ANTITUMOR AGENTS FROM BOHEMIC ACID COMPLEX, III. THE ISOLATION OF MARCELLOMYCIN, MUSETTAMYCIN, RUDOLPHOMYCIN, MIMIMYCIN, COLLINEMYCIN, ALCINDOROMYCIN, AND BOHEMAMINE

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ABSTRACT.—Six anthracycline antitumor agents, marcellomycin, musettamycin, rudolphomycin, alcindoromycin, collinemycin, and mimimycin, have now been isolated from bohemic acid complex. This has been achieved by classical column chromatography with Sephadex LH-20 and ammonia-neutralized silica and by analytical and preparative hplc techniques with normal phase systems containing aqueous ammonia.

The discovery of the anthracycline antitumor agent adriamycin (1) has led to an intensive effort to discover additional members of this class of compounds both in our own laboratories (2, 3) and elsewhere (4–10). Previously we reported the isolation of three new pyrromycin-based antitumor agents from bohemic acid complex[2] (2, 3), musettamycin (**1**) marcellomycin (**2**) and rudolphomycin (**3**) (figure 1). In this paper we report the separation techniques developed in the course of our work as well as the isolation of three minor anthracycline components of the mixture, mimimycin (**4**) collinemycin (**5**) and alcindoromycin (**6**). In addition, we have also isolated the novel 6-oxo-2H-oxireno[α]pyrrolizine, bohemamine (**7**). Details of the structure elucidations of compounds **1–7** will be published elsewhere (11, 12). The preliminary biological profiling of compounds **1–6** is included in this communication.

RESULTS

Bohemic acid complex is produced by fermentation of *Actinosporangium* sp. strain C36,145 (ATCC 31127) under the conditions described earlier (2). Methyl isobutyl ketone was used to extract the crude complex from the whole broth under neutral or slightly basic conditions. Concentration of the extracts to a low volume followed by precipitation with petroleum ether gave the complex as an oily solid. The fats and oils were removed by ether washing of the solids.

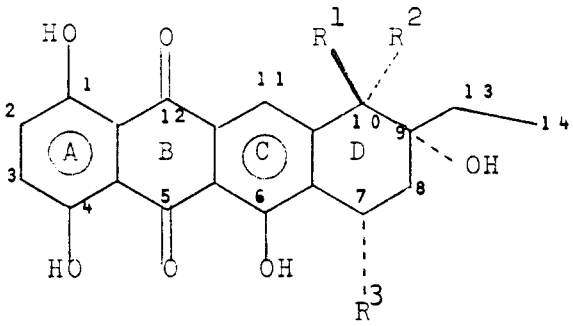
Initial attempts to fractionate the crude complex were made with preparative layer chromatography. Either toluene-methanol (4:1) or toluene-acetone-methanol (3:1:1) was used for development of the plates. The zones appearing at R_f 0.28–0.30 and 0.30–0.32 were shown to be biologically active. While preparative layer chromatography did yield enriched material, the yields were very low. Silica gel chromatography in columns was also unsatisfactory due to severe tailing and mixing of fractions.

In view of the unsatisfactory results with silica gel chromatography, Sephadex LH-20 chromatography, in either chloroform or methylene chloride, was carried

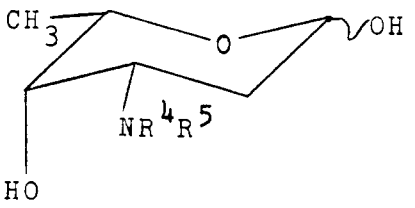
[1]Parts I and II, *J. Antibiot.*, **30**, 519, 525 (1977).

[2]It is the practice in these laboratories to give trivial names, in many cases derived from various operas, to antibiotic complexes early in the isolation effort. In view of its colorful nature, this particular antibiotic complex has been named bohemic acid (after La Bohème) and is produced by an actinosporagium sp. strain C36145 (ATCC 31127). Individual components of the complex, when these are unknown, are given names based on characters in the opera.

FIGURE 1. Structures.

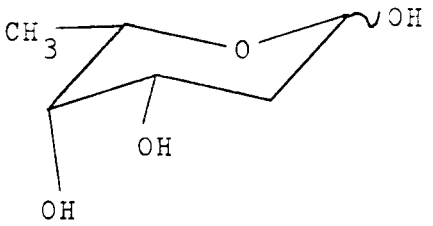


	R^1	R^2	R^3	
1	CO_2CH_2	H	α-Rh-4'-α-DF	musettamycin, *1*
2	CO_2CH_3	H	α-Rh-4'-α-DF-4"-α-DF	marcellomycin, *2*
3	CO_2CH_3	H	α-Rh-4'-α-DF-4"-α-R	rudolphomycin, *3*
4	H	CO_2CH_3	α-Rh-4'-α-DF-4"-α-DF	mimimycin, *4*
5	H	CO_2CH_3	α-Rh-4'-α-DF	collinemycin, *5*
6	CO_2CH_3	H	α-NDMRh-4'-α-DF-4"-α-DF	alcindoromycin, *6*
8	CO_2CH_3	H	α-Rh	pyrromycin
9	CO_2CH_3	H	α-Rh-4'-α-DF-4"-α-Cin	cinerubin A

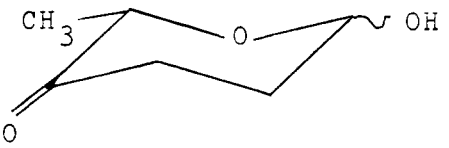


$R^4 = R^5 = CH_3$ Rhodosamine (Rh)

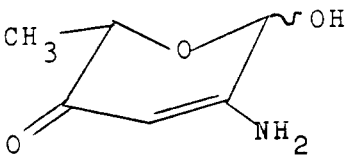
$R^4 = H$ $R^5 = CH_3$ N-desmethylrhodosamine (NDMRh)



2-deoxy-L-fucose (DF)

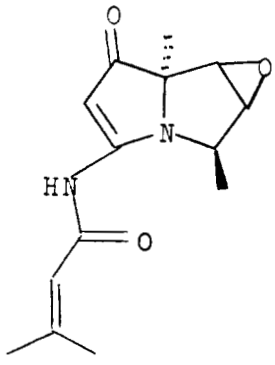


Cinerulose (Cin)



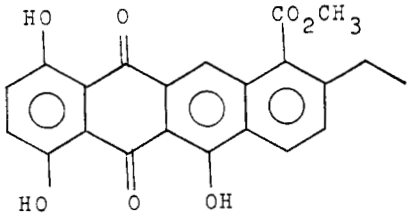
Rednose (R)

FIG. 1. Structures.



bohemamine

7



η-pyrromycinone

10

out. The complex was resolved into four fractions (fig. 2). Fraction 1, eluting at the front, was a complex mixture of compounds which was shown subsequently to contain pyrromycin (6, 13) and cinerubins A and B (6, 14, 15).

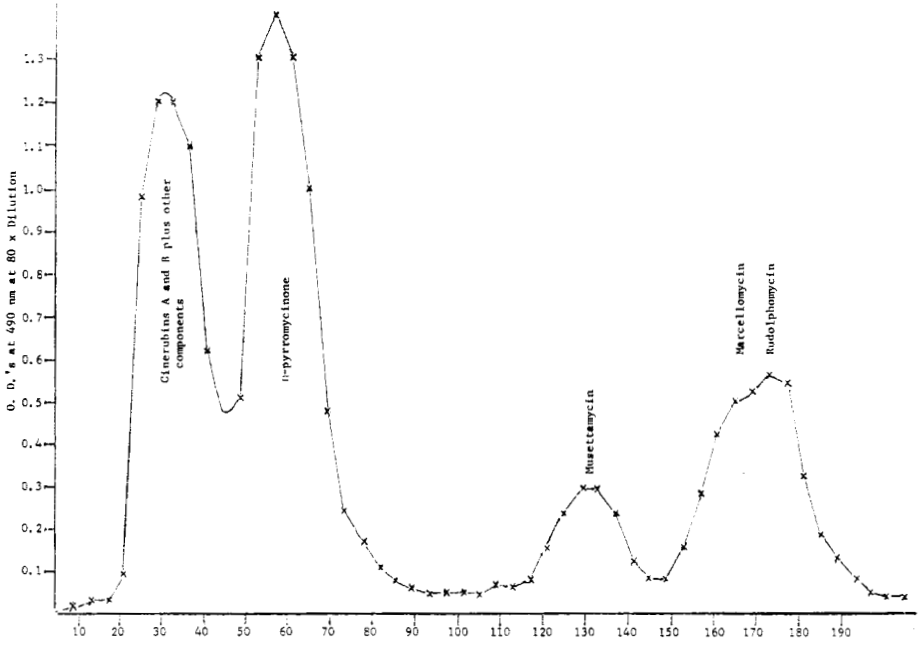


FIG. 2. (LH-20 Sephadex Chromatography of crude Bohemic Acid Complex).

η-Pyrromycinone, **10**, crystallized from the second fraction. It was identified by comparison of its ir spectrum and other physical data (13). The third fraction was crystalline and contained only minor impurities according to tlc. The crystalline compound was named musettamycin (**1**). Initially the fourth fraction was thought to consist of a single material, marcellomycin (**2**); however, tlc showed the presence of an impurity having the same R_f as musettamycin. When the fourth fraction was rechromatographed on LH-20, no additional musettamycin was obtained, thus leading to the conclusion that the impurity was a compound other than musettamycin.

At this time, reverse phase hplc on C_{18}-Porasil B columns were used to obtain both musettamycin (**1**) and marcellomycin (**2**) in a pure state as described in our earlier communication (2). Unfortunately, this procedure was quite lengthy and afforded only a few hundred milligrams of each compound after several weeks work. Accordingly, chromatography was carried out on iron-free silica gel to which sufficient ammonium hydroxide had been added to neutralize the acidic binding sites and to minimize potential hydrolysis of the glycosidic linkage in musettamycin (**1**) and marcellomycin (**2**). Chromatography of fraction four with toluene-methanol (19:1) afforded rudolphomycin (**3**) and marcellomycin (**2**).

Because of its antitumor profile and an apparent lack of leukopenia in a small animal toxicological model (17, 18), it was decided to prepare additional quantities of marcellomycin (**2**) for toxicological profiling. This required considerable improvement in the isolation techniques in order to give adequate supplies of the compound for testing.

In order to simplify the mixture, a bulk purification step was introduced. Even though the glycosidic linkages in compounds **1–3** were labile to acids, it was possible to dissolve the complex in methylene chloride and distribute the aminoglycosides into a 0.1N acetic acid solution (pH 4). The layers were separated immediately; the aqueous phase was made neutral and reextracted into methylene chloride. The material obtained was shown to contain all of the biological activity of the starting material but represented only 20% of the weight.

Hplc separation of the aminoglycoside components in the active fraction was carried out on a μ-Porasil column with methylene chloride-methanol-ammonium hydroxide (96:4:1) as solvent. The excellent resolution achieved revealed the presence of cinnerubins **9** and **11**, rudolphymycin (**3**), musettamycin (**1**) and marcellomycin (**2**) as well as a number of minor components (figure 3).

Translation of the analytical solvent system to the Prep LC/500 resulted in long retention times for the more polar components of the mixture. Decreasing the proportion of alcohol to 4.5 parts or lowering the ammonia concentration by using 6% ammonium hydroxide in lieu of the concentrated ammonium hydroxide gave excellent results as illustrated in figure 4. The first run on the set of two PrepPak cartridges gave poorer resolution than subsequent runs. This may have been due to the relatively high iron content of the silica packing. Following the first run, the column was stripped with methanol, reequilibrated with the chromatographic solvent and a second run was made. Following the second and subsequent runs, the cartridges were cleaned with solvent in which the proportion of methanol had been increased to 10 parts. In this manner, 12 successive runs were made with the same set of cartridges. The loadings were gradually increased until 25 g runs were being made. Fractions were analyzed by hplc and combined where appropriate. Marcellomycin (**2**) was found in fractions 10–15 in a yield from 6–8 g of material, of approximately 90% purity, in each 25 g run. When all

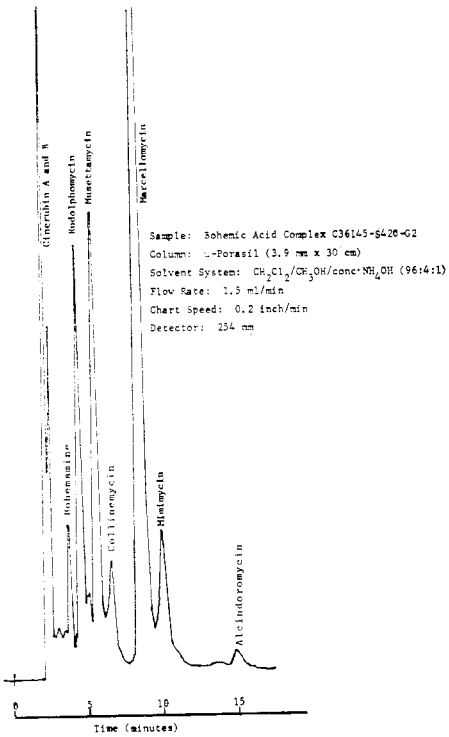


FIG. 3.

of the crude material had been processed, the accumulated marcellomycin-rich fractions were rechromatographed on the same two PrepPak cartridges. The practical load limit was found to be 6–8 g per run. The central portion of the main peak was recycled once and collected in fractions 16–22 (fig. 5). From a

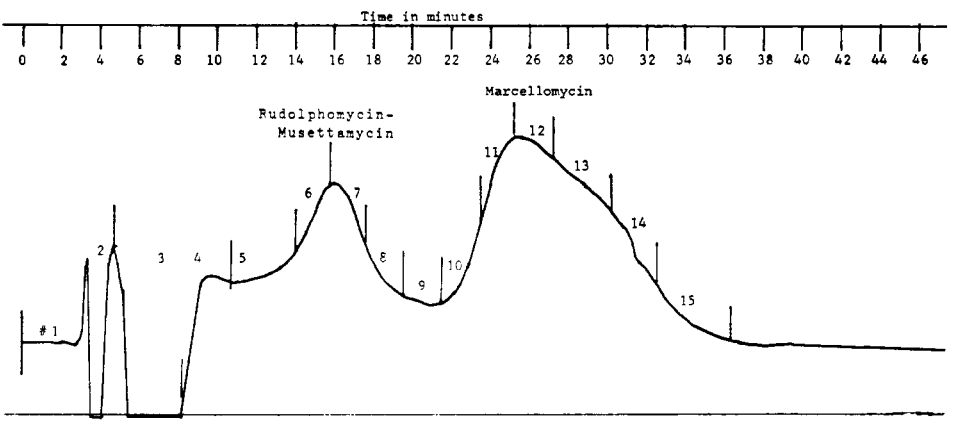


FIG. 4. Prep LC/500 run 25 g Bohemic Acid Complex on 2 PrepPak cartridges.
Flow Rate: 200 ml/min
Solvent System: $CH_2Cl_2/CH_3OH/6\%$ NH_4OH (95:5:1)
Relative Response (RI detector): 5
Chart speed: 0.5 cm/min

6.0 g run a total of 3.8 g of marcellomycin of 99+% purity was obtained. During the course of this work, a number of fractions greatly enriched in the minor components of the complex were obtained. These were subjected to further preparative hplc. In this manner were obtained mimimycin (**4**) and collinemycin (**5**), the C_{10} epimers of marcellomycin (**2**) and musettamycin (**1**), respectively. During concentration of the fractions containing **4**, it was observed that **4** partially epimerized to **2**. Consequently the fractions from chromatography were washed with water prior to concentration in order to prevent base-catalyzed epimerization. The sixth anthracycline we have isolated was alcindoromycin (**6**), which was concentrated in the tailing fractions from the Prep LC/500 runs and in the column washes. These were combined and chromatographed on Sephadex LH-20 with toluene-methanol (9:1) as the eluting solvent. Similarly the mixture of musettamycin and rudolphomycin from the Prep LC/500 chromatogram was chromatographed on LH-20 Sephadex with methylene chloride as eluent. The mixture was cleanly resolved into pure musettamycin and rudolphyomcin. Bohemamine (**7**) (12) was obtained from the late fractions of this column.

