5:5:6:4); stationary, lower phase; load sample, 670 mg/10 ml; stationary phase retained, 83%; fraction volume, 16-18 ml.

B. ^1H NMR of material from fractions 49-52.

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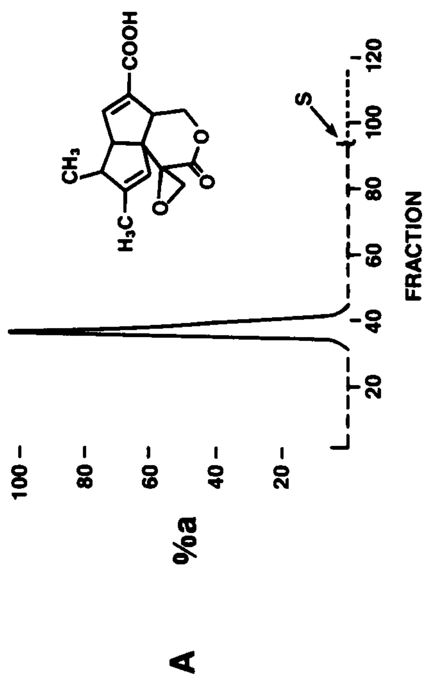

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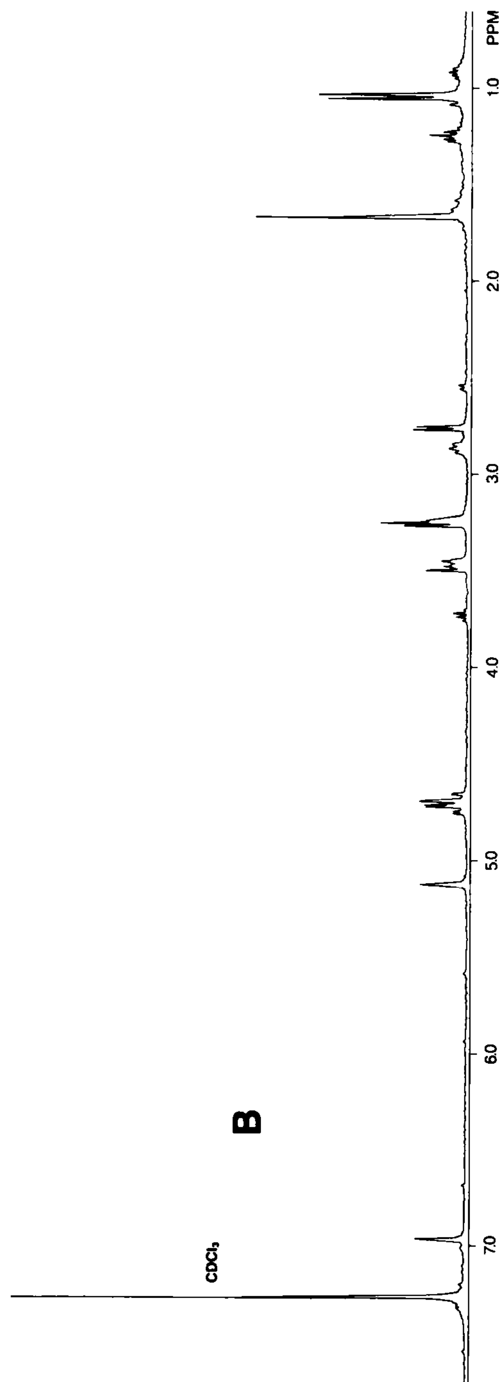

Figure 4. Pentalenolactone

A. CPC separation elution profile. Solvent system, $CHCl_3$-MeOH-H_2O (1:1:1); stationary, lower phase; load sample, 50 mg/5 ml; stationary phase retained, 86%; fraction volume, 15 ml.

