ISOLATION AND CHARACTERIZATION OF A41030, A COMPLEX OF NOVEL GLYCOPEPTIDE ANTIBIOTICS

APPLICATION OF THE MICHEL-MILLER HIGH PERFORMANCE LOW PRESSURE LIQUID CHROMATOGRAPHY SYSTEM

J. H. EGGERT and K. H. MICHEL

Lilly Research Laboratories, Eli Lilly and Company,
Indianapolis, Indiana 46285, U.S.A.

(Received for publication February 7, 1986)

A new antibiotic complex, designated A41030, has been isolated from the fermentation broth of *Streptomyces virginiae*. Factors A, B, C, D, E, F and G were separated by an efficient preparative high performance low pressure liquid chromatography system. The apparatus offers economic reversed phase separations on glass columns. The A41030 factors are members of the general class of glycopeptide antibiotics and are active *in vitro* and *in vivo* vs. Gram-positive bacteria.

A broad screening program for antibiotics produced by soil microorganisms led to the discovery of the A41030 antibiotic complex produced by *Streptomyces virginiae* NRRL 12525[1]. The complex is composed of factors A, B, C, D, E, F and G. The factors are members of the general class of glycopeptide antibiotics[2,3]. All factors contain a chlorinated ristocetin-like peptide core. Factors A, B, D and E are unique, among antibiotics of this type, in that they contain no sugar moieties. Each factor is active *in vitro* and *in vivo* vs. Gram-positive bacteria. The complex demonstrates growth-promoting activity and increased feed efficiency in animals.

Isolation and Purification

Following fermentation of *S. virginiae*, the majority of the antibiotic was determined to be in the broth. The procedure for isolating the crude complex and purifying the individual factors is diagramed in Fig. 1. Details are reported in the experimental section.

Physical and Chemical Characterization

The analytical separation of the factors by silica gel TLC is described in Fig. 2. The analytical reversed-phase HPLC separation of the factors is described in Fig. 3 and the preparative High Performance Low Pressure Liquid Chromatography (HPLPLC) system is diagramed in Figs. 5~8. All of the factors displayed significant end absorption plus UV λ_{max} nm 278 (neutral, acid), 298 (base), and all demonstrated three *pKa*'s in the range of 5.4~>10. The factors are soluble in dilute base, dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF), and alcohol - water mixtures. The molecular formulas, molecular weights, and structures of the factors are shown in Fig. 4[2,3].

Experimental

The analytical HPLC results shown in Fig. 3 were obtained using a Constametric III pump (LDC, Division of Milton Roy Co., U.S.A.), LDC Spectromonitor III variable wavelength UV detector, and a Rheodyne Model 7120 injection valve (Rheodyne Inc., U.S.A.). Bioassay was performed by a paper disc assay on agar plates seeded with *Bacillus subtilis*.

Fig. 1. Isolation, purification and separation of the A41030 complex.

*Michel-Miller High Performance Low Pressure Liquid Chromatography Columns packed with LiChroprep RP-8, 25~40 μm[6].

Solvent: H_2O - CH_3CN - dibutylamine, H_3PO_4 to pH 2.5. See Experimental details for specific solvent compositions.

