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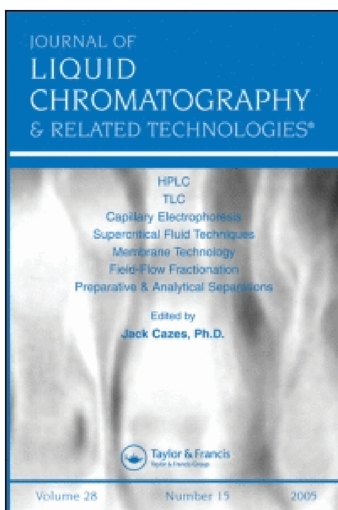

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SEPARATION AND PURIFICATION OF MACROLIDES USING THE ITO MULTI-LAYER HORIZONTAL COIL PLANET CENTRIFUGE

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ABSTRACT

Four different microbially produced macrolide complexes have been separated and purified by chromatography on the Ito Horizontal Coil Planet Centrifuge. These antibiotics represent different subclasses which present a variety of separation problems. The separations required high resolution capabilities under mild chromatographic conditions.

INTRODUCTION

Macrolides are an important therapeutic class of antibiotics and several new members of this class have been discovered in screening programs recently (1-5). These compounds frequently are produced as a family of closely related biogenetic congeners, the separation of which provides a major challenge to the isolation chemist. Moreover, many of these compounds are complex, polyfunc-

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tional molecules which will not withstand the rigors of harsh chromatographic methods.

In the course of our efforts to discover new therapeutically useful antibiotics we have encountered several families of macrolides of various structural subclasses. Countercurrent chromatographic systems have been developed using the Ito multi-layered horizontal Coil Planet Centrifuge (CPC) which allow for the separation and purification of individual compounds from these. These chromatographic methods are the subject of this communication.

The macrolides encountered include 2-norerythromycins (6) and niddamycins, two families which belong to the classical subclasses of 14- and 16-membered basic macrolides, respectively. A novel family of 18-membered neutral macrolides, the tiacumicins (7), comprise 6-congeners; three of these which differ only in the site of esterification by short chain fatty acid moieties of various sugar hydroxyl groups were separated by countercurrent chromatography. Coloradocin (8) is a complex structure involving both a 10- and 14-membered macrolide rings. Although the compound is neutral, it is labile and difficult to purify.

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MATERIALS AND METHODS

Countercurrent chromatography was carried out on an Ito multi-layered horizontal Coil Planet Centrifuge purchased from P.C. Incorporated, Potomac, Maryland. The #14, 1.6 mm ID column was used at a speed of 700 RPM. Flow rates of approximately 300 ml/hr were employed. Solvent was delivered with a Milton Roy miniPump® and sample was applied through a Rheodyne injection loop. Commercial "HPLC Grade" solvents were used. Solvent systems were chosen for appropriate partition coefficients by the method previously described (9). In the case of the ionizable macrolides, 2-norerythromycins and niddamycins, an aqueous buffer at 0.01 M concentration was employed. Eluate was collected in a Gilson FC-100 microfractionator and fractions were analyzed by bioactivity and by TLC on Merck Kieselgel 60 F-254 Silica Gel

plates. ^{1}H NMR spectra were measured on appropriately combined fractions by either a General Electric GN300 or GN500 spectrometer equipped with 5 mm probes. ^{1}H NMR spectra were used as a criterion of purity and as the initial tool in structure determination.

RESULTS

2-Norerythromycins

The 2-norerythromycins are a complex of 14-membered basic macrolides isolated from a genetically engineered construct in which genetic information from the oliandomycin producer, *Streptomyces antibioticus* were inserted into a mutant of *Saccharapolyspora erythrea* which was blocked in its ability to produce macrolides. The resulting macrolide complex produced, contained, in addition to the 2-norerythromycins, several other erythromycin derivatives. Many of these including the four 2-norerythromycins A, B, C, and D (1 to 4, respectively) were isolated by countercurrent chromatography. The crude basic organic extractables from a 750 liter fermentation of our genetic construct was first purified by chromatography over Sephadex® LH-20 in chloroform-methanol-n-heptane (1:1:1) to yield 2.5 g of syrup. This was chromatographed, in 0.5 g batches, on the CPC in a system consisting of n-heptane-benzene-acetone-isopropanol-0.01 M aqueous citrate buffer pH 6.3 (5:10:2:3:5) with the upper layer as the mobile phase. Fractions were pooled following TLC analysis on plates developed with chloroform-methanol-conc. ammonium hydroxide (90:10:1). Spots were visualized by spraying with a mixture of p-anisaldehyde-95% ethanol-conc. sulfuric acid (1:9:1, v/v). Analogous pools from different batches were combined to give eight crude fractions designated A through H.

Fractions B (392 mg) and C (280 mg) were separately chromatographed on the CPC in a system carbon tetrachloride-methanol-0.01 M aqueous potassium phosphate buffer pH 7.0 (1:1:1) with the lower phase mobile to yield 192 mg and 62 mg, respectively, of

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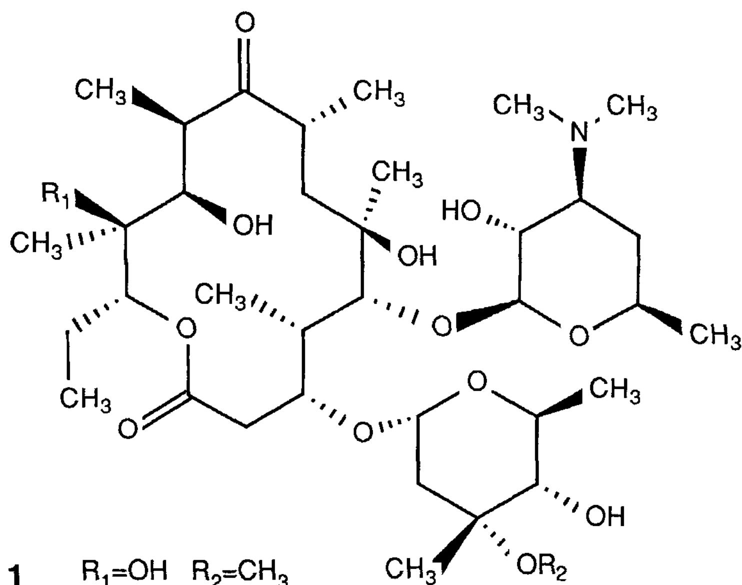

1 R_1=OH R_2=CH_3

2 R_1=H R_2=CH_3

3 R_1=OH R_2=H

4 R_1=H R_2=H

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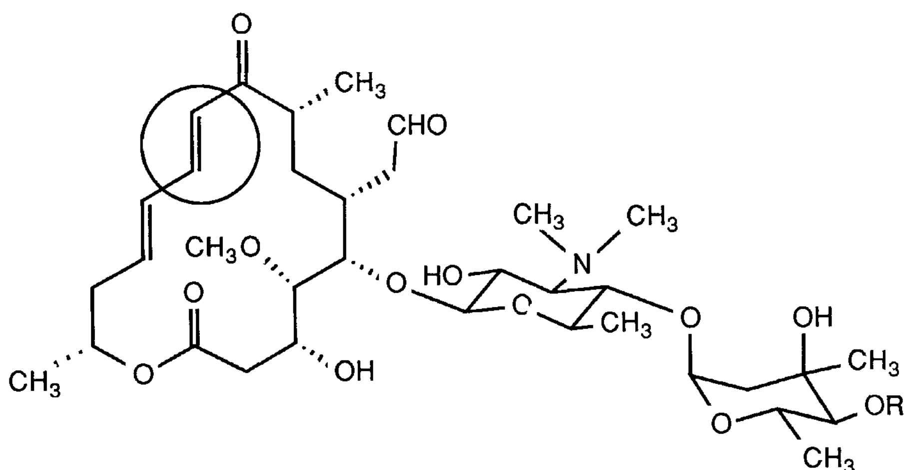
2-norerythromycin B, 2. Fraction D (342 mg) was chromatographed on the CPC in the same system, except that the phosphate buffer was at pH 7.3. This yielded 2-norerythromycin A, 1 (123 mg). 2-Norerythromycin D, 4 (54 mg), was obtained by rechromatography of fraction E (282 mg), in the five component system used initially, but in which the buffer had been replaced with 0.01 M phosphate at pH 7.0. An additional 200 mg of 2-norerythromycin D, 4, was obtained from fraction F (256 mg) by CPC chromatography in hexane-ethyl acetate-0.01 M aqueous phosphate buffer pH 7.5 (3:7:5) with the upper phase mobile. Fraction H (789 mg) was desalted by chromatography on Sephadex® LH-20 in methanol to give 172 mg of crude 2-norerythromycin C, 3. This was purified by

chromatography on the CPC in chloroform-methanol-0.01 M aqueous citrate buffer pH 6.0 (1:1:1) with the lower phase mobile to give 46 mg of the pure material.

Niddamycins

The niddamycins are a family of basic 16-membered macrolides produced by *Streptomyces djakartensis*. The exact composition of the complex varies with different fermentation conditions and producing strains; however, the major component is usually niddamycin B, 5.



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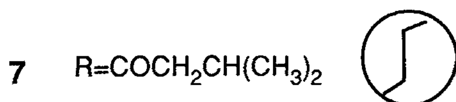
5 R=$COCH_2CH(CH_3)_2$

6 R=$CO(CH_2)_2CH_3$


7 R=$COCH_2CH(CH_3)_2$

Crude niddamycin was prepared by acidification and filtration of the fermentation broth. The filtrate was readjusted to alkaline pH and extracted with amyl acetate; this organic phase was back extracted with aqueous acid. Crude material was precipitated at pH 8 and collected by filtration. A 200 mg sample of crude niddamycin was chromatographed on the CPC in CCl_4-MeOH-0.01 M

aqueous potassium phosphate buffer, pH 7 (2:3:2) in which the upper phase was used as mobile. Fractions were analyzed by bioassay or by HPLC (C-18 in 43% acetonitrile-water, 0.2 M NaCl, pH 4) and appropriately combined to give three pure compounds. Eluting first was the novel congener, niddamycin F, **6** (30 mg); this was followed by the major component, niddamycin B, **5** (60 mg). The third congener did not elute in the mobile phase, but was recovered when the stationary phase was forced from the column with a stream of nitrogen. This material was the 10,11 dihydro derivative, niddamycin A_1, **7** (30 mg).

Tiacumicins

The tiacumicins are a novel series of 18-membered macrolide antibiotics in which the macrolide ring is glycosidically attached to one or two sugars. A seven carbon sugar is esterified at various positions with small fatty acids. The other sugar, when present is esterified with an isomer of the fully substituted benzoic acid, everninic acid. These antibiotics were recovered from the ethyl acetate extract of the fermentation broth of *Dactylosporangium aurantiacum* subsp. *hamdenensis*. The extract from 16 liters of broth was partially purified on a Sephadex® LH-20 column eluted with CH_2Cl_2-MeOH (2:1). Active fractions were pooled and concentrated to an oil (200 mg) which was chromatographed on the CPC in CCl_4-$CHCl_3$-MeOH-H_2O (7:3:7:3) with the lower phase used as stationary. Sample was applied to the column in a mixture of equal parts upper and lower phase. The solvent front was observed after 30 ml, whereupon collection of 2 ml fractions began. Fractions were tested for antibiotic activity against *Clostridium difficile* and active fractions were examined by ^1H NMR spectroscopy. Pure tiacumicin C, **8** (19 mg), was eluted in fractions 6-14 and tiacumicin B, **9** (33 mg), in fractions 18-26. A third component, tiacumicin F, **10** (approx. 2 mg), was eluted in fractions 15-17 and contained up to 20% tiacumicin B as contaminant.

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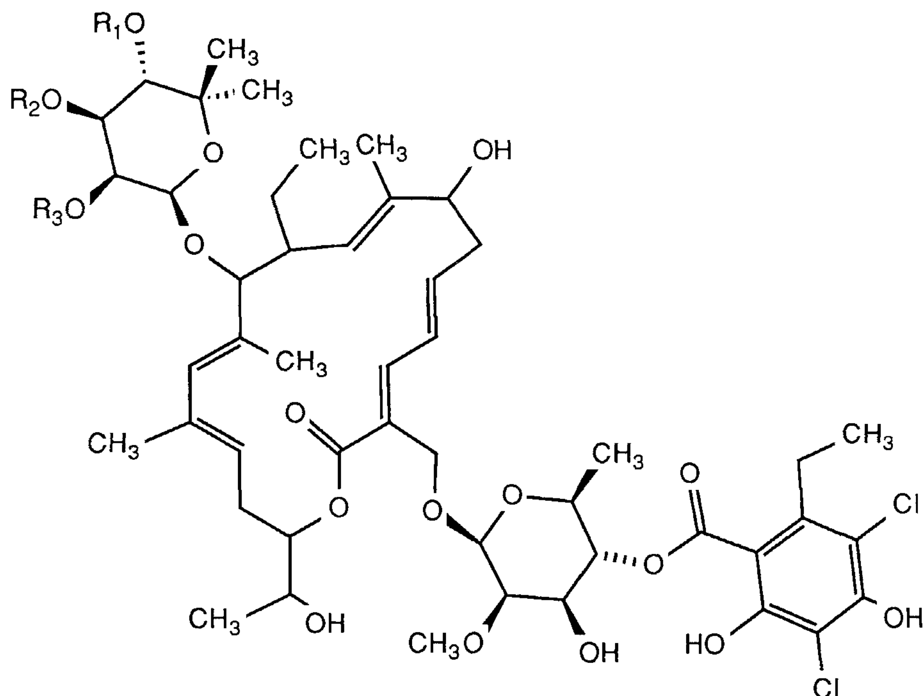

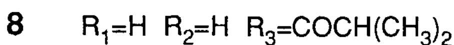

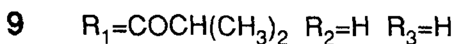

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Coloradocin

Coloradocin, **11**, is a complex macrolide produced by a novel *Actinoplanes* species, *A. coloradoensis* and structurally related to the nargenicins and nodusmicins. It has been shown to be identical to the antibiotic, luminamicin, produced by the *Norcardiodes* strain OMR-59.

The lability of coloradocin under alkaline conditions and/or in the presence of nucleophiles, dictated the development of the

following mild isolation and purification procedure. Supernatant broth (49.8 L) obtained from a submerged fermentation with *A. coloradoensis* was adjusted to pH 4 and stirred with 2.5 kg of Amberlite® XAD-2. A dark brown oil (60 g) was obtained by MeOH elution of the XAD^{-2}. Chromatography of this material on Sephadex® LH-20 in MeOH (4.5 L bed vol.) reduced the crude weight to 19 g with no loss in biopotency. This material was further purified in two successive low-pressure diol-LC (10) steps utilizing biphasic solvent systems originally developed for the CPC. The first diol-LC column was run in CCl_4-$CHCl_3$-MeOH-H_2O (5:5:8:2) and the second in CCl_4-MeOH-H_2O (5:4:1). The recovered bioactive material (4.7 g, 25% pure) was then purified to 90% homogeniety by successive chromatography of 400 mg samples in the CPC using $CHCl_3$-MeOH-H_2O (1:1:1) with the lower layer as the stationary phase. This afforded 1.1 g of coloradocin, **11**.

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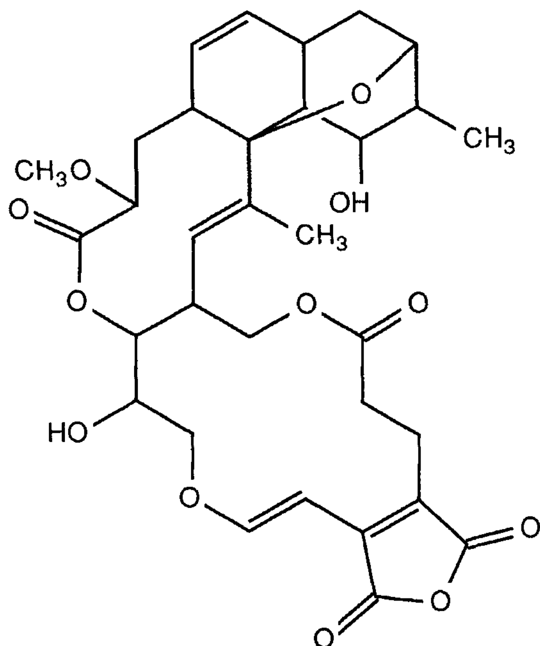

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DISCUSSION

Macrolide antibiotic complexes have traditionally proved to be difficult to separate. In the case of the classical basic macrolides, as exemplified here by 2-norerythromycins and niddamycins, the polarity of the molecule is largely determined by the amino sugar. Minor structural differences in the other areas of the molecule do little to affect the chromatographic mobility of the various congeners. Moreover, solid packings such as silica gel tend to hold tenaciously to the amino sugar moiety and solvents containing amines or concentrated ammonium hydroxide are required to elute the macrolide. The base sensitive nature of the macrolide ring itself probably accounts for the poor recovery yields of basic macrolides from silica gel chromatography. Countercurrent chromatography provides the required high resolving power combined with mild conditions to allow for virtually quantitative separation of these compounds. Several different solvent systems have been developed for the basic macrolides and partition ratios within these are strongly dependent on pH. Citrate or phosphate buffers were used to maintain constant pH in the 6 to 6.5 region or 7 to 7.5 region, respectively. The strength of the buffer must be sufficient to account for the column load, but high levels of salt tend to cause precipitation problems. We experienced this effect using 0.1 M buffer, but had no problems with buffers at 0.01 M.

The separations achieved in some cases are striking. The 2-norerythromycins differ structurally only in the neutral sugar and on the macrolide ring at C-12. In the case of 2-norerythromycin A, 1, the contribution to polarity in these regions is a 1,2 glycol on the macrolide and a hydroxyl at 4". In 2-norerythromycin D, 4, there is a glycol at 3" 4" and a lone hydroxyl at 11. None-the-less these two compounds are well separated in the initial CPC chromatography. Similarly in the niddamycins the simple reduction of the 10,11 double bond represents the structural difference between niddamycin B, 5 and niddamycin A_1, 7. Yet

in the systems used the latter was retained in the stationary phase whereas the former was eluted in two column volumes.

Among the neutral macrolides, the tiacumicins B **8**, C **9**, and F, **10** differ only on the positioning of the isobutyrate ester on the seven carbon sugar. Yet these three are separated, albeit not to baseline, by a single chromatographic step on the CPC. The power of the method is further demonstrated by the purification of the labile macrolide, coloradocin, **11**. This compound was unstable outside of the pH range 3 to 8 and also underwent Michael additions with nucleophiles within that pH range.

These four examples from the macrolide field clearly demonstrate the power of countercurrent chromatography to effect high resolution separation under extremely mild conditions. The Ito Coil Planet Centrifuge with its lack of moving seals assures quantitative recovery from these small scale, preparative chromatographies.

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REFERENCES

1. Tomoda, H., Iwata, R., Takahashi, Y., Iwai, Y., Oiwa, R., and Omura, S., Lustromycin, a new antibiotic produced by *Streptomyces* sp., J. Antibiot., 39, 1205, 1986.

2. Mizobuchi, S., Mochizuki, J., Soga, H., Tanba, H., and Inoue, H., Aldgamycin G, a new macrolide antibiotic, J. Antibiot., 39, 1776, 1986.

3. Sasaki, T., Furihata, K., Shimazu, A., Seto, H., Iwata, M., Watanabe, T., and Otake, N., A novel macrolide antibiotic, notonesomycin A, J. Antibiot., 39, 164, 1986.

4. Rodphaya, D., Sekiguchi, J., and Yamada, Y., New macrolides from *Penicillium urticae* mutant S11R59, J. Antibiot., 39, 516, 1986.

5. Fauth, U., Zähner, H., Miihlenfeld, A., and Achenbach, H., Galbonolides A and B - Two non-glycosidic antifungal macrolides, J. Antibiot., 39, 1760, 1986.

6. McAlpine, J.B., Tuan, J.S., Brown, D.P., Grebner, K.D., Whittern, D.N., Buko, A.M., and Katz, L., New antibiotics from genetically engineered actinomycetes 1. 2-Norerythromy-

cins, isolation and structural determinations, J. Antibiot., 40, in press.

7. Hochlowski, J.E., Swanson, S.J., Ranfranz, L.M., Whittern, D.N., Buko, A.M., and McAlpine, J.B., Tiacumicins, A novel complex of 18-membered macrolides. II. Isolation and structure determination, J. Antibiot., 40, 575, 1987.

8. Rasmussen, R.R., Scherr, M.H., Whittern, D.N., Buko, A.M., and McAlpine, J.B. Coloradocin, An antibiotic from a new *Actinoplanes*. II. Identity with luminamicin and elucidation of structure, J. Antibiot., in press.

9. Brill, G.M., McAlpine, J.B., and Hochlowski, J.E., Use of coil planet centrifuge in the isolation of antibiotics, J. Liquid Chromatogr., 8, 2259, 1985.

10. Rasmussen, R.R. and M.H. Scherr, Preparative low-pressure chromatography of antibiotics on a column of diol-bonded silica gel, J. Chromatogr., 386, 325, 1987.

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