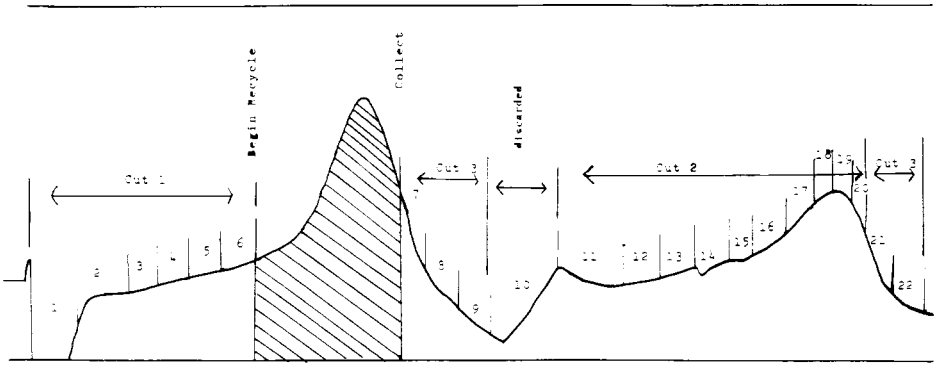


FIG. 5. A representative refractive index chromatogram from LC/500 chromatography with recycle of 6.0 g of marcellomycin-rich material containing 5% mimimycin. Response rate 10. System 95:5:1 CH_2Cl_2:MeOH:6% concd. NH_4OH. Flow rate 200 ml/min. (cf. experimental section, purification of marcellomycin by shave and recycle.)

BIOLOGICAL TESTS

Biological data for musettamycin and marcellomycin was reported in an earlier publication (19). Data for the other new anthracyclines isolated from bohemic acid is given in this publication.

IN VITRO TESTS.—Antimicrobial tests revealed some activity against gram positive bacteria with rudolphomycin being the most potent. None of the products was effective in inhibiting gram negative bacteria, yeasts, or fungi (table 1).

IN VIVO TESTS.—Of the four bohemic acid products, rudolphomycin was the most potent and toxic in a test against L-1210 leukemia (table 2). Of the remaining products, collinemycin and alcindoromycin gave slightly superior survival characteristics compared to mimimycin. Rudolphomycin was also tested against B16 melanoma and produced moderate inhibition at several doses (table 3).

DISCUSSION

The use of Sephadex LH-20 chromatography for the separation of complex natural product mixtures has received much attention in the past several years. Several modes of separation may occur depending upon the running conditions;

molecular sieving, partition, and adsorption effects have all been noted (20–23). The application of LH–20 chromatography to the separation of glycosides of anthracyclines has been reported by Fleck and co-workers (24).

In our case, it is quite evident that the gel filtration and partitioning effects were not important in achieving the separation and that the dominant effect was adsorption presumably via hydrogen bonding between polar functions in the gel matrix and the anthracycline glycosides. Indeed, no separation was achieved when the chromatography was attempted with methanol as the eluting solvent. This was due presumably to supression of the adsorption effects by the solvent. The most important factors in achieving good separations on Sephadex LH–20 have proven to be flow rate and proper cleaning of the gel between columns.[3]

In the case of the bohemic acid complex, the use of LH–20 and silica gel has been complementary. While there is relatively poor resolution of rudolphomycin and musettamycin on silica gel, the use of LH–20 chromatography to resolve rudolphomycin-musettamycin mixtures has proved to be quite successful.

The most interesting finding with respect to our hplc procedure, both on microparticulate packings and the Prep LC/500 preparative work, has been the importance of adding water to achieve good resolution. During the course of this study a number of microparticulate packings were examined. Partisil 10 and Licrosorb-Si 60 proved considerably more active than μ-Porasil but gave slightly poorer resolution. This was corrected by deliberate deactivation of the columns with water. The use of mobile phases containing ammonium hydroxide results in slow deactivation of the columns, which requires the daily use of standards and periodic regeneration. These systems also shorten column life, presumably due to leaching of the silica gel. Some problems with detectors occurred due to deposition of a white film on lens surfaces when columns containing the methylene chloride-methanol-concentrated ammonium hydroxide systems were used. It is recommended that these columns be reserved solely for work with the alkaline systems.

It was found that when one is dealing with polyphenolic quinones such as anthracyclines, the amount of ammonia is important. In initial attempts to scale the analytical hplc systems to preparative loads, excessive amounts of ammonia were used. This gave apparent separation, as shown by the presence of several bands on the refractive index detector. However, when the samples were analyzed by analytical hplc, all samples were identical. It is speculated that ionization of one or more of the phenolic functions led to a separation by ionic species rather than one based on the differing absorption effects of the sugar side chains.

The high metal content of the preparative silica gel was also troublesome. However, since the resolution improved in the second and subsequent runs, the practice of prewashing the columns with ϵ-pyrromycinone to remove iron was adopted.

MATERIALS AND EQUIPMENT

For column chromatography, either Glenco Universal LC glass columns, (Glenco Scientific, Inc.) or Lab-Crest columns with Solv-Seal type joints for low dead-volume connections (Fischer & Porter, Lab-Crest Scientific Div.) were used. In the case of the latter, for solvents which attack neoprene, the O-rings were removed from the Teflon bushings, which were then wrapped snugly with several (usually two) layers of 0.5 in. Teflon tape. For large scale work, a 6″

[3]After two large scale runs the failure of the musettamycin and marcellomycin bands to resolve could be traced to insufficient cleansing of the gel. However, reduction of the flow rate from 16 to 8 ml/min served to restore the resolution.

diameter glass column equipped at the base with a no-clog filter on which was laid a layer of glass wool and a circular polyethylene disk cut to a diameter of 5⅞ in. to allow for swelling was used.

Sephadex LH 20 was purchased from Pharmacia Fine Chemicals. Silica gel Woelm, 63–200μ or silica gel Woelm for dry column chromatography, activity III, 30μ, obtained from ICN Pharmaceuticals, Inc., was used for silica chromatography. Fractions from chromatography were read using a Bausch & Lomb Spectronic 20 colorimeter or a Brinkmann PC/600 colorimeter having an adjustable gap probe connected to the electronic system by a glass fiber optic bundle.[4]

Thin layer chromatography was performed on Quantagram LQDF plates from Quantum Industries, Silica Gel 60 F-254 plates from EM Laboratories, Inc. For micro-slide tlc, prescored Uniplate silica gel HLF plates from Analtech, Inc. were used.

Generally ACS grade solvents were used without further treatment for both classical and preparative liquid chromatography. For hplc work, either analytical or semipreparative, solvents from Burdick and Jackson were used as is, while solvents from Fisher Scientific Co. or from Matheson, Coleman & Bell were filtered through a 10 μm Millipore filter, RH type.

Various hplc column packings were used. μ-Styragel, Bondapak Phenyl/Porasil B, and μ-Porasil were obtained from Waters Associates. Partisil 10 and, for semi-preparative work, Partisil 20 were purchased from Whatman, Inc. Li-Chrosorb SI-60 was purchased from EM Laboratories through suppliers of its products.

A variety of hplc apparatus was used; but for more critical analytical and all semi-preparative work, the model M-6000A solvent delivery system and model U6K injector with 2 ml loop, both from Waters Associates, were used. Detection at 254 nm for analytical work and at 254 nm or 460 nm for semi-preparative work was made with either a Variscan model 635M spectrophotometer equipped with flow-through micro cells from Varian Instrument Division or with a Monochromotor GM 770 with Spectroflow Monitor SF 770 variable wave length detector from Schoeffel Instrument Corp. In semipreparative work, a differential refractometer model R401 was also used. For less critical work, a Milton Roy Co. Minipump with 290 ml/hr upper capacity and a model 7120 injector, Rheodyne, Inc., were used with either a model 1222 duo Monitor from the Laboratory Data Control Division of Milton Roy Co. or a homemade black box measuring at 254 nm. Larger preparative separations were made on the Prep LC/System 500 from Waters Associates using one or two PrepPak-500/Silica cartridges as needed.

EXPERIMENTAL[5]

A. INITIAL EXTRACTION AND DISTRIBUTIONS OF CRUDE MATERIAL

ISOLATION OF BOHEMIC ACID COMPLEX FROM FERMENTATIONS OF ACTINOSPORANGIUM SP. STRAIN C-36,145 (ATCC 31127).—The whole broth was stirred vigorously for 20–30 min with an equal volume of methyl *iso*-butyl ketone at broth pH (~8.0) and ambient temperature. The mixture was then filtered; copious amounts of diatomaceous earth was used as a filter aid. The organic phase was separated, concentrated to a minimal volume, and diluted with petroleum ether to give an oily red mass which, after decantation, was stirred with excess diethyl ether and filtered to afford the complex as a dark red amorphous solid. Yields varied in the range 110–270 mg/liter whole broth from broth volumes of 7–3000 liters.

SEPARATION OF BASIC COMPONENTS FROM NON-BASIC COMPONENTS IN BOHEMIC ACID COMPLEX. 1. *On a Laboratory Scale.* The crude complex, 10 g as extracted from broth, was dissolved in one liter of methylene chloride and extracted twice with one liter portions of 0.1 N aqueous acetic acid. The aqueous extracts were brought to pH 7.5 with solid potassium carbonate as quickly as they could be separated from the organic phase. The extracts were then combined and extracted with methylene chloride, and the organic phase was evaporated to give 2.07 g of product. Oily material (3.46 g) having little or no basic glycoside content was obtained by evaporation of the acid-extracted methylene chloride.

2. *On a large scale.* Crude bohemic acid complex, 760 g in 53 liters of methylene chloride, was stirred with 50 liters of 0.1 N acetic acid at 19–20°, and 0.5 kg of diatomaceous earth filter aid was then added. The mixture was filtered on a 24-inch precoated Nutsche filter; the mat was rinsed with four liters of methylene chloride. The aqueous phase, now 47 liters, was separated and neutralized to pH 7.0 with sodium bicarbonate. The methylene chloride solution was extracted again as before and the process repeated. Extraction and work-up, as in

[4]This apparatus has considerable advantages over other colorimeters in that measurements may be made directly in the collection tubes, and in that dilution of samples is necessary only for very concentrated fractions, adjustments in the gap width being made to change sensitivity.

[5]All melting points are uncorrected. Infra-red spectra were obtained on a Beckman IR model 4230 instrument, and ultraviolet spectra were obtained on a Beckman Acta III uv instrument. ^{1}H nmr spectra, 100 mHz, were obtained on a Varian model HA-100 instrument, except mimimycin, collinemycin, and alcindoromycin, which were examined on a Varian XL-100 instrument. Optical rotations were determined with a Perkin-Elmer 241-MC polarimeter.

the small scale run, afforded an oily material which, in turn, upon treatment with ether, gave 210 g of complex containing 40% marcellomycin as determined by hplc analysis.

B. CLASSICAL, OR OPEN, COLUMN TECHNIQUES ON CRUDE MATERIAL

FRACTIONATION OF BOHEMIC ACID COMPLEX ON SEPHADEX LH-20.—Sufficient Sephadex LH-20 to fill, when swollen, a Pharmacia SR 25/100 column (25 mm I.D. x 100 cm height) was stirred gently in excess chloroform for several hours and then left standing in the solvent for a total of 68 hours. The column was slurry packed, downwashed until completely settled, and the upper teflon tip was adjusted to just contact the top of the gel bed. This resulted in an effective bed height of 90–95 cm.

Bohemic acid complex (500 mg) was dissolved in chloroform (10 ml) and applied at the top of the bed through the tip, and the column was then developed with chloroform at a flow rate of 1 ml/min. Fractions of 6 ml were collected by an automatic collector. Samples from alternate tubes were diluted 80 times with chloroform and read for adsorbance at 490 nm in a Bausch & Lomb Spectronic 20 apparatus. In later work, a Brinkmann PC/600 colorimeter having a solvent resistant probe was used to read the tubes directly.

Readings began with the first fraction having color discernable to the eye; that fraction was designated number one. In all, four major peaks were found (fig. 2), and the fractions representative of these peaks were pooled and evaporated. The results are given in Table 1. Fractions between peaks were discarded.

TABLE 1. Small scale Sephadex LH-20 chromatography.

Cut #	Fraction #	wgt/mg	Composition (by hplc)
1	5–11	66	complex mixture
2	15–21	36	η-pyrromycinone
3	36–44	18	musettamycin
4	46–57	48	marcellomycin-rudolphomycin mixture

LARGE SCALE FRACTIONATION OF BOHEMIC ACID COMPLEX ON SEPHADEX LH-20.—The Sephadex LH-20, 8.73 kg dry weight, was prepared as in the preceding experiment and slurry-packed into a glass column (6 in. diameter by 77 in. height) equipped at the base from the bottom up with a no-clog filter, a layer of glass wool, and a polyethylene disk cut to a diameter of 5⅞ in. to allow for swelling. A 25 g sample was heated in 1.5 liters of chloroform for 15 min and then stirred for 16 hr. The mixture was filtered to remove 3.6 g of insoluble matter and then charged to the column. In later runs, the use of 10–30% methanol in the sample solution was found to vastly improve solubility. The column was developed at a flow rate of 16 ml/min. After elution of a void volume of 1445 ml, collection of 200 ml fractions was begun and continued to a total of 180 fractions. Every other fraction was analyzed spectrophotometrically as described in the previous experiment (fig. 2). Fractions were pooled as guided by this analysis, evaporated *in vacuo*, and except for the η-pyrromycinone-rich cut which crystallized directly, were diluted with ether to precipitate the products. Results are tabulated in table 2.

TABLE 2. Large scale Sephadex LH-20 chromatography.

Cut #	Fraction #	wgt/g	Composition (by hplc)
1	1–10	1.72	oily, low anthracycline content
2	11–40	5.47	complex mixture
3	41–50	1.56	mixture
4	51–70	1.35	η-pyrromycinone
5	71–118	1.98	mixture: η-pyrromycinone and several components
6	119–142	1.01	musettamycin
7	143–154	0.44	mostly musettamycin
8	155–190	4.58	marcellomycin-rudolphomycin mixture
9	191–397	1.44	mixture of compounds

PURIFICATION OF MUSETTAMYCIN FROM THE THIRD PEAK ELUATE OF SEPHADEX LH-20 COLUMNS.—The ether-precipitated solid (421.4 mg) was dissolved in excess boiling chloroform; the solution, while still hot, was filtered through fluted filter paper. The filtrate was then concentrated by boiling on a steam bath to 20 ml. Petroleum ether (Skellysolve B) was added dropwise to the warm concentrate just to the cloud point followed by 2–3 drops of chloroform. After being allowed to cool slowly to ambient temperature, the mixture was left overnight at −20°. The deep red crystalline platelets were collected and dried *in vacuo* to afford 358.8 mg musettamycin: mp 162–3° dec.: $[\alpha]^{23}_{578}$+258°, $[\alpha]^{23}_{589}$+222° (c 0.05, $CHCl_3$); ir νmax (KBr) 3480, 2970, 2930, 2820, 2770, 1735, 1600, 1450, 1320, 1295, 1220, 1160, 1010, 990 cm^{-1}; uv λmax (MeOH) 233 nm (ε 4.13 x 10^4), 256 (ε 2.38 x 10^4), 284 (ε 1.04 x 10^4), 466 (ε 1.02 x 10^4), 490 (ε 1.25 x 10^4), 510 (ε 1.04 x 10^4), 524 (ε 9.23 x 10^3), and 570 (ε 2.36 x 10^3); ^1H nmr (c 50 mg/ml, $CDCl_3$) δ (1.05) (t, 3H), 1.17 (d, 3H), 1.24 (d, 3H), 2.16 (s, 6H), 1.3–2.5 (m, 8H), 3.60 (bs, 1H), 3.68 (s, 3H), 3.75 (bs, 1H), 3.8–4.15 (m, 2H), 4.10 (s, 1H), 4.48 (m, 2H), 4.48 (m, 2H), 5.00 (bs, 1H), 5.24 (bs, 1H), 5.50 (bs, 1H), 7.21 (s, 1H), 7.23 (s, 1H), and 7.66 (s, H).

Anal. Calcd for $C_{36}H_{45}NO_{14}$: C, 60.41; H, 6.34; N, 1.95. Found: C, 60.27; H, 6.50; N, 1.99.

SEPARATION OF MARCELLOMYCIN AND RUDOLPHOMYCIN ON NEUTRALIZED SILICA GEL

A. LABORATORY SCALE SEPARATION.—Silica gel (Grace Davidson grade 62) was stirred in 6N hydrochloric acid for 1–2 hours at 90–100° then filtered and washed with ionized water until neutral. The gel was then oven-dried overnight at 110°.

The dried silica (770 g) was stirred in an excess of toluene-methanol (8:2) mixture. Ammonium hydroxide solution was then added with stirring until a pH of 8 was read on indicator paper or by the electrode of a pH meter. Generally about 12 ml of concentrated base was required for this amount of adsorbent.

The silica was then slurry-packed into a glass column equipped at the bottom with a Teflon frit and a stopcock fitted with Teflon tubing. At the top, a short column extender was attached, through which enough slurry was poured to provide a bed which, when settled, would extend above the connection. When the slurry was completely settled and after some downwashing, the solvent level was drawn down to the top of the bed. The extender was carefully removed with that portion of adsorbent in it and replaced by the same type of end fitting as on the bottom. A direct line from a solvent reservoir was connected, and the column was washed with toluene-methanol (19:1) until fully equilibrated.

A sample of the marcellomycin-rudolphomycin mixture (1.02 g) from Sephadex LH-20 chromatography was dissolved in a mixture of 30 ml of the solvent system and 3 ml methanol. The solution was then applied through the top fitting onto the frit via a long syringe needle. After drawing the charge down onto the top of the bed, development was begun at a flow rate of 0.9 ml/min. Collection of 20 ml fractions was initiated just before the void volume had eluted as evidenced visually by the approach of the colored front to the bottom of the bed.

Elution of components was evaluated spectrophotometrically as described in previous experiments and by thin-layer chromatography of 20 μl portions on silica with a toluene-acetone-methanol (6:2:2) system. Fractions were pooled as indicated and evaporated to dryness. Results are as tabulated in table 3. Stripping the column with pure methanol afforded an additional 240 mg of pigmented material with low biological activity.

TABLE 3. Small scale fractionation of rudolphomycin and marcellomycin.

Cut #	Fraction #	wgt/mg	Composition (by tlc)
1	—[a]	160	η-pyrromycinone
2	1–20	7	η-pyrromycinone
3	21–120	161	rudolphomycin
4	121–160	19	rudolphomycin and marcellomycin
5	161–238	278	marcellomycin

[a]A forerun of four liters containing slight coloration was collected in one portion.

B. LARGE SCALE SEPARATION.—Silica gel (7.1 kg) was prepared as above and charged to a 6 in. diameter chromatographic column. A charge of 10 g of the marcellomycin-rudolphomycin mixture from Sephadex LH-20 chromatography was applied. After three days, the methanol content was increased by 1% daily to a level of 10% on day 8, at which time the desired anthracyclines eluted from the column.

Fractions of 200 ml were collected and analyzed spectrophotometrically at 490 nm. The fractions were pooled accordingly. Upon concentration, precipitates formed in a number of cuts and were collected separately. The mother liquors were evaporated *in vacuo* to dryness

and stirred with petroleum ether, and the solids were collected and dried. Results are tabulated in table 4.

TABLE 4. Large scale fractionation of rudolphomycin and marcellomycin.

Cut #	Fraction #	wgt/mg	State	Constituents (by tlc)
Foreshot	—	104	crystn. ppt.	η-pyrromycinone
1	1–240	504	crystn. ppt.	"
1	"	150	amorph.	η-pyrromycinone and 3 minor components
2	241–257	108	amorph.	η-pyrromycinone and rudolphomycin (wk)
3	258–283	352	crystn. ppt.	rudolphomycin
3	"	218.5	crystn.	"
4	284–314	929	amorph.	"
5	315–339	663	amorph. pp.	marcellomycin and rudolphomycin
5	"	1125	amorph.	" "
6	340–365	1330	amorph. ppt.	marcellomycin
6	"	237	amorph.	"
7	366–440	1512	amorph. ppt.	"
7	"	192	amorph.	"
postcut	—	1735	amorph. ppt.	unknown
postcut	—	1600	amorph.	"

Marcellomycin was obtained as a dark red amorphous solid: mp 175–6° dec.; ir .max (KBr) 3450, 2960, 2940, 2820, 2790, 1730, 1615, 1600; 1450, 1260, 1095, 1010, and 800 cm^{-1}; uv λmax (MeOH) 233 nm (ϵ 4.02 x 10^4), 256 (ϵ 1.24 x 10^4, shoulder), 294 (ϵ 8.97 x 10^3, shoulder), 490 (ϵ 1.34 x 10^4), 510 (ϵ 1.06 x 10^4), 524 (8.97 x 10^3), and 580 (ϵ 9.3 x 10^2); ^{1}H nmr (c 50 mg/ml, CD_2Cl_2) δ 1.0–1.4 (m, 12H), 2.2 (s, 6H), 1.5–2.7 (m); 3.68 (s, 3H), 3.7 (m), 4.12 (s, 1H), 4.5 (m, 2H), 4.90 (bs, 1H), 5.05 (bs, 1H), 5.32 (bs, 1H), 5.52 (bs, 1H), 7.24 (s, 2H), and 7.63 (s, 1H).

Anal. calcd. for $C_{42}H_{55}NO_{17}$: C, 59.64; H, 6.55; N, 1.65. Found: C, 58.77; H, 6.77; N, 1.82.

Rudolphomycin was obtained as a dark red amorphous solid: mp 171–5° (dec.); ir νmax (KBr) 3460, 3410, 2980, 2940, 2820, 2770, 1735, 1600, 1450, 1315, 1295, 1220, 1160, 1118, 1040, and 1010 cm^{-1}; uv λmax (MeOH) 233 nm (ϵ 4.46 x 10^4), 257 (ϵ 2.79 x 10^4), 280 (ϵ 2.98 x 10^4,) 490 (1.38 x 10^4), shoulders at 466, 480, 511, and 523 nm; ^{1}H nmr (c 56 mg/ml; $CDCl_3$) δ 1.39 (t, 3H), 1.45 (d, 3H), 1.54 (d, 3H), 1.71 (d, 3H), 2.13 (s, 6H), 2.0–2.5 (m), 2.5–3.0 (m, 3H), 3.72 (s, 3H), 3.93 (bs, 1H), 4.23 (bs, 1H), 4.37 (m, 1H), 4.48 (s, 1H), 4.74 (m, 1H), 4.84 (m, 1H), 5.29 (bs, 1H), 5.45 (m, 1H), 5.52 (q, 1H), 5.71 (s, 1H), 5.82 (bs, 1H), 5.88 (s, 1H), 7.39 (s, 2H), 7.88 (s, 1H).

Anal. calcd. for $C_{42}H_{52}N_2O_{16}\cdot 1.5H_2O$: C, 58.12; H, 6.38; N, 3.23. Found: C, 58.22, 58.15; H, 6.33, 6.29; N, 3.05, 3.06.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF BOHEMIC ACID COMPONENTS

A. BY REVERSE PHASE, SEMIPREPARATIVE.—Marcellomycin (1 g) obtained from Sephadex LH–20 chromatography in chloroform was washed with 0.001 M EDTA in 0.01 phosphate (Na) buffer at pH 6.8 to remove traces of iron. The material was then passed through a four-column bank of μ-Styragel, 500–100–100–100 Å, (from Waters Associates) in 100 mg batches in chloroform at a flow rate of 0.6–0.7 ml/min as an initial clean-up. The main peak was eluted at 26–31 min.

The pooled main peak material, after removal of solvent *in vacuo*, was divided into 25 samples, each 30–35 mg, which were then chromatographed successively on a phenyl/Porasil B, 37–75 μ column (Waters Associates), 1 *M* by 4.6 mm ID. The running solvent was acetonitrile-0.01 M sodium acetate (45:55), pH 4.0, at a flow rate of 3.0 ml/min. Fractions were collected at 1 min intervals. Monitoring was done by a refractive index detector (Waters Associates) and by hplc analysis of every fifth fraction on a 61 cm x 21.1 mm I.D. column packed with phenyl/Porasil, 37–50 μ (Waters Assoc.) with monitoring at 254 nm in a uv detector; the same solvent system was used. The preparative column was washed with acetonitrile at 4.0 ml/min and then with the running solvent for 15 min each between injections.

Pure marcellomycin was found in fractions 18–35 which, when combined from the 25 runs, afforded 245 mg of product. Musettamycin was purified further in the same way except that an acetonitrile-0.01 M sodium acetate (35:65), pH 4.0, running solvent was used for the preparative column. The analytical column system was not changed.

B. NORMAL PHASE CHROMATOGRAPHY, ANALYTICAL SCALE.—Samples of bohemic acid, fractions and complex, were all dissolved at 1–10 mg/ml in methylene chloride containing 5–20% methanol and were filtered through a solvent resistant 0.5 μm filter. Filtrates were stored in

sealed ampoules. A solvent system containing methylene chloride (960 ml), methanol (40 ml), and aqueous ammonium hydroxide solution (10 ml) was stirred for 5–10 min and then allowed to stand until the mixture cleared and an undissolved aqueous phase formed a puddle on top. The solvent was used as it was without separation of the aqueous layer. Care was taken to replenish the solvent before the reservoir level became so low that some of the aqueous phase would be pumped onto the column.

Two microporous silicas, μ-porasil and Partisil 10, were used in respective 30 cm x 4.6 mm id and 25 or 30 cm x 4.1 id columns. The columns were preequilibrated with 40 volumes (ca 160 ml) of the solvent system. New columns required preequilibration with systems containing an excess of polar constituents. For example, for μ-porosil, 40 volumes of a methylene chloride-methanol-ammonium hydroxide solution (90:10:1) system was required. Samples of 1–20 μg, depending on complexity, were injected. Retentions of the various components varied somewhat, but the relative positions remained the same (cf fig. 3). Over a period of time, column activity gradually diminished, and required regeneration and reequilibration.

PREPARATIVE SEPARATION OF RUDOLPHOMYCIN AND MARCELLOMYCIN ON THE PREP LC/SYSTEM 500 APPARATUS.—One column chamber of the apparatus was loaded with a Prep PAK-500 Silica cartridge containing 325 g of silica and placed under a radial pressure of 40 atm. The column was then preequilibrated by pumping the majority of a five-liter batch of methylene chloride-methanol-ammonium hydroxide solution (90:10:2) through at 200 ml/min and then recycling the remainder for about 20 min. This was then displaced with the running solvent, methylene chloride-methanol-ammonium hydroxide solution (95.5:4.5:0.5). Several liters were passed through to waste and then the remaining solvent was recirculated to the reservoir. With successive runs on the same columns, the reequilibration step was generally omitted as unnecessary. However, at the end of the first run only the columns were washed with three liters of methanol and reactivated with methlene chloride.

The marcellomycin-rudolphomycin mixture (3.0 g), prepared as described above by Sephadex LH-20 chromatography, was dissolved in 30 ml of the running system and filtered. It was then applied to the column either by being pumped on via one of the solvent inlet ports or by injection with a gas-tight syringe through the injection port. The column was then developed by solvent pumped through at 250 ml/min and monitored by the built-in refractive index (RI) detector to which 1–2% of the eluate stream was diverted. A chart speed of 0.5 cm/min and sensitivity setting of 20 were maintained for the recorder, whereby two peaks were observed.

Fractions of varying amounts were collected and analyzed by hplc, as described in the previous experiment. There was some color eluted at the front, detected as a negative off-scale response by the refractive index detector. The first positive peak, fractions 4–12, corresponded to rudolphomycin (1.036 g). The second peak corresponded to marcellomycin, but fractions in the first half of it, i.e. 13–19, were contaminated with rudolphomycin. Except for fractions 13 and 14, there was very little of the latter present, but these were combined and, without concentration, pumped back through the column with the same solvent system. The RI response was raised to a sensitivity of 50, but otherwise conditions were the same as above. The front registered in this case as a positive rather than negative off-scale displacement, and the two peaks were closer together. However, resolution was excellent affording another 35 mg of rudolphomycin and 81 mg of an approximately 1:1 mixture of the latter with marcellomycin from the valley between the peaks. The second peak afforded 122 mg of marcellomycin contaminated with a small amount of rudolphomycin plus 711 mg of pure marcellomycin. Including the 791 mg from fractions 20–26 of the first pass, a total of 1.5 g of marcellomycin was obtained. This represented 50% of the starting material.

SEPARATION OF MARCELLOMYCIN FROM OTHER BASIC COMPONENTS OF BOHEMIC ACID COMPLEX.—Both chambers of a Prep LC/System 500 apparatus were loaded with Prep PAK-500 silica cartridges and placed under a radial pressure of 40 atm. Reequilibration was achieved with 10 liters of a methylene chloride-methanol-ammonium hydroxide solution (90:10:2) mixture. Five liters of the solution were passed through the columns, and the rest was recirculated until the chart recorder for the RI detector showed a stable baseline. This was then displaced with the running solvent, methylene chloride-methanol-6% aqueous ammonium hydroxide[6] (95:5:1). Five liters were passed to waste, and the remainder was recirculated (ca. 19 liters) to the reservoir until a stable baseline was again achieved.

A mixture (25 g) of the basic components of bohemic acid complex, prepared as described earlier, was dissolved in methylene chloride-methanol (95:5) with sonication and filtered through a 40 M sintered glass Buchner funnel. This solution was then loaded into the system and the chromatograph run as described in the previous experiment at 200 ml/min with the detector response rate lowered to 5.

The chart recorder tracing showed a characteristic negative and two positive peaks, the second appearing to leave a trailing shoulder (fig. 4). Results are tabulated in table 5.

[6]This is concentrated ammonium hydroxide diluted to 6% of its original strength.

TABLE 5. Prep lc fractionation of bohemic acid complex.

Cut #	Fraction #	wgt/g	Composition (by hplc)
1	2–3	3.16	frontal material:cinerubins, aglycones, etc.
2	4–5	1.86	1:1 rudolphomycin:musettamycin
3	6–7	2.62	1:1 rudolphomycin:musettamycin
4	8	0.76	15% rudolphomycin, 50% musettamycin, 35% collinemycin
5	9	0.55	50% collinemycin, 40% marcellomycin
6	10–15	9.42	≥95% marcellomycin
7	16–17	0.98	40% marcellomycin, 40% mimimycin
8	18–21	0.47	~70% alcindoromycin

Because of epimerization in the concentrates, cuts were washed with water to remove ammonia before *in vacuo* evaporation of the solvents was begun.

SEPARATION OF MUSETTAMYCIN AND RUDOLPHOMYCIN BY SEPHADEX LH–20 CHROMATOGRAPHY.—Sephadex LH–20 (900 g) was stirred gently in excess methylene chloride for 1–2 hr and left to stand overnight. A glass column, 115 cm in height by 5 cm in dimaeter, was prepared as described above in the separation of marcellomycin and rudolphomycin. The gel was slurry packed into the column and the bed prepared as in previous experiments described above.

A sample (3 g) of the musettamycin-rudolphomycin mixture from a Prep LC/System 500 fractionation of the basic components of bohemic acid complex was dissolved in 50 ml methylene chloride and applied to the column. Development was carried out with the same solvent at 0.5 ml/min for the first 24 hr and, thereafter, at 1 ml/min. Fractions of 15 ml were collected. Selected fractions were analyzed by hplc and tlc in order to pool the cleanest fractions. The first band (fractions 88–104) yielded 1.25 g of musettamycin, and the second band (fractions 125–142) yielded 1.32 g of rudolphomycin. In a larger scale (12 g) run, 477 mg of a uv-absorbing, non-colored compound was isolated from a band eluting shortly after rudolphomycin and was subsequently identified as bohemamine.

CRYSTALLIZATION OF MARCELLOMYCIN SOLIDS.—A sample (711 mg), prepared as above from the fourth peak eluate of a Sephadex LH–20 column, was dissolved in 5 ml of methylene chloride with additions of 1.0–1.5 ml methanol. The solution was diluted with a large excess, about 40 ml, of acetonitrile, whereupon, crystals began to form immediately. After two hours at ambient temperature and two at 5–8°, the product was collected and dried *in vacuo* to give 352 mg of marcellomycin. Another 139 mg of 95% pure material was obtained from the mother liquor.

PREPARATION OF MARCELLOMYCIN TARTRATE.—Marcellomycin, 14.6 g (16.8 mmole), from Prep LC/500 chromatography was dissolved in 200 ml of methylene chloride-methanol (1:1). 40 ml, of acentonitrile, whereupon, crystals began to form immediately. After two hours at To this was added a solution of 2.53 g (16.8 mmole) of l-tartaric acid in 125 ml of methylene chloride-methanol (1:1). After thorough mixing, the solution was diluted with 300 ml of ether to precipitate the salt, which was collected by filtration and dried for 14 hr under high vacuum. The yield was 15.1 g marcellomycin l-tartrate, mp 142–4° (dec.), 99% pure by hplc analysis; $[\alpha]^{23}D+216°$, $[\alpha]^{23}_{578}+311°$ (c 0.58, H_2O).

Anal. calcd for $C_{42}H_{55}NO_{17}\cdot C_4H_6O_6$: C, 55.47; H, 6.17; N, 1.41. Found: C, 55.21; H, 6.45; N, 1.66.

PURIFICATION OF MARCELLOMYCIN BY SHAVE AND RECYCLE ON THE PREP LC/SYSTEM 500.—Marcellomycin, 6 g, from Prep LC/System 500 chromatography of the basic components of bohemic acid and of better than 95% purity was dissolved by sonication in 75 ml of methylene chloride-methanol (95:5). The solution was loaded on a chromatograph. The column was developed at a flow rate of 200 ml/min. After a forerun of 2.56 liters from injection, color appeared and collection of fractions was begun. Following elution of another 1.5 liters, at which point experience had taught that marcellomycin elution should have begun, the apparatus was put into the recycle mode. When the detector showed the hind side of the major peak to be eluting, collection was again begun. Fractions were pooled according to hplc analysis (cf. figure 5) and worked up as described above. Results are given in table 6.

TABLE 6. Prep lc purification of marcellemycin with recycle.

Composite #	*Fractions #*	wgt/g	Composition
1	3–6	0.498	≥75% marcellomycin+faster moving components
2	11–20	3.11	≥98% marcellomycin
3	7–9, 21–22		~75% marcellomycin, ~25% mimimycin

The yield of marcellomycin ≥98% pure was 3.78 g, having been collected from the front portions of the main peaks (fractions 5–6, 11–16, and 22–27). Another 710 mg of an approximately 6:4 marcellomycin-mimimycin mixture was obtained from fractions collected from the hind side of these (fractions 7–9, 17–18, and 28–29). Also, two early fractions (3–4) afforded ≥90% marcellomycin (250 mg) contaminated with a trace of musettamycin. All other fractions contained very little pigment and were discarded.

ISOLATION OF MIMIMYCIN BY PREP LC/SYSTEM 500 CHROMATOGRAPHY.—A composite (9.3 g) of mimimycin-enriched (about 30%) material from a series of marcellomycin purifications was processed with recycling as described in the preceding experiment. In this case, recycling was performed four times with no shaving on the first peak (cf. fig. 5). Data are tabulated in table 7.

TABLE 7. Prep lc purification of mimimycin.

Cut #	Fractions #	wgt/g	Composition (hplc anal.)
1	10–14, 20–27	3.00	≥95% marcellomycin, trace mimimycin
2	8–9, 28–29, 35–36	0.81	~60% marcellomycin
3	4–7, 15	1.70	30% marcellomycin, 70% mimimycin
4	37–39	0.31	~70% mimimycin
5	16–18	0.80	≥90% mimimycin

The cut 5 product in 50 ml of the running solvent was injected and chromatographed as described in the preceeding experiments through two cycles (i.e. one recycle). From the heart of the recycled peak 364 mg of mimimycin ≥97% purity was obtained. Side fractions yielded additional amounts of less pure mimimycin, 35 mg 90% pure and 217 mg about 80% pure.

The purest mimimycin fraction was shown to still contain some aliphatic impurities by pmr analysis. To remove these, 293 mg of the compound was washed four times with 30 ml portions of diethyl ether, and the insoluble material was dried *in vacuo* to a red powder, 202 mg, mp 154–6° dec.; ir νmax (KBr) 3460 (broad), 2982, 2948, 2829, 2775, 1740, 1648, 1604, 1455, 1420, 1407, 1387, 1321, 1298, 1265, 1222, 1188, 1165, 1118, 1088, 1039, 1010, and 994 cm^{-1}; uv λmax ($CHCl_3$) 258 nm (ε 2.33×10^4), 296 (ε 9.30×10^3), 495 (ε 1.54×10^4), 515 (ε 1.17×10^4), and 529 (ε 9.98×10^3); ^1H nmr (c 2 mg/ml, $CDCl_3$) δ 1.0–1.4 (m, 12H), 2.2 (s, 6H), 1.5–2.7 (m), 3.7 (m), 3.88 (s, 3H), 4.0 (s, 1H), 4.15 (m), 4.5 (m, 2H), 5.0 (bs, 2H), 5.28 (bs, 1H), 5.50 (bs, 1H), 7.32 (s, 2H), 7.63 (s, 1H).

Anal. calcd for $C_{42}H_{55}NO_{17}$: C, 59.64; H, 6.55; N, 1.65. Found: C, 59.14; H, 6.58; N, 1.29.

ISOLATION OF COLLINEMYCIN BY PREP LC/SYSTEM 500 CHROMATOGRAPHY.—A composite (4.9 g) of fractions eluted from silica in the Prep LC runs between the musettamycin and rudolphomycin cuts (e.g. cut 4 in table 5) was dissolved in 50 ml of methylene chloride-methanol (10:1). The solution was filtered and chromatographed through two cycles as described in the preceding experiment. Data is tabulated in table 8.

TABLE 8. Prep lc fractionation of musettamycin-collinemycin-rudolphomycin mixtures.

Cut #	Fraction #	wgt/mg	Composition (hplc anal.)
1	3	316	30% unknown,[a] 30% rudophomycin, 30% musettamycin
2	4–5	485	45% rudolphomycin, 50% musettamycin
3	6	163	5% musettamycin, 85% collinemycin, 5% marcellomycin
4	7–8	546	5% musettamycin, 50% collinemycin, 40% marcellomycin
5	9	347	≥80% marcellomycin
6	10–15	1960	20% rudolphomycin, 40% musettamycin, 40% marcellomycin
7	16–17	510	30% musettamycin, 70% collinemycin
8	18	214	≥90% collinemycin

[a]This unknown has a lower retention time than rudolphomycin.

The collinemycin of 90% purity accumulated from several runs (469 mg) was dissolved in a minimal volume of methylene chloride-methanol (19:1) with sonication and filtered through sintered glass as in previous experiments. It was then chromatographed through two cycles,

as above, but with a slightly less polar solvent system of methylene chloride-methanol-6% aqueous ammonium hydroxide (19.5:4.5:1.25). From the final fractions of both peaks, a total of 167 mg of collinemycin, 95% pure, was realized. The major portion of the peak gave an additional 195 mg of collinemycin containing about 25% musettamycin. The interim fractions between the cycles gave 32 mg of collinemycin of ≥90% purity.

Collinemycin is a red solid: mp 139–41° dec.; ir νmax (KBr) 3490 (broad), 2980, 2943, 2825, 2778, 1741, 1649, 1606, 1455, 1421, 1408, 1322, 1300, 1268, 1226, 1203, 1167, 1120, 1094, 1040, 1015, 995, 973, and 912 cm^{-1}; uv λmax ($CHCl_3$) 257 nm (ε 2.26 x 10^4), 288 (ε 9.30 x 10^3), 296 (ε 9.09 x 10^3), 484 (ε 1.38 x 10^4), 494 (ε 1.47 x 10^4), 514 (ε 1.12 x 10^4), 528 (ε 9.60 x 10^3); ^1H nmr (1 mg/ml, $CDCl_3$) δ 1.04 (t, 3H), 1.22 (d, 3H), 1.30 (d, 3H), 2.20 (s, 6H), 1.60–2.60 (m, 8H), 3.65 (bs, 1H), 3.75 (bs, 1H), 3.88 (s, 3H), 4.00 (s, 1H), 4.10 (m, 2H), 4.49 (m, 2H), 5.02 (bs, 1H), 5.27 (bs, 1H), 5.50 (bs, 1H), 7.32 (s, 2H), and 7.64 (s, 1H).