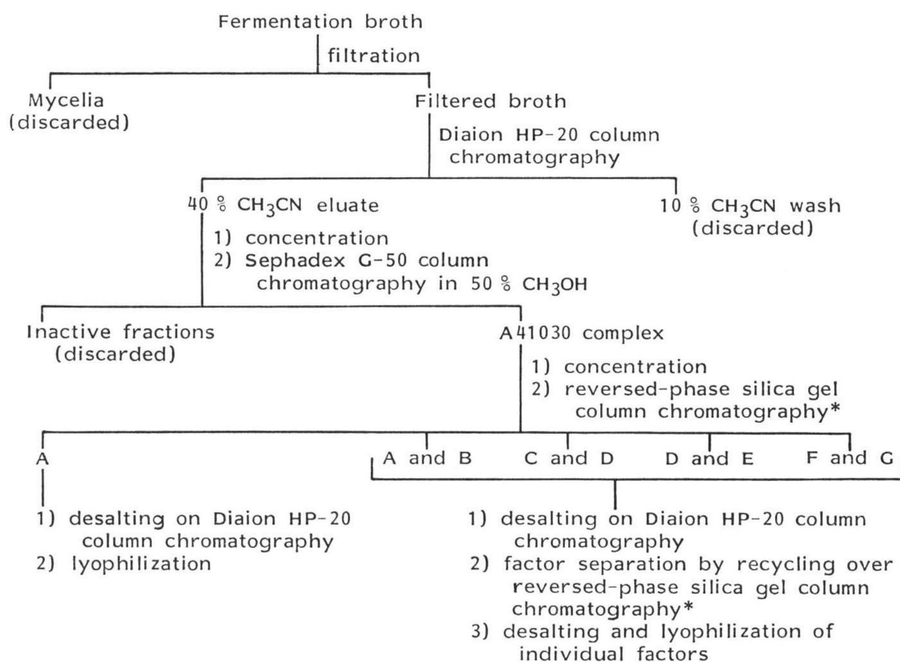


Fig. 2. Thin-layer bioautography of the individual A41030 factors.

Support: Silica gel 60 (Merck-Darmstadt), solvent: CH_3CN - EtOH - H_2O (80: 10: 15), detection organism: *Bacillus subtilis*.

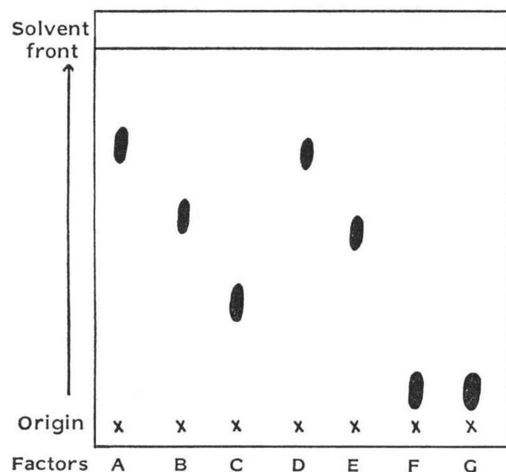
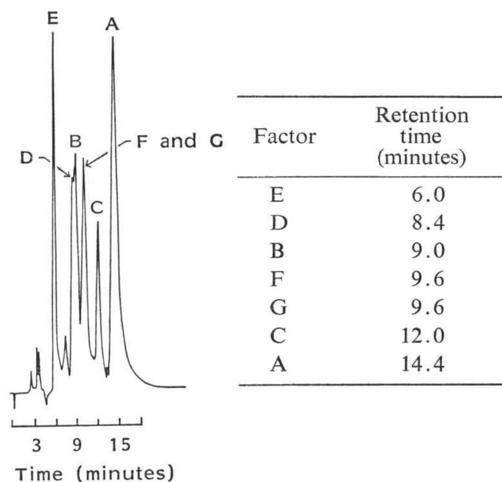


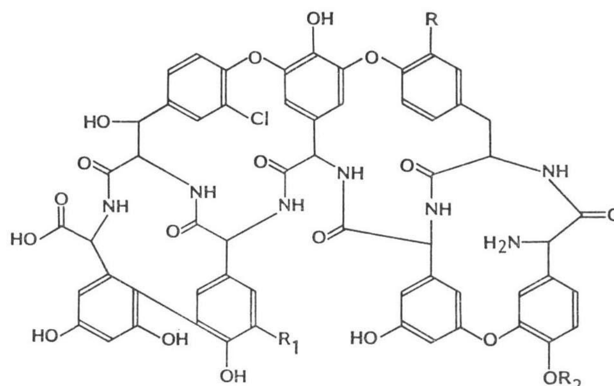
Fig. 3. Analytical HPLC of the A41030 complex.

Column: Stainless steel, 4.6×250 mm, resin: LiChrosorb RP-18 (10 μm), solvent: H_2O - CH_3CN - dibutylamine (81.5: 18: 0.5) adjusted to pH 2.5 with H_3PO_4, flow rate: 1 ml/minute, detection: 210 nm, injection: 1 μg each in 10 μl.



Factor	Retention time (minutes)
E	6.0
D	8.4
B	9.0
F	9.6
G	9.6
C	12.0
A	14.4

Fig. 4. Physical-chemical properties and structures of the A41030 factors.



Factor	Empirical formula	MW (integral)	R	R_1	R_2
A	$C_{58}H_{44}N_7O_{18}Cl_3$	1,231	Cl	Cl	H
B	$C_{58}H_{45}N_7O_{18}Cl_2$	1,197	Cl	H	H
C	$C_{64}H_{54}N_7O_{23}Cl_3$	1,393	Cl	Cl	Gal
D	$C_{66}H_{64}N_8O_{18}Cl_2$	1,326	H	Cl	H
E	$C_{58}H_{46}N_7O_{18}Cl$	1,163	H	H	H
F	$C_{70}H_{64}N_7O_{28}Cl_3$	1,555	Cl	Cl	Gal-Gal
G	$C_{78}H_{83}N_8O_{28}Cl_3$	1,684	Cl	Cl	Gal-Gal

Factors D and G have two equivalent butyl groups attached to the peptide nucleus at an undetermined location[2,3].

Construction of the Preparative Chromatography System

The system is shown in Fig. 5. Valveless piston pumps from Fluid Metering, Inc., Oyster Bay, N.Y., U.S.A., producing flow rates up to 40 ml/minute were used. The Teflon tubing, used to connect all of the components, and 3-way valves (No. 1 and 2) were from Omnifit, Inc., Cedarhurst, N.Y., U.S.A. The tube-end fittings, stainless steel pressure gauge (300 psi) with adapter, and 4-way Tefzel slider injection valve (No. 3) were from Altex Scientific, Inc., Berkeley, CA., U.S.A. The Michel-Miller HPLPLC glass columns[4,5], column end fittings[5], and other accessories were obtained from Ace Glass, Vineland, N.J., U.S.A. Two slurry packing procedures for packing columns with reversed phase resins are also available from Ace Glass[4] or from the authors. The columns were packed with 25~40 μm LiChroprep RP-8 or RP-18[6], or with a 10~20 μm LP-1/C18 reversed-phase silica gel which was prepared in our laboratories by a special procedure described in examples 6 and 7 of U.S. No. 4,299,763[7]. Loops holding different volumes of solutes were constructed from Teflon tubing (1.6 mm or 3.2 mm ID) and Altex tube end fittings. The eluates were monitored using ISCO Model UA-5 UV monitors with Type 6 optical units (Instrumentation Specialties Co., U.S.A.).

Note: The mention of manufacturers and suppliers is for identification of parts only and is not intended to deter substitution of other components.

Operation of the Preparative Chromatography System

Loop Loading Phase (Fig. 6): Valve No. 3, the 4-way slider injection valve, consists of two 4-way valves connected to form a sample injection valve. This valve is designed to connect the pump directly to the column in the loop load position, thus allowing the sample to be drawn into a loop of fixed volume by *e.g.* the use of a vacuum line, while the column is equilibrated with the solvent at a predetermined flow rate. During this operation the connection between valves No. 1 and 2 is closed.

Column Loading Phase (Fig. 7): When the loop is filled, and just before any sample can exit from valve No. 3 into the vacuum line, the valve is manually actuated, thereby forcing the solvent through the sample loop and introducing the sample onto the column as an undiluted slug without interruption

Fig. 5. Preparative high performance low pressure liquid chromatography.

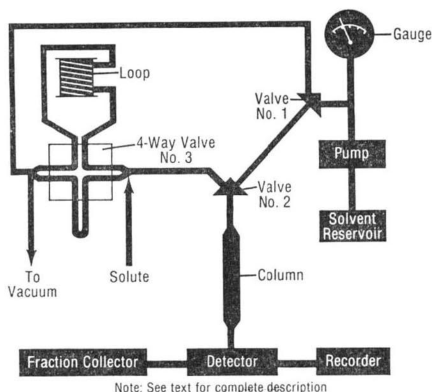


Fig. 6. Loop loading.

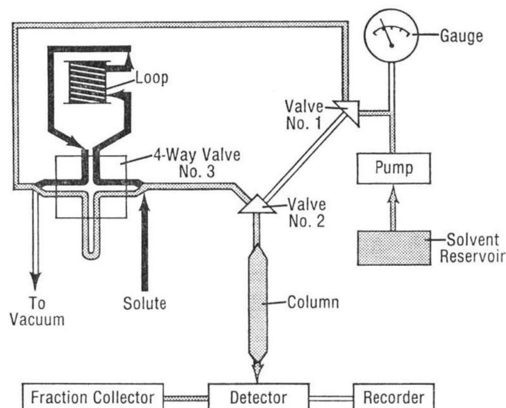


Fig. 7. Column loading.

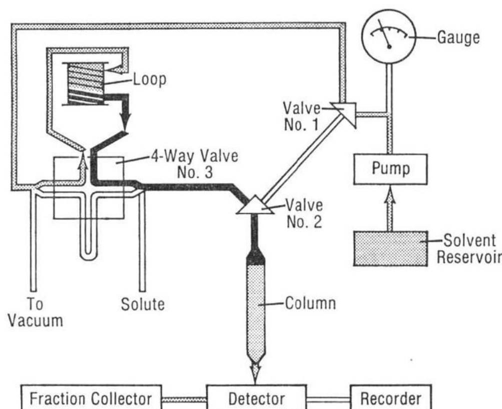
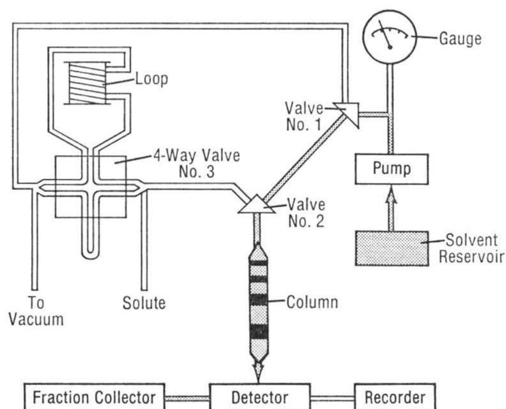


Fig. 8. Separation.



of the flow rate.

Separation Phase (Fig. 8): Solvent now flows from the reservoir through the loop into the loaded column separating the sample components. The passage between valves No. 1 and 2 can now be opened, thereby shortening the solvent pathway. The isolated loop and valve No. 3 can now both be used to load other columns.

Isolation of the A41030 Complex

The whole fermentation broth (4,215 liters) was filtered using a filter aid. The filtered broth was applied to a column containing 100 liters of Diaion HP-20 (Mitsubishi Chemical Industries, Ltd.) at a flow rate of 4 liters/minute. The column was washed with 300 liters of H_2O and 1,000 liters of MeOH - H_2O (1 : 3) at 4 liters/minute. Elution was performed with MeOH - H_2O (1 : 1) at 6 liters/minute, collecting 100-liter fractions. Fractions were monitored for biological activity using a standard disc assay against *B. subtilis*. Fractions 2~15 were combined, concentrated, and lyophilized to give 220 g of crude antibiotic complex.

A 110 g portion of the crude complex, was dissolved in 5 liters of MeOH - H_2O (1 : 1) by adjustment to pH 10 with NaOH. The solution was applied at 50 ml/minute to a 30-liter column (0.2 × 1 m) of coarse Sephadex G-50 (Pharmacia Fine Chemicals) equilibrated with MeOH - H_2O (1 : 1). The column was eluted at 50 ml/minute, collecting 3-liter fractions. Fractions 13~24, which were biologically active, were combined, concentrated and lyophilized to give 35.7 g of the antibiotic complex.

Desalting Solutions of A41030 Factors

NaCl was added (1 mg/ml final concentration) as an ionic marker to solutions of the antibiotic complex or individual factors that did not already contain NaCl. The solution was applied to a 100-ml column of Diaion HP-20 previously equilibrated with H_2O. The column was washed with H_2O, adjusted to pH 2.5 with formic acid, until no chloride was detected in the wash by precipitation as silver chloride (approximately 500 ml required). Elution was performed with 1 liter of H_2O - CH_3CN (6 : 4) at 20 ml/minute.

Purification of A41030A

An 8.0 g portion of the antibiotic complex was dissolved in 200 ml of H_2O - CH_3CN - NaCl (84 : 16 : 2 g/liter) and filtered. The filtrate was applied to a 4-liter stainless steel column (8×100 cm, Chromatospac Prep-100, Jobin-Yvon) packed with 10~20 μm LP-1/C18 reversed-phase silica gel (U.S. No. 4,299,763)[7]. The column was eluted at 60 ml/minute with H_2O - CH_3CN - NaCl (84 : 16 : 2 g/liter); 480 ml fractions were collected. The eluate was monitored at 254 nm. Selected fractions were analyzed for the presence of factor A by analytical HPLC (Fig. 3). Fractions 52~79 were combined and concentrated to a volume of 500 ml. The concentrate was adjusted to pH 8.2 with NaOH and filtered.

The filtrate was desalted, as previously described, except that the column was eluted with H_2O - CH_3CN (8 : 2) at 15 ml/minute, collecting 1-liter fractions. Fractions were assayed for biological activity. Upon refrigeration, 389.6 mg of factor A precipitated from fraction 2. Fraction 1 and the filtrate from fraction 2 were concentrated and lyophilized to give 731.8 mg and 514 mg of factor A, respectively.

Purification of A41030B

A 1.0 g portion of the antibiotic complex was dissolved in 35 ml of H_2O - CH_3CN - NaCl (85 : 15 : 2 g/liter). The solution was applied to a 4.7×45 cm HPLPLC glass column[4] packed with 25~40 μm LiChroprep RP-18[6]. The column was eluted at 21 ml/minute, with the same solvent combination used for sample dissolution, collecting 21 ml fractions. The eluate was monitored at 280 nm. Fractions 184~245 were combined and concentrated under reduced pressure to 25 ml. Concentrates from seven similar purifications were combined, diluted to 1.4 liters with H_2O, and desalted as previously described. The eluate was concentrated and lyophilized to give 523 mg crude factor B.

A 550 mg portion of two combined crude factor B preparations was dissolved in 10 ml of H_2O - CH_3CN - dibutylamine (74.5 : 25 : 0.5), adjusted to pH 7.8 with phosphoric acid, by addition of tetrabutylammonium hydroxide. The solution was applied to a 2.8×59 cm glass column[4] packed with 25~40 μm LiChroprep RP-8[6].

The column was eluted at 5 ml/minute with the same solvent used for sample dissolution, collecting 27 ml fractions. The eluate was monitored at 254 nm. Selected fractions were monitored for the presence of factor B by analytical HPLC (Fig. 3). Fractions 37~48 were combined and concentrated to 200 ml. The concentrate was diluted to 500 ml with H_2O and adjusted to pH 2.0 with phosphoric acid. This solution was desalted as previously described. The eluate was concentrated and lyophilized to give 295.6 mg of crude factor B.

A 285 mg portion of this preparation was dissolved in 30 ml DMF - H_2O (4 : 6) with heat, cooled to room temperature, and refrigerated, resulting in precipitation of factor B. The precipitate was recovered by filtration, washed with Me_2CO, and dried under vacuum to yield 84 mg of pure factor B.

Purification of A41030C

A 9.0 g portion of the antibiotic complex was dissolved in 200 ml H_2O - CH_3CN - NaCl (83 : 17 : 2 g/liter). The solution was chromatographed on the 4-liter Chromatospac Prep-100 reversed-phase column, described in the purification of factor A. The column was eluted at 60 ml/minute with the same solvent combination used for sample dissolution, collecting 480 ml fractions. The eluate was monitored at 254 nm. Selected fractions were analyzed for the presence of factor C by analytical HPLC (Fig. 3). Fractions 28~52 were combined and concentrated to 500 ml.

Concentrates from two similar purifications were combined and desalted as previously described.

The eluate was concentrated and lyophilized to give 2.75 g of a factor C enriched mixture of factors.

A 1.25 g portion of this mixture was dissolved in 25 ml of H_2O - CH_3CN - dibutylamine (79.5 : 20 : 0.5), adjusted to pH 7.8 with phosphoric acid, by addition of tetrabutylammonium hydroxide. The sample was applied to a 2.8×59 cm column[4] packed with 25~40 μm LiChroprep RP-8 and eluted at 4 ml/minute with the same solvent combination used for sample dissolution. The eluate was monitored at 254 nm and 28 ml fractions were collected. Selected fractions were analyzed for the presence of factor C by analytical HPLC (Fig. 3). Fractions 150~182 were combined and concentrated to 500 ml.

Concentrates from three similar purifications were combined, adjusted to pH 1.7 with phosphoric acid, and desalted as previously described. The eluate was concentrated and lyophilized to give 0.87 g of partially purified factor C.

This preparation was dissolved in 20 ml of H_2O - CH_3CN - dibutylamine (79.5 : 20 : 0.5), adjusted to pH 7.8 with phosphoric acid, by addition of tetrabutylammonium hydroxide. The sample was chromatographed on a 2.8×59 cm column[4], packed with 25~40 μm LiChroprep RP-8, as previously described. Fractions 87~114 were concentrated to 500 ml.

Concentrates from two similar purifications were combined and desalted as previously described. The eluate was concentrated and lyophilized to give 688 mg of factor C. A 678 mg portion of this preparation was dissