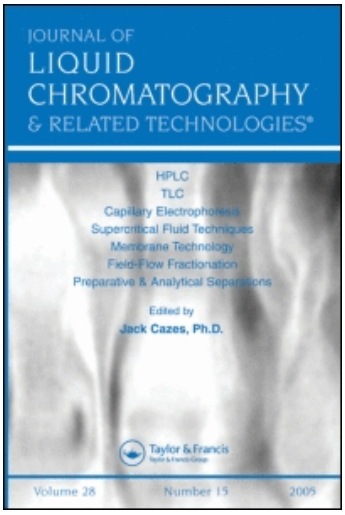

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

APPLICATION OF pH-ZONE-REFINING CCC TO THE ISOLATION OF ANTIFUNGAL FERMENTATION PRODUCTS

Guy H. Harris[a]; Ping Dai[a]
[a] Department of Natural Products Drug Discovery, Merck Research Laboratories, Rahway, NJ, U.S.A.

Online publication date: 30 June 2001

J. LIQ. CHROM. & REL. TECHNOL., 24(11&12), 1775–1790 (2001)

EXTRACTIONS AND PURIFICATIONS

APPLICATION OF pH-ZONE-REFINING CCC TO THE ISOLATION OF ANTIFUNGAL FERMENTATION PRODUCTS

Guy H. Harris* and Ping Dai

Merck Research Laboratories, Department of Natural Products Drug Discovery, R80Y-360, P.O. Box 2000, Rahway, NJ 07065, USA

ABSTRACT

The application of pH-zone-refining countercurrent chromatography (CCC) to the purification and bioassay guided isolation of novel antifungal agents from fungal fermentation extracts is demonstrated with separations of three families of acidic natural products. The separations were carried out using two types of commercially available multilayer coil planet centrifuges. Using this methodology as the final isolation step, three new glycosylated polyketides, arthrinosides A-C, were isolated from fermentation extracts of an *Arthrinium* sp. The three compounds differ in the length and hydroxylation of the polyketide chain and were not easily separable using normal or reverse phase column chromatography. Mycoparasitic acids A and B, new natural products differing only in epoxidation of an endocyclic double bond, were separated directly from crude fungal culture extracts. This CCC method was applied to the purification of a new biotransformation product of sordarin resulting from hydroxylation at C-11. Several

*Corresponding author. E-mail: guy_harris@merck.com

hundred milligrams of this new derivative were easily separated in a single chromatographic run.

INTRODUCTION

The bioactivity-guided isolation of antifungal natural products from fungal and bacterial sources can present challenging problems for the natural products chemist because of the physical nature of the molecules and the milieu in which they are produced.[1,2] They are frequently produced as complex families differing only by minor changes in hydroxylation, oxidation, and methylation patterns. Purification of individual members of a complex can, thus, require careful selection of chromatographic methods. The presence of reactive functional groups can cause solvent and pH stability problems, as well as poor chromatographic behavior, limiting the choice of chromatographic methods. In our own work, we have encountered highly lipophilic compounds possessing an acidic or basic moiety, which are difficult to chromatograph.

High speed countercurrent chromatography (HSCCC) has been used to solve some of these chromatography problems.[3] For example, keto-enol tautomerization of the β-ketoaldehyde and α-diketo moieties of the ceramide synthase inhibitor australifungin, caused poor chromatographic behavior with preparative reverse phase methods. HSCCC provided a solution for final, preparative purification of australifungin.[4] A family of alkyl citrates identified as potent inhibitors of serine palmitoyl transferase, the viridiofungins, were produced as a complex mixture differing in carbon chain length, hydroxylation and oxidation of the aliphatic chain, and aromatic amino acid acylated to the citrate portion.[5] HSCCC provided a normal phase chromatographic step for resolution of the viridiofungin complex prior to final purification, using preparative reverse phase HPLC. As an extension of our application of HSCCC, we describe here the use of the recently described technique of pH-zone-refining CCC,[6-9] to solve similar problems encountered during the isolation of three families of antifungal natural products from fungal fermentations.

The recent discovery that the antifungal natural product sordarin (**1**) is a potent inhibitor of the binding of elongation factor 2 (EF2) to the ribosome[10] has been made to develop derivatives for agricultural and human antifungal use.[11] Biotransformation of **1,** using an actinomycete culture, resulted in the production of 4'-O-demethylsordarin (**2**) and a new analog, 11-hydroxysordarin (**3**).[12,13] pH-Zone-refining CCC was successfully applied to the preparative separation of compounds **1-3** (Figure 1).

A screening program to identify new broad spectrum antifungal agents resulted in the isolation of two new families of natural products. Mycoparasitic acids A and B (**4**, **5**) were obtained from fermentation extracts of the mycopara-

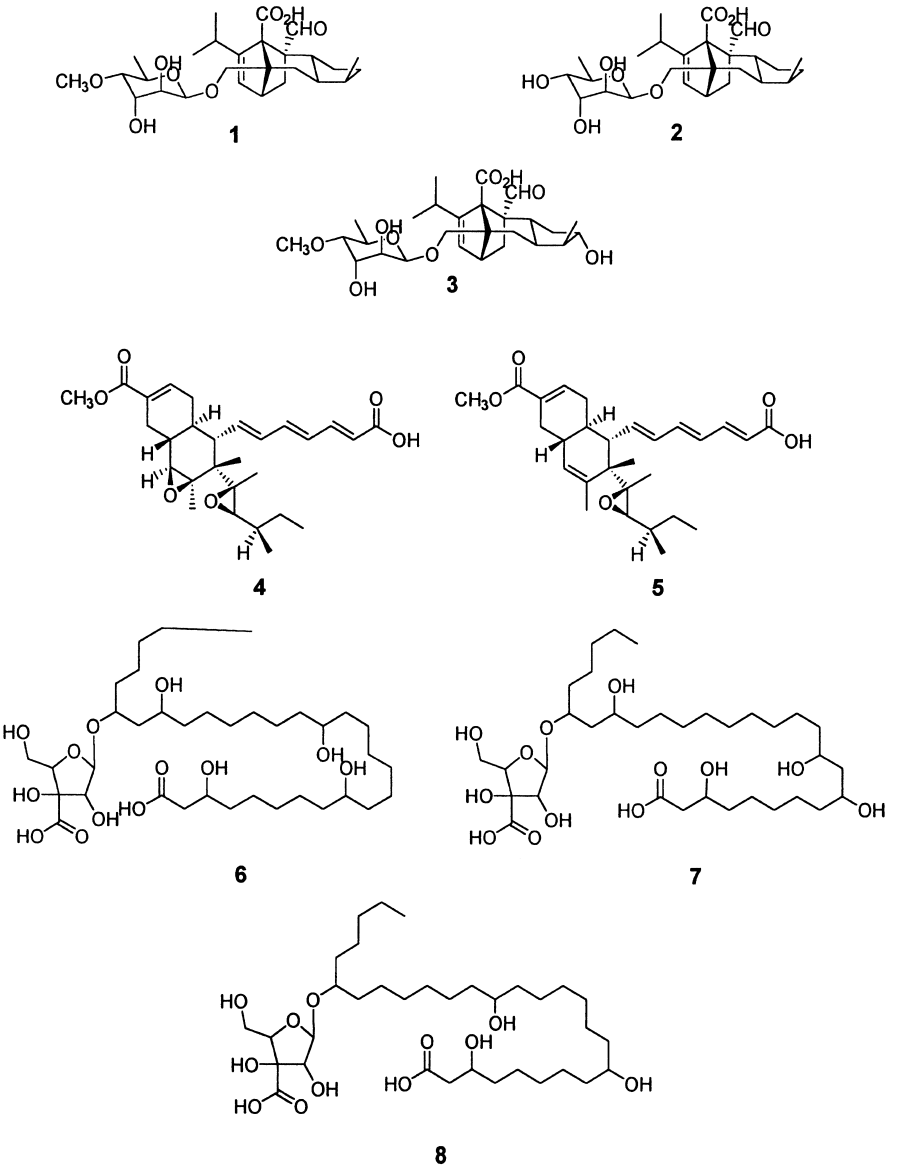

Figure 1. Structures of natural products separated using pH-zone-refining CCC. Sordarin (1), 4'-O-demethylsordarin (2), 11-hydroxysordarin (3); mycoparasitic acids A (4) and B (5); arthrinosides A (6), B (7), and C (8).

sitic fungus *Hypomyces polyporinus*.[14] Arthrinosides A - C (**6** – **8**) are glycosylated, linear polyketides isolated from fermentation extracts of an *Arthrinium sp.*[15] Both compound families possess a free carboxylic acid(s), which was exploited for the pH-zone-refining CCC separation.

EXPERIMENTAL

General Methods and Equipment

Separations were carried out on one of the following two instruments. A Multi Layer Coil Planet Centrifuge (MLCPC) obtained from P.C., Inc. (Potomac, Md., USA), containing a single coil of #14 teflon tubing (1.68 mm I.D.) with a volume of 300 mL, or a Tripple® coil consisting of three separate coils of #14 teflon tubing (1.68 mm I.D.) with volumes of 14.6 mL, 76 mL, or 223 mL. The β values ranged from 0.57 to 0.85 for both coils. A CCC-1000 was obtained from Pharma-Tech Research Corporation, Inc. (Baltimore, Md., USA) containing 3 - 100 mL, 1.68 mm I.D. teflon tubing coils. The β values ranged from 0.45 to 0.75. Solvent pumps were either Waters 510 or Knauer 64, and a flow through pH monitor was obtained from Pharmacia Inc. (Upsala, Sweden). Solvent systems used for CCC separations were equilibrated and the phases separated prior to addition of the retainer acid or eluant base. Samples were injected directly onto the column using the "Ito technique" or using a Rheodyne 7125 sample injection valve. The amount of stationary phase displaced from the coil at the beginning of each separation was measured and used to calculate the stationary phase fraction (S_f), as described by Conway.

Preparation of Crude Sordarins Fraction

A methanol extract (50 L) of a fermentation of culture MA7235,[13] which had been fed 0.5 g/L sordarin, was diluted with an equal volume of H_2O and passed through a bed of brominated polystyrenedivinylbenzene resin (Mitsubishi SP207) in upflow mode at 400 mL/min. The resin was then washed with 25% methanol followed by elution of the crude sordarin mixture with 75% methanol in downflow mode. The crude sordarin fraction was concentrated *in vacuo* to remove most of the methanol, adjusted to 0.1 N NaOH, and twice extracted with CH_2Cl_2. The aqueous layer was adjusted to pH 2.5 with H_2SO_4 and extracted twice with CH_2Cl_2. The combined CH_2Cl_2 layers were washed with H_2O, brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to yield a crude sordarins fraction as a yellow oil (12.2 g).

pH Zone-Refining Separation of Crude Sordarins Mixture

The 76 mL Tripple® coil in a P.C. Inc. MLCCC was charged with a stationary phase of CH_2Cl_2 containing 0.04% trifluoroacetic acid (TFA). The crude sordarin fraction, 0.2 g or 1.5 g, was dissolved in stationary phase and loaded onto the tail of the coil. The coil was eluted tail to head with 10 mM NH_4OH at 2.0 mL/min., 800 rpm, collecting 1.5 min. fractions. Separation of 5 g of the crude sordarin fraction was similarly carried out on the 227 mL Tripple® coil with a mobile phase flow rate of 6.0 mL/min. Fractions from each separation were analyzed for sordarin, 4'-O-demethylsordarin, and 11-hydroxysordarin using analytical RP HPLC.

Preparation of Crude Mycoparasitic Acids A and B

A solid fermentation (2 L liquid equivalent) of fungal culture GB5217 was extracted with methyl ethyl ketone (MEK), filtered, and the solvent removed *in vacuo* to yield a crude culture extract (5.8 g).

pH Zone-Refining CCC Separation of Crude Mycoparasitic Acids A and B

A solvent system of methyl isobutyl ketone (MiBK) : acetonitrile : H_2O (4:1:5) was equilibrated and the phases separated. TFA (10 mM final concentration), was added to upper (organic) phase used to fill a 300 mL PharmaTech CCC-1000 coil. The crude mycoparasitic acid mixture, 1.6 g, was dissolved in stationary phase, 15 mL, and loaded onto the head of the coil. The column was then eluted head to tail at 2.5 mL/min, 1015 rpm, with the lower phase, to which NH_4OH (10 mM final concentration), was added and 4 min. (10 mL) fractions collected. Approximately 170 mL of stationary phase was lost prior to column equilibration, S_f 0.43. Elution was continued until the pH of the eluate was equal to that of the mobile phase. Fractions were assayed for mycoparasitic acid A and B using analytical RP HPLC. Fractions 28–42 contained mycoparasitic acid A, 110 mg, and fractions 45–70 contained mycoparasitic acid B, 254 mg.

Preparation of Crude Arthrinoside Complex

A liquid fermentation of culture MF6512 (2 L) was extracted with an equal volume of methanol and the extract clarified by filtration through celite. The

methanol was removed *in vacuo*, the remaining aqueous solution acidified to pH 3.15 with 1 N HCl, and then extracted with ethyl acetate (11.5 g). This extract was further purified on diol silica gel using a mobile phase of CH_2Cl_2:MeOH (9:1) to yield a crude complex of arthrinosides (530 mg).

pH Zone-Refining CCC Separation of Diol Purified Crude Arthrinoside Complex

A solvent system of methyl isobutyl ketone (MiBK):H_2O (1:1) was equilibrated and the phases separated. Trifluoroacetic acid (TFA), 0.1% final concentration, was added to upper (organic) phase used to fill a 300 mL PharmaTech CCC-1000 coil. The diol silica gel purified arthrinoside complex, 480 mg, was dissolved in stationary phase, 15 mL, and loaded onto the head of the coil. The column was then eluted head to tail at 2.5 mL/min, 1050 rpm, with the lower phase, to which NH_4OH (10 mM final concentration) was added and 5 min. (10 mL) fractions collected. Elution was continued until the pH of the eluate was equal to that of the mobile phase. Fractions were assayed for antifungal activity using an agar diffusion assay versus *Candida albicans*. Arthrinosides were recovered from the fractions by adjustment of the pH to 3 with H_3PO_4 and extraction into ethyl acetate. Fractions 30-34 contained arthrinoside B (26 mg), 37-42 contained arthrinoside A and an unknown component (45 mg), and 43-46 contained arthrinoside C (59 mg).

pH Zone-Refining CCC Separation of Crude Extract of MF6512

The crude fermentation extract (1.4 g) of MF6512, after extraction with ethyl acetate, was separated, as described above, for the diol silica purified complex. Fractions 34-41 contained arthrinoside A (129 mg), 44-45 contained arthrinoside B (27 mg), and 46-50 contained arthrinoside C (58 mg).

RESULTS AND DISCUSSION

Separation of Sordarin, 4'-O-Demethyl Sordarin and 11-Hydroxysordarin

Biotransformation of **1** using actinomycete culture MA7235 resulted in the production of two products, one resulting from 4'-O-demethylation and the other from 11-hydroxylation, each in approximately 30% yield.[14] The reaction products, as well as unreacted **1**, were recovered from methanol extracts of two 23 L

scale fermenter biotransformations by solid phase adsorption onto SP207. Compounds **1**–**3** had good solubility in CH_2Cl_2 at acidic pH and good aqueous solubility at alkaline pH. These solubility properties allowed further purification of the mixture using differential pH extraction to provide a crude acids fraction, which was dominated by **1**–**3**, and also suggested the feasibility of separating the three components using pH zone-refining CCC.

A stationary phase of CH_2Cl_2 containing 0.04% TFA and a mobile phase of 10 mM aqueous NH_4OH, was chosen for the pH zone-refining CCC separation of the crude acids fraction containing **1**–**3** based upon the above solubility observations, and from literature descriptions by Ito and coworkers for this method. An initial separation of 200 mg of this material on a 76 mL #14 coil yielded three distinct pH plateaus (Figure 2). The first of these corresponded to the most polar of the three compounds, **3**, eluting at 0.43 – 0.71 column volumes. The next compound to elute was **2**, the next most polar compound on reverse phase, at 0.91 – 1.26 column volumes. The last of the three compounds to elute was **1** at 1.42 – 1.58 column volumes. Following elution of **1,** the pH of the eluant rapidly increased to that of the eluting mobile phase. Good retention of stationary phase was observed with an initial S_f of 0.72.