Anal. calcd for $C_{36}H_{45}NO_{14}\cdot H_2O$: C, 58.93; H, 6.46; N, 1.91. Found: C, 58.97; H, 6.36; N, 2.24.

ISOLATION OF ALCINDOROMYCIN BY SEPHADEX LH-20 CHROMATOGRAPHY.—A composite of alcindoromycin-rich cuts (e.g. cut 8 in table 5) were pooled to give 861 mg of starting material. This was dissolved in 50 ml of a toluene-methanol (9:1) mixture and chromatographed with the same solvent system on a column containing Sephadex LH-20 preequilibrated with toluene-methanol (8:2). Bed dimensions were 150 cm height by 5 cm diameter. The flow rate was 4.8 ml/min, and 25 ml fractions were collected in an automatic collector to a total of 200, starting after elution of the void volume.

The major peak eluted with fractions 126–136 resulted in 250.6 mg of alcindoromycin: mp 148–50° dec.; $[\alpha]^{23}D$ (c=0.05 in $CHCl_3$)+13°, $[\alpha]^{23°}_{578}$+15.5°. Analysis and cmr showed one half molecule of toluene per molecule of dried compound. Alcindoromycin is a red solid: ir νmax 3460 (broad), 2980, 2940, 1740, 1650, 1620, 1608, 1455, 1433, 1423, 1408, 1392, 1322, 1298, 1222, 1200, 1188, 1168, 1120, 1012, 996, and 958 cm^{-1}; uv λmax ($CHCl_3$) 258 nm (ε 2.12 x 10^4), 288 (ε 9.40 x 10^3), 484 (ε 1.41 x 10^4), 495 (ε 1.51 x 10^4), 515 (ε 8.48 x 10^{-3}); ^1H nmr (c 2 mg/ml, $CDCl_3$) δ 1.0–1.4 (m, 12H), 2.37 (s, 3H), 1.5–2.7 (m), 3.70 (m), 3.73 (s, 3H), 4.14 (s, 1H), 4.15 (m), 4.96 (bs, 2H), 5.29 (bs, 1H), 5.49 (bs, 1H), 7.34 (s, 2H), and 7.74 (s, 1H).

Anal. calcd for $C_{41}H_{53}NO_{17}\cdot\frac{1}{2}C_7H_8$: C, 60.88; H, 6.54; N, 1.60. Found: C, 60.46, 60.51; H, 6.65, 6.72; N, 1.59, 1.65.

ANTIMICROBIAL ACTIVITY

The standard 2-fold tube dilution procedure (25) was used to determine the minimum inhibitory concentration (MIC) of the bohemic acid products for several microorganisms. Results are given in table 9.

TABLE 9. Minimum inhibitory concentration (μg/ml) of bohemic acid products.

Organisms		Rudolphomycin	Collinemycin	Mimimycin	Alcindoromycin
Streptococcus pneumoniae	A-9585	.06	1	2	1
Streptococcus pyogenes	A-9604	0.25	2	8	2
Staphylococcus aureus	A-9497	1	8	16	8
Staphylococcus (Pen-Res)	A-9606	32	63	63	63
Streptococcus faecalis	A20688	1	4	63	8
Escherichia coli	A15119	>125	>125	>125	>125
Escherichia coli	A20341-1	>125	>125	>125	>125
Klebsiella pneumoniae	A15130	>125	>125	>125	>125
Proteus mirabilis	A-9900	>125	>125	>125	>125
Proteus vulgaris	A21559	>125	>125	>125	>125
Serratia marcescens	A20019	>125	>125	>125	>125
Enterobacter cloacae	A-9659	>125	>125	>125	>125
Pseudomonas aeruginosa	A-9843A	>125	>125	>125	>125
Candida albicans	A-9540	>16	>16	>16	>16
Candida albicans	A15049	>16	>16	>16	>16
Candida albicans	A15050	>16	>16	>16	>16
Candida tropicalis	A15051	>16	>16	>16	>16
Candida krusei	A15052	>16	>16	>16	>16
Trichophyton mentagrophytes	A-9870	>16	>16	>16	>16
Microsporum canis	A-9872	>16	>16	>16	>16

ANTITUMOR EFFECTS IN VIVO

Tests for inhibition of ascitic L-1210 leukemia and B16 melanoma in mice were performed according to methods previously described (26, 27). Experimental conditions and results are listed in the tables 10 and 11.

TABLE 10. Effect of bohemic acid products on L-1210 leukemia.

Material	Dose mg/kg	MST days	Effect MST % T/C	Average weight change, g	Survivors day 5
Rudolphomycin (S420-G27)	12.8	Tox	Tox	Tox	0/6
	6.4	Tox	Tox	Tox	3/6
	3.2	8.0	107	−2.2	5/6
	1.6	10.0	133	−1.2	5/6
	0.8	9.0	120	−1.6	6/6
	0.4	8.0	107	−0.9	6/6
Mimimycin (S420-G45)	51.2	10.0	133	−3.2	5/6
	25.6	8.0	107	+0.1	6/6
	12.8	8.5	113	−0.3	6/6
	6.4	9.0	120	−1.3	6/6
	3.2	8.0	107	−0.3	6/6
	1.6	7.5	100	+0.5	6/6
Collinemycin (S420-G44)	51.2	11.0	147	−2.5	5/6
	25.6	11.0	147	−4.2	6/6
	12.8	10.0	133	−2.3	6/6
	6.4	8.0	107	−1.2	6/6
	3.2	8.0	107	+1.3	6/6
	1.6	8.0	107	−0.3	6/6
Alcindoromycin (S420-G42)	51.2	11.0	147	−2.7	6/6
	25.6	10.0	133	−1.9	6/6
	12.8	9.5	128	−0.8	6/6
	6.4	8.0	107	+0.6	6/6
	3.2	8.5	113	+0.5	6/6
	1.6	7.0	93	+1.3	6/6
Control	Saline	7.5	—	+0.2	10/10

Tumor inoculum: 10^6 ascites cells implanted i.p.
Host: BDF_1 ♀ mice.
Treatment: Once, Day 1, i.p.
Tox: Toxicity, <4/6 survivors, Day 5
Evaluation: MST = median survival time.
Effect: % T/C = MST treated/MST control x 100.
Criteria: T/C ≦ 125 considered significant antitumor effect.

TABLE 11. Effect of rudolphomycin on B16 melanoma.

Material	Dose mg/kg/day	MST days	Effect MST % T/C	Average weight change, g	Survivors day 5
NSC-293858	3.2	23.0	115	−0.3	9/10
	1.6	32.0	160	−2.5	10/10
	0.8	29.0	145	−0.2	10/10
	0.4	26.5	133	−0.8	10/10
	0.2	26.0	130	−1.0	10/10
	0.1	27.5	138	−0.4	10/10
	0.05	22.0	110	−0.8	10/10
	0.025	22.5	113	−0.9	10/10
Control	Saline	20.0	—	−1.0	10/10

Tumor inoculum: 10^6 tumor brei cells.
Host: BDF_1 ♂ mice.
Treatment: Once, Day 1.
Tox: Toxicity, 6/10 survivors, Day 5.
Evaluation: MST = median survival time.
Effect: % T/C = MST treated/MST control x 100.
Criteria: T/C 125 considered significant antitumor effect.

Biological data on musettamycin and marcellomycin, and techniques used to obtain it, have already been reported in an earlier publication (19).

ACKNOWLEDGMENTS

This work was supported in part by NCI contract N01-CM-77147.

The authors thank Dr. Masataka Konishi of Bristol-Banyu Research Institute for sharing his techniques of pretreating silica with ammonia. We thank Mr. Joel Ganotti for his suggestions on the use of Sephadex LH-20. We also thank Miss Elizabeth Ragen for microanalyses and Dr. Martin Misiek for the microbiological findings.

Received 17 May 1979.

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