B. ^1H NMR of material from fractions 37-40.

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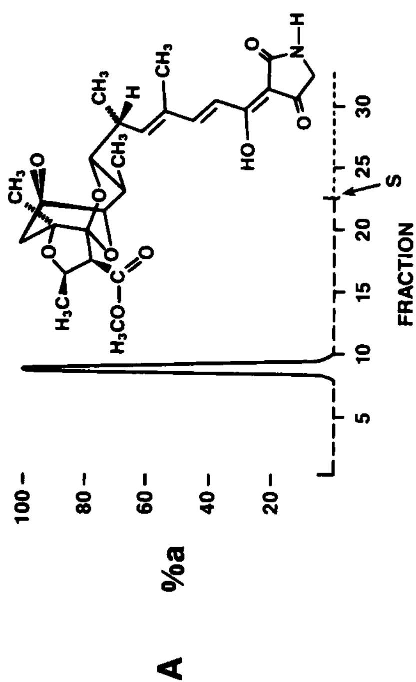

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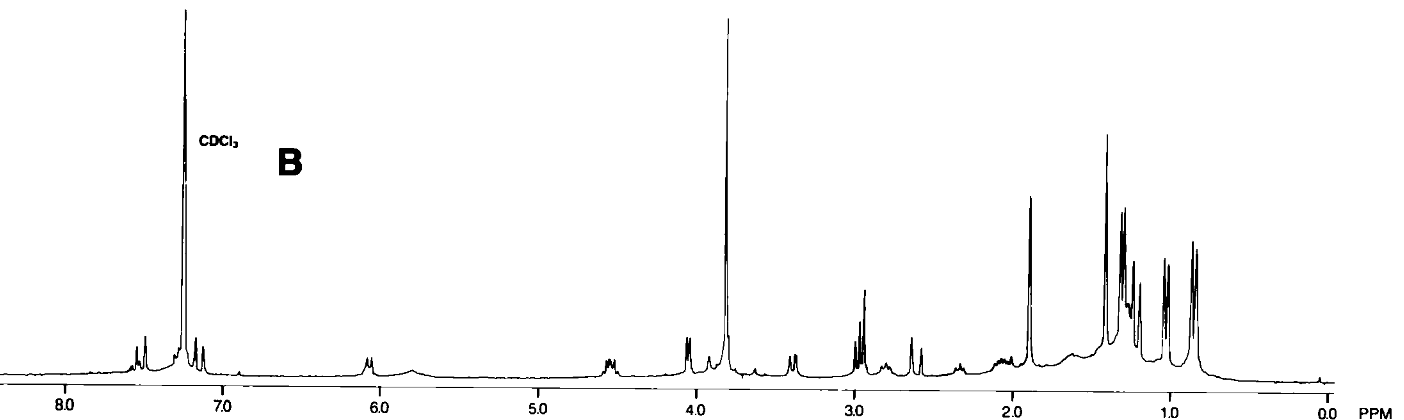

Figure 5. Bu 2313 B

A. CPC separation elution profile. Solvent system, n-hexane-CH_2Cl_2-MeOH-H_2O (5:1:1:1)*; stationary, upper phase; load sampie, 200 mg/5 ml; stationary phase retained, 82%; fraction volume, 15 ml; motor, reverse (800 RPM); flow rate, 2 ml/min.

* A vapor trap should be used between the pump and the column inlet when employing this system.

B. ^1H NMR of material from fractions 8-9.

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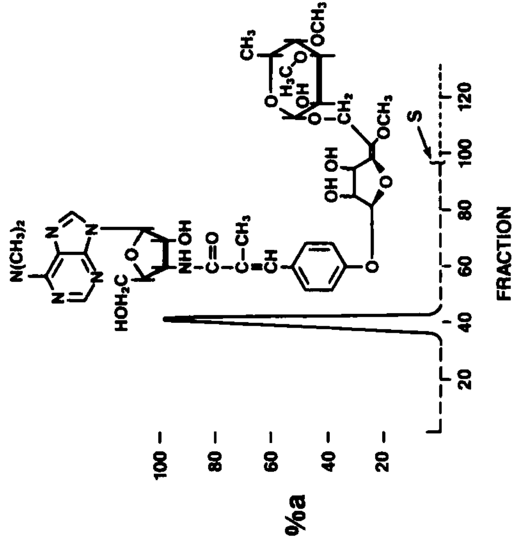

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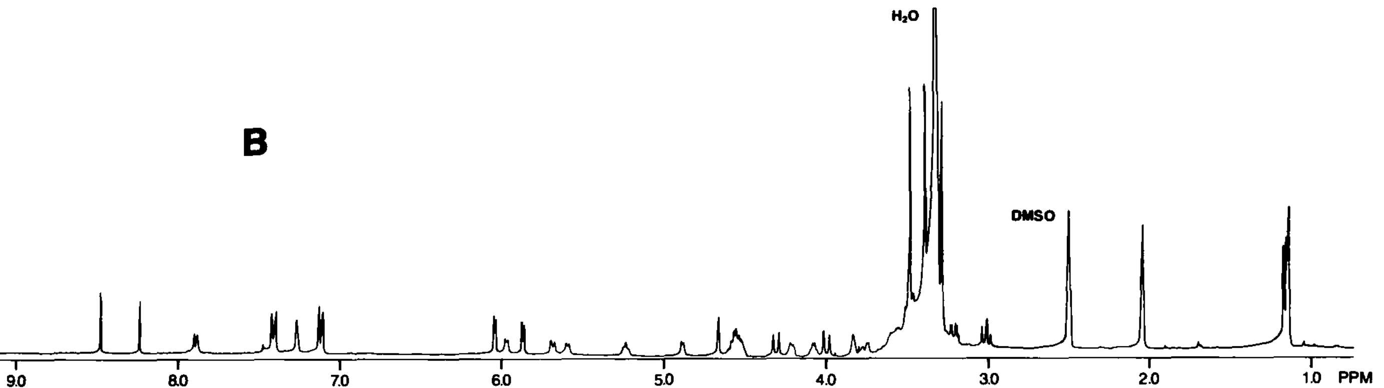

Figure 6. A 201 E

A. CPC separation elution profile. Solvent system, CCl_4-$CHCl_3$-MeOH-H_2O (2:5:5:5); stationary, lower phase; load sample, 350 mg/5 ml; stationary phase retained, 77%; fraction volume, 15 ml.

B. ^1H NMR of material from fractions 38-41.

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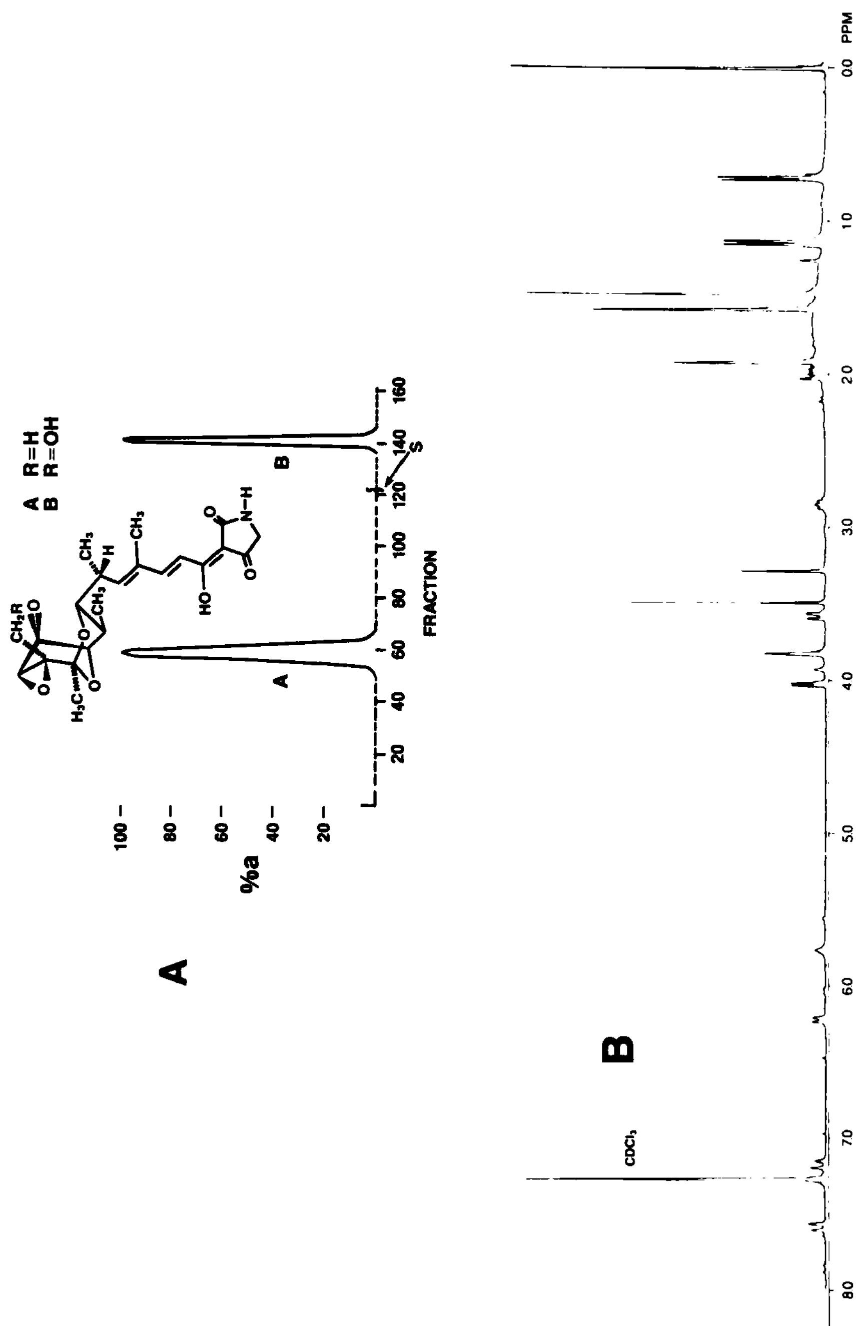

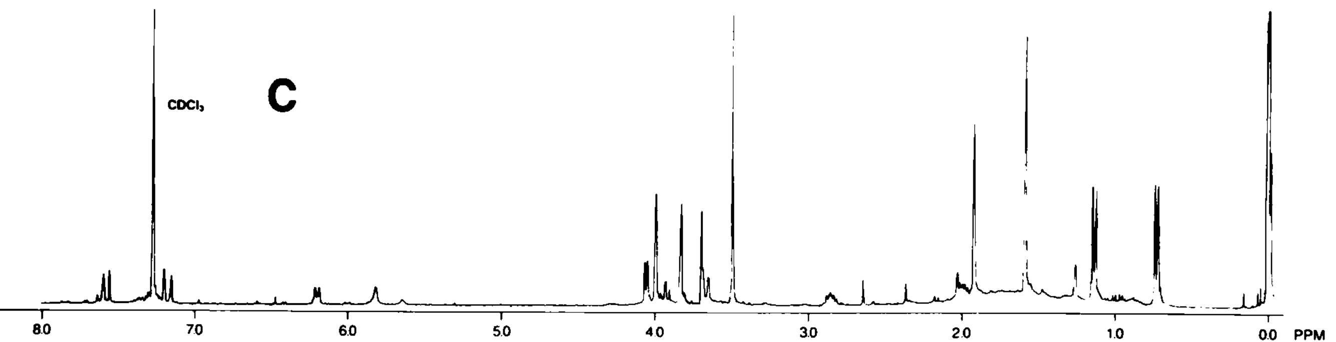

Figure 7. Tirandamycin A and B

A. CPC separation elution profile. Solvent system, n-hexane-EtOAc-MeOH-H_2O (70:30:15:6)*; stationary, lower phase; load sample, 134 mg/5 ml; stationary phase retained 89%; fraction volume, 10 ml.

B. ^1H NMR of material from fractions 48-61.

C. ^1H NMR of material from fractions 140-142.

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TABLE 3

Useful Isolation CPC Systems

MeOH	H_2O	$CHCl_3$	CCl_4	Average Stationary Displaced (ml/325 ml #14 Coil) on Actual Runs
1	1	1	1	50-60 L
7	3	3	7	30-60 U,L
6	4	5	5	30-40 L
3	2	2	3	40-60 L
6	4	5	5	30-60 L
3	2	3	2	30-40 L
4	1	1	4	160-170 U,L
8	2	5	5	150-180 L
5	5	5	2	60-80 L
5	2	1	4	30-40 L
10	4	3	7	30-40 L
5	2	2	3	30-40 L
10	4	5	5	30-40 L
1	1	1	-	50-60 L
13	8	7	-	50-60 L
4	1	-	5	30-40 L
hex	**EtOAc**	**MeOH**	**H_2O**	
3	7	5	5	60-70 L
70	30	15	6	30-40 L
60	40	15	6	30-40 L
MeOH/H_2O/$CHCl_3$/$EtCl_2$/hex 2:1:1:1:1				90-100 L
IPA/hep/benzene/acetone/pH 7.0 aqueous phos. buffer 2:5:10:3:5				90-120 U
n-PrOH/n-BuOH/H_2O 1:2:3				190-210 U
MeOH/H_2O(0.005% EDTA)/CCl_4/hep 8:2:9:1				35-45 U

Abbreviations

hex = n-hexane
hep = n-heptane
IPA = 2-propanol
n-PrOH = 1-propanol
n-BuOH = 1-butanol
$EtCl_2$ = 1,1-Dichloroethane
L = lower phase stationary
U = upper phase stationary

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CONCLUSION

The limited set of examples presented in this paper outlines the value of CPC chromatography as a method in the isolation of antibiotics from crude fermenation extracts. The versatility of the method is enhanced by the variety of possible two-phase solvent systems and the sensitivity of partition coefficients to the particular solvent combination ratios (Table 3).

It must be noted in this particular situation, that the use of certain acids, bases, and high salt concentrations in our solvent systems is often prohibited because of their adverse effects when bioassaying collected fractions. This problem becomes minor as we develop more solvent systems, such as those that contain carefully selected buffers.

The CPC counter-current system provides highly efficient chromatographic separations which are carried out on an analytical or preparative scale in the same relative time frame (10). In addition, we have found the method to be one of the least degradative of any chromatographic technique we employ. Our use of CPC chromatography is expected to cover a wider range of structural types as investigations continue.

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REFERENCES

1. Ito, Y., J. Chromatogr., 301, 405, 1984.

2. P.C. Inc., 11805 Kim Place, Potomac, Maryland 20854.

3. Lin, W., Fisher, S., Wells, Jr., J., Ricca, C., Principe, P., Trejo, W., Bonner, D., Gougoutos, J., Toeplity, B., and Sykes, R., J. Antibiot., 34, 791, 1981.

4. Wax, R., Maiese, W., Weston, R., and Birnbaum, J., J. Antibiot., 29, 670, 1976.

5. Takeguchi, S., Ogawa, Y., and Yonehara, H., Tetrahedron Lett., 2732, 1969.

6. Tsukiura, H., Tomita, K., Hanada, M., Kobaru, S., Tsunakawa, M., Fugisawa, K., and Kawaguchi, H., J. Antibiot., 33, 157, 1980.

7. Kirst, H., Antibiotic A201C, A201D, and A201E, U.S. Patent, 4,205,164, May 27, 1980.

8. Meyer, C., J. Antibiot., 24, 558, 1979.

9. Hagenmaier, H., Jaschke, K., Santo, L., Scheer, M., and Zahner, H., Arch. Microbiol., 109, 65, 1976.

10. Ito, Y., Sandlin, J., and Bowers, W., J. Chromatogr., 244, 247, 1982.

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