The same conditions were applied to 1.5 g of sample with better results (Figure 3). Compound **3** eluted at 1.65–3.16 column volumes, **2** at 3.95–5.92 column volumes, and **1** at 6.79–7.82 column volumes. The resulting longer CCC separation is consistent with a 7.5-fold increase in sample loading. Good retention of stationary phase was still observed with a S_f of 0.64. Resolution of the three compounds improved with the increase in feed sample, resulting in almost one column volume of mobile phase separating each of the three zones.

Further scale-up of the separation on a 227 mL #14 coil with 5.2 g of sample was successful at separating **2** and **3** (Figure 4). Compound **3** eluted at 1.82–3.25 column volumes and **2** at 3.96–5.75 column volumes. Compound **1** began eluting at 6.34 column volumes, however, stationary phase bleed throughout the run lead to complete loss of stationary phase after 6.62 column volumes of eluant. Whitish, lipid-like, material was observed at the phase interface in several of the fractions, perhaps an indication of sample component insolubility at this loading. A lower initial S_f (0.41) was observed for this coil/loading than that observed for the smaller coil. A second equivalent run yielded nearly identical results, with compound **3** eluting at 1.58–2.89 column volumes and **2** at 3.52–5.11 column volumes. As in the previous run, compound **1** began eluting just as stationary phase was completely lost.

The recovered zone of each compound from each run was generally 70–80% pure based upon weight, ^{1}H NMR, and HPLC analysis. Each sordarin zone was completely free of the other two sordarin derivatives present in the feed sample. The impurity weight for each zone appeared to be due to other acidic, non-sordarin components of the feed sample. This was not unexpected since the

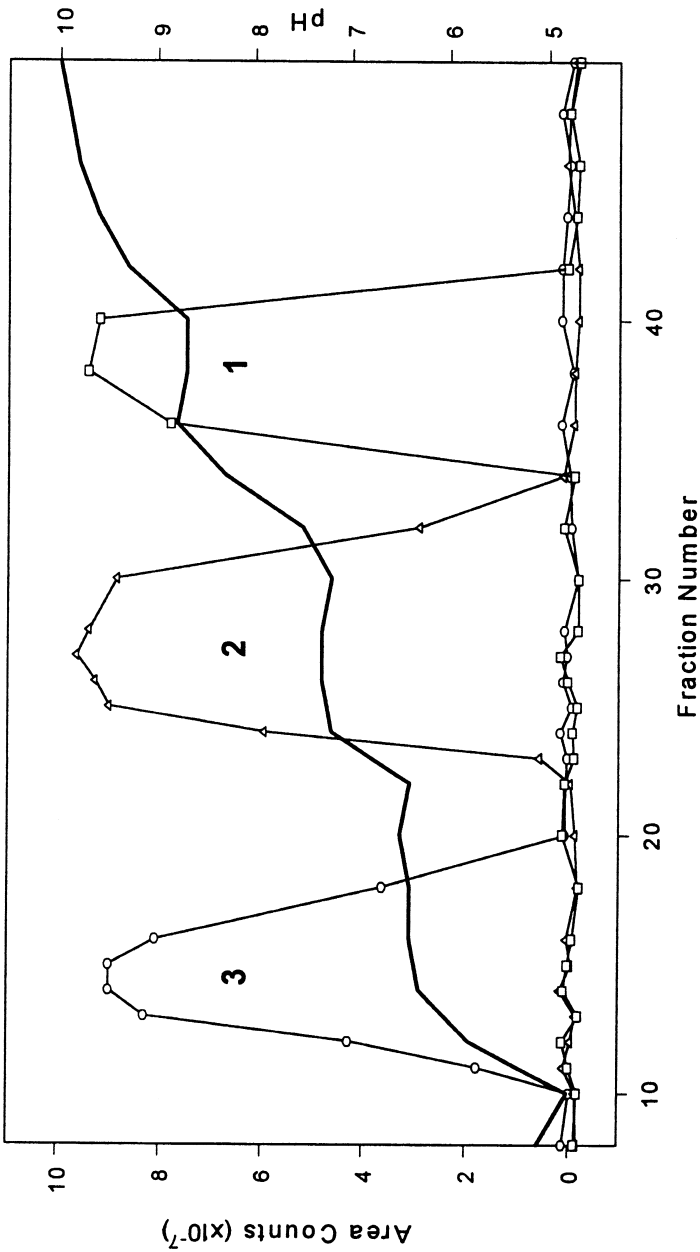

Figure 2. pH-Zone-refining CCC separation of 200 mg of crude sordarins fraction. The elution profile of 11-hydroxysordarin (**3**), 4'-O-demethylsordarin (**2**), and sordarin (**1**) relative to the fraction pH (thick line) is shown.

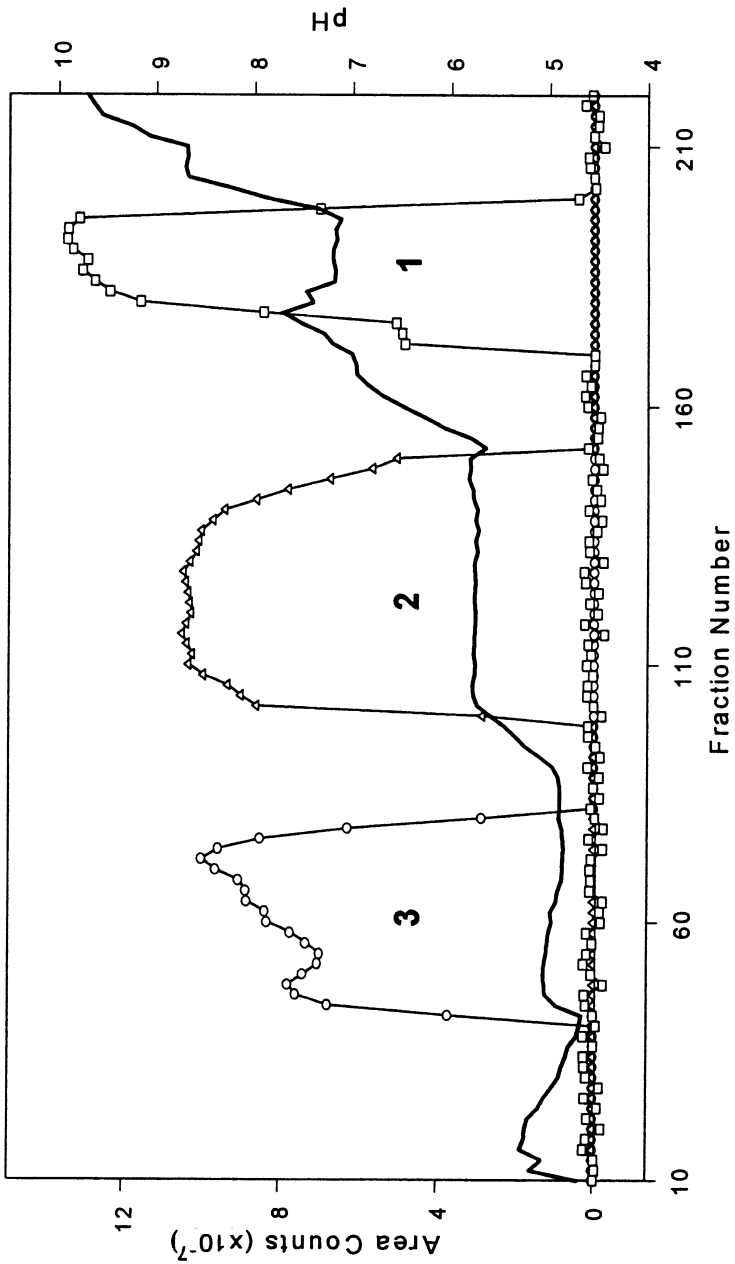

Figure 3. pH-Zone-refining CCC separation of 1.5 g of crude sordarins fraction. The elution profile of 11-hydroxysordarin (**3**), 4'-O-demethylsordarin (**2**), and sordarin (**1**) relative to the fraction pH (thick line) is shown.

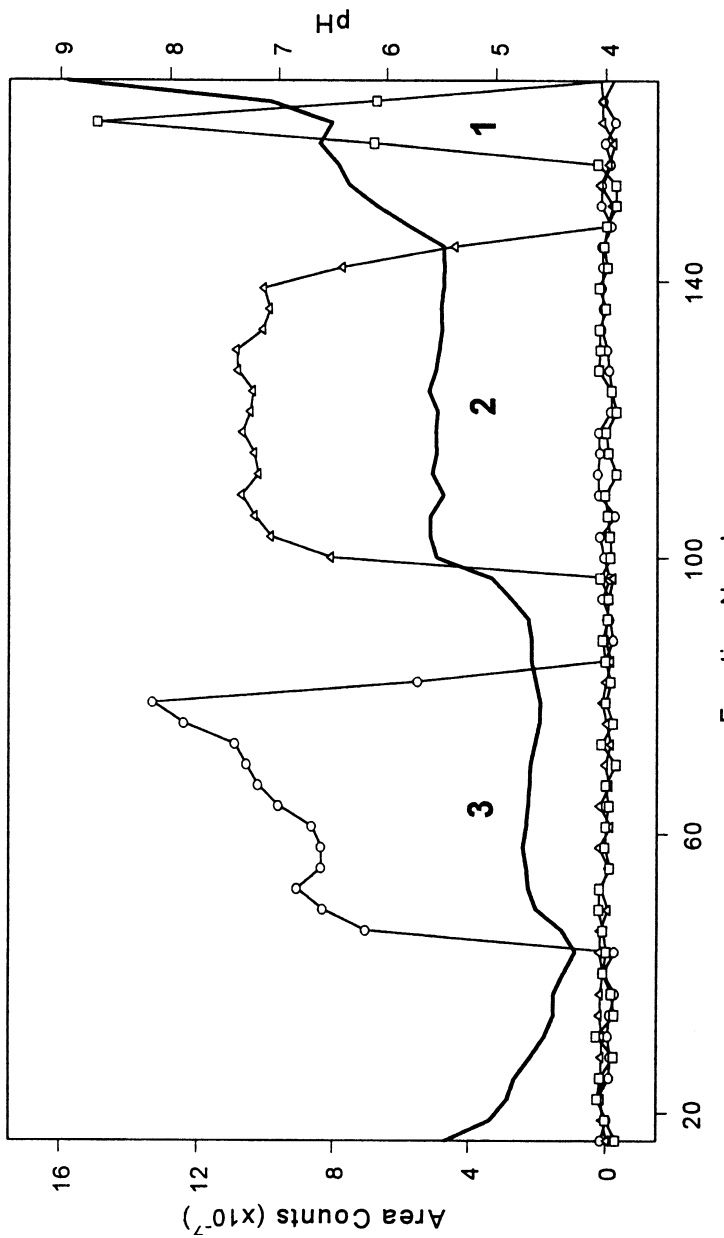

Figure 4. pH-Zone-refining CCC separation of 5.2 g of crude sordarins fraction. The elution profile of 11-hydroxysordarin (**3**), 4'-O-demethylsordarin (**2**), and sordarin (**1**) relative to the fraction pH (thick line) is shown.

feed sample was still a relatively crude mixture of sordarin derivatives, minor fermentation products, and media components. Compounds **2** and **3** were easily purified following the CCC separation using preparative RP HPLC.

Separation of Mycoparasitic Acids A and B

Mycoparasitic acids A and B differ only in the epoxidation of a endocyclic double bond and both contain a terminal carboxylic acid. Separation of these two compounds directly from a methyl ethyl ketone fermentation extract was attempted using pH-zone-refining CCC methodology. A solvent system consisting of MiBK:acetonitrile:H_2O (4:1:5) with the upper layer adjusted to 0.04% TFA and lower layer adjusted to10 mM NH_4OH, was used to separate the crude extract. The pH of the CCC separation was monitored online and the fractions were analyzed for **4** and **5** using RP HPLC, as well as for bioactivity using antifungal agar diffusion assays (Figure 5). Two major pH plateaus were evident, with the first corresponding to **4** and the second to **5**. While the two components were clearly separated, the concentration of **4** and **5** within their respective elution zones was not uniform and increased with elution volume. The pH of the two plateaus was only slightly different and, at approximately pH 8.9, appeared high relative to the other separations reported here. Nevertheless, the separation was successful and yielded 110 mg of **4** and 254 mg of **5**, both at a purity of 70–80%. Each compound was further purified to homogeneity using preparative RP HPLC.

Separation of Arthrinosides A-C

The initial sample of arthrinoside A, **6**, was obtained after normal phase chromatography on diol silica followed by semi-preparative RP HPLC. Neither chromatographic step was optimal however. Diol silica gel did not resolve members of the family, and scaling of the RP HPLC yielded broad peaks making it difficult to separate the other members of the complex.

The diacid character of the arthrinosides was successfully exploited for pH zone-refining CCC. A solvent system MiBK containing 0.1% TFA: aqueous 10 mM NH_4OH was used to separate 480 mg of the crude diol column eluate. The pH of the eluant was monitored online and the fractions assayed for antifungal activity. The complete lack of a chromophore made RP HPLC analysis of the fractions difficult. Three distinct pH plateaus were observed implying three different major components (Figure 6). The middle zone contained **6** and an unrelated compound. The first and last zones contained two new members of the family, **7** and **8,** respectively. The new compounds were essentially pure and the samples were used for structure elucidation.

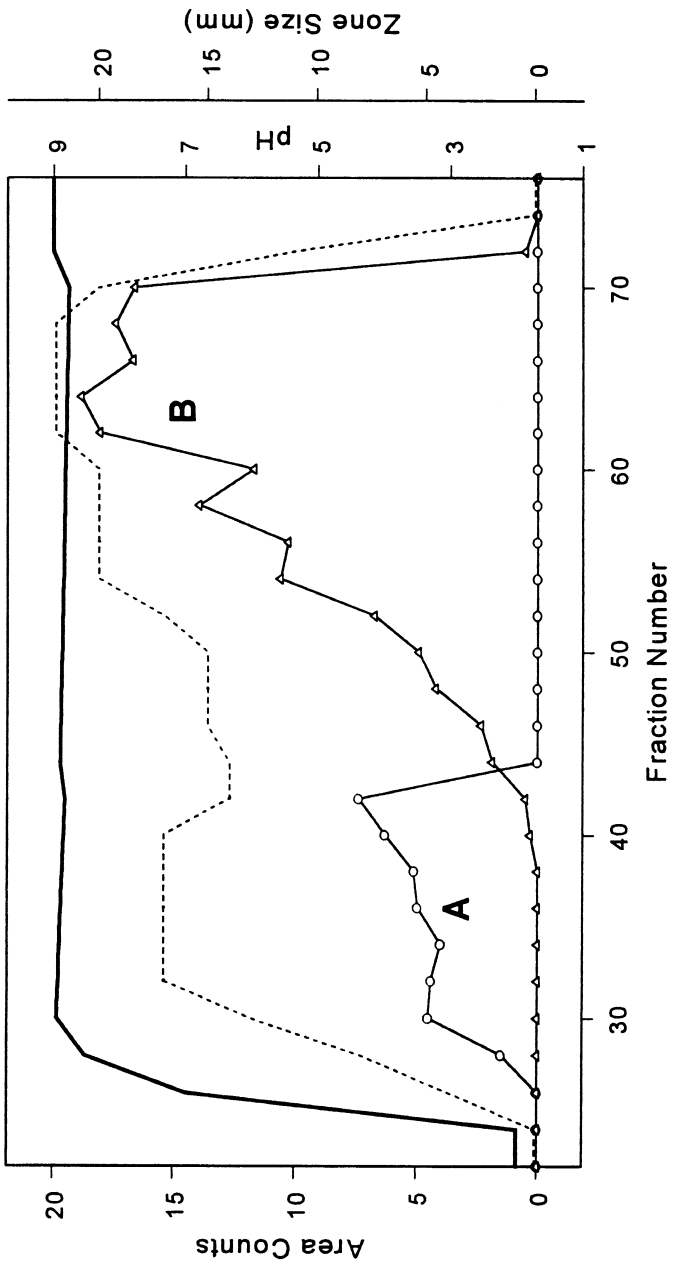

Figure 5. pH-Zone-refining CCC separation of 1.6 g of GB5217 fermentation crude extract. The elution profile of mycoparasitic acids A (o) and B (△) is shown relative to the fraction pH (thick line) and antifungal activity (dashed line).

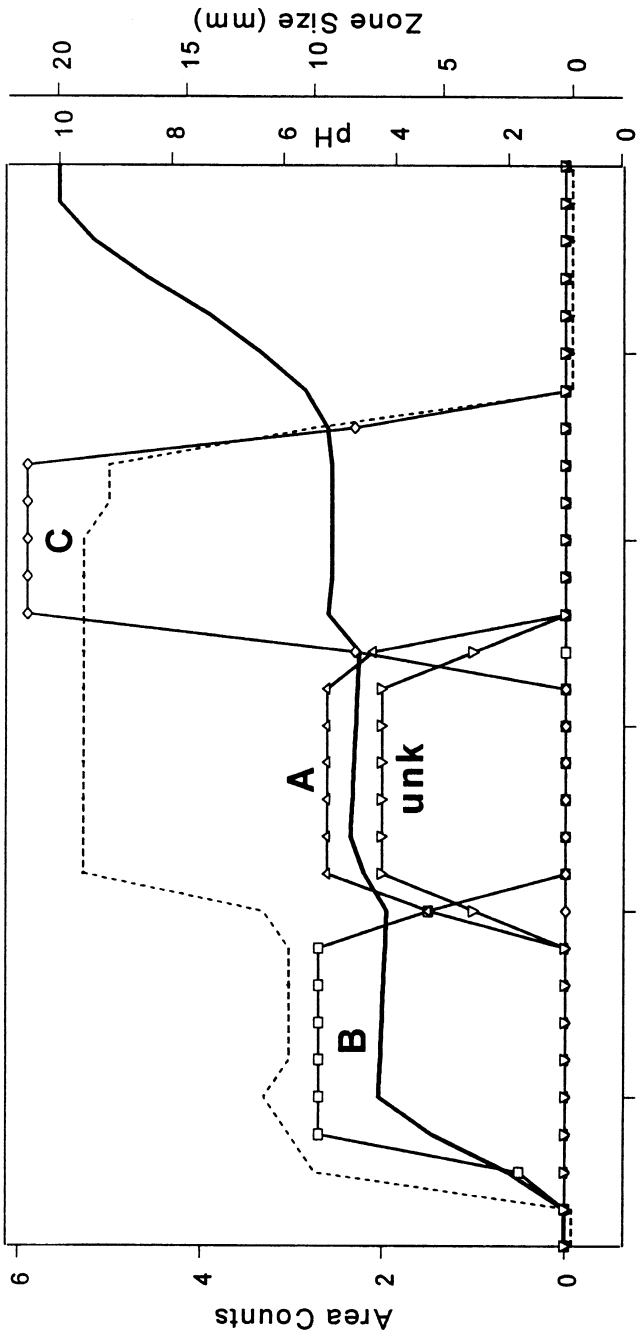

Figure 6. pH-Zone-refining CCC separation of 480 mg of diol silica purified arthrinoside complex. The elution profile of arthrinoside A (△), B (□), and C (◇) is shown relative to the fraction pH (thick line) and antifungal activity (dashed line). (▽) unknown compound.

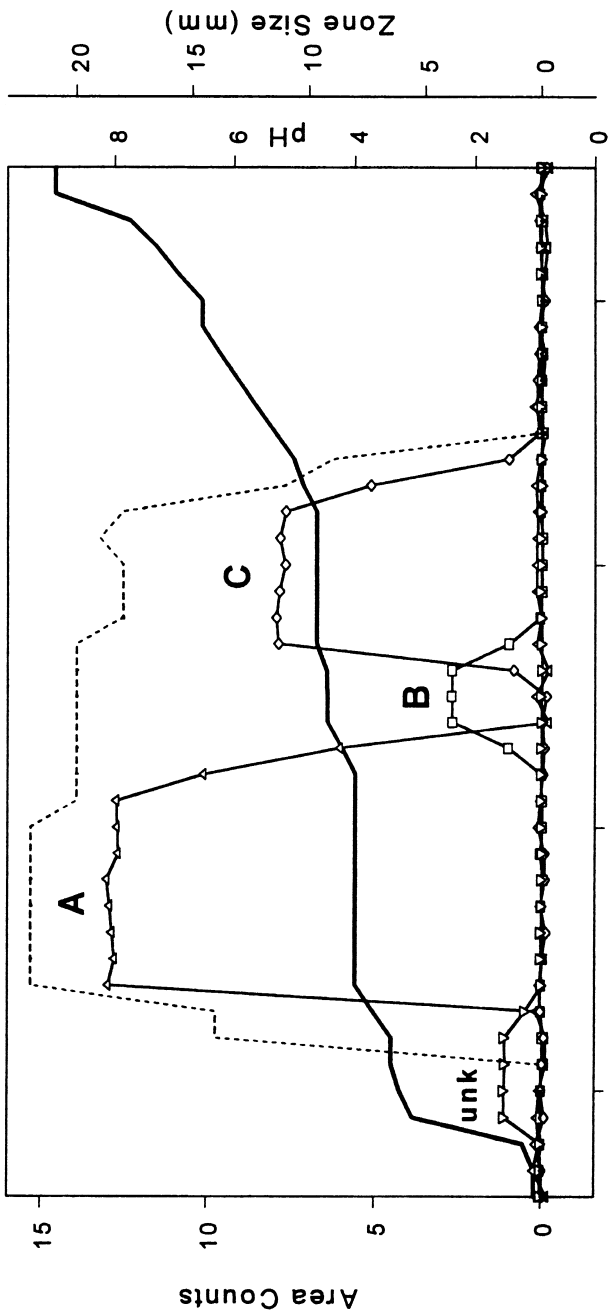

Figure 7. pH-Zone-refining CCC separation of 1.4 g of crude MF6512 fermentation extract. The elution profile of arthrinoside A (△), B (□), and C (◇) is shown relative to the fraction pH (thick line) and antifungal activity (dashed line).

To further explore the utility of pH-zone-refining CCC for arthrinoside isolation, a 1.4 g portion of the crude ethyl acetate fermentation extract was separated, as described above, for the diol silica fraction (Figure 7). This separation yielded **6** (129 mg) at a purity of >90% and compounds **7** (27 mg) and **8** (78 mg) at a purity of 70–80%. Several aspects of this separation are of interest. First, the order of elution of compounds **6** and **7** was reversed when compared with that obtained using the diol silica purified complex as a feed sample. Second, the unknown component, which co-eluted with **6** in the separation of the diol silica purified complex, was now completely resolved from **6** and related components. The difference in elution order might have been due to a difference in the ionic state of the arthrinosides in the two feed samples or caused by different amounts of non-arthrinoside impurities. Third, **6-8** accounted for only 17% of the weight of the CCC feed sample but dominated the separation, and were recovered at excellent purity.

CONCLUSIONS

The work described here demonstrates that pH-zone-refining CCC is a very useful alternative to classical chromatographic methods for the purification of sordarin and its derivatives, as well as for the mycoparasitic acids. The use of pH-zone-refining CCC in combination with bioassay is certainly the best, if not the only, method to isolate the arthinosides, especially B and C. The application of the pH-zone-refining CCC to crude or semi-pure mixtures has been demonstrated previously for phenolic compounds and alkaloids.[16,17] The separations described above were all performed using crude or semi-pure mixtures. The separation of the sordarin analogs was highly reproducible and scaleup was predictable. Almost identical elution profiles were observed at the highest loading attempted. pH-zone-refining CCC was useful for increasing the purity of the of 11-hydroxysodarin. The mass of the crude sordarin fraction would have required multiple runs, perhaps as many as 10-20, to process using typical laboratory scale (5.0 cm diameter or less) preparative reverse phase columns. The bulk of this material was processed in two CCC separations, and final purification using RP HPLC required just two runs. The separations of crude mycoparasitic acids and arthrinosides were not repeated beyond that reported here because of lack of sample. The elution order reversal of arthrinosides A and B is interesting and warrants further investigation.

REFERENCES

1. *Natural Products Isolation*, Wagman, G.H., Cooper, R., Eds.; Elsevier: N.Y., N.Y., 1989.

2. Mandala, S.M.; Harris, G.H. *Methods in Enzymology*; Merrill, A.H., Hannun, Y.A., Eds.; Academic Press: N.Y., N.Y., 1999; vol. 311, 335-348.
3. Harada, K. High Speed Countercurrent Chromatography**.** *Chemical Analysis***;** Ito, Y., Conway, W.D., Eds.; Wiley: New York, 1996; vol. 132, 301-319.
4. Mandala, S.M.; Thornton, R.A.; Frommer, B.R., Curotto, J.E.; Rozdilsky, W.; Kurtz, M.B.; Giacobbe, R.A.; Bills, G.F.; Cabello, M.A.; Martin, I., Pelaez, F.; Harris, G.H. J. Antib. **1995**, *48*, 349-356.
5. Harris, G.H.; Jones, E.T.T.; Meinz, M.S.; Nallin-Omstead, M.; Helms, G.L.; Bills, G.F.; Zink, D.; Wilson, K.E. Tetr. Lett. **1993**, *34*, 5235-5238.
6. Weisz, X.; Scher, A.L.; Shinomiya, K.; Fales, H.M.; Ito, Y. J. Am. Chem. Soc. **1994**, *116*, 704-708.
7. Ito, Y.; Shinomiya, K.; Fales, H.M.; Weisz, A.; Scher, A.L. *Modern Countercurrent Chromatography*; Conway, W.D., Petroski, R.J., Eds.; ACS Symposium Series, 593; American Chemical Society: Washington, DC, 1995; 156-183.
8. Ito, Y. High Speed Countercurrent Chromatography. *Chemical Analysis*; Ito, Y., Conway, W.D., Eds.; Wiley: New York, 1996; vol. 132, 121-178.
9. Ito, Y.; Ma, Y. J. Chromatog. A **1996**, *753*, 1-36.
10. Justice, M.C.; Hsu, M.-J.; Tse, B.; Ku, T.; Balkovec, J.; Schmatz, D.; Nielsen, J. J. Biol. Chem. **1998** *273*, 3148-3151.
11. Tse, B.; Balkovec, J.M.; Blazey, C.M.; Hsu, M.-J.; Nielsen, J.; Schmatz, D. Bioorg. Med. Chem. Lett. **1998**, *8*, 2269-2272.
12. Sturr, G.; Harris, G.H.; Chartrain, M.M.; Greasham, R. J. Ind. Micro., In preparation.
13. Sturr, G.; Chartrain, M.M.; Harris, G.H.; Nielsen-Kahn, J.; Heimbuch, B. WO20000504 **1999**.
14. Harris, G.H.; Dai, P.; Dombrowski, A.W.; Rosenbach, M.; Thompson, J.R.; Flattery, A.; Abruzzo, G.; Bills, G.F. J. Nat. Prod., In preparation.
15. Harris, G.H.; Dai, P.; Guan, Z.; Dombrowski, A.; Rosenbach, M.R.; Thompson, J.R.; Basilio, M.A.; Pelaez, F.; Vicente, F. J. Nat. Prod., In preparation.
16. Ma, Y.; Qi, L.; Gnabre, J.N.; Huang, R.C.C.; Chou, F.E.; Ito, Y. J. Liq. Chrom. & Relat. Technol. **1998**, *21*, 171-178.
17. Ma, Y.; Ito, Y.; Sokolosky, E.; Fales, H.M. J. Chromatog. A **1994**, *685*, 259-262.

Received September 11, 2000 Author's Revision November 30, 2000
Accepted December 3, 2000 Manuscript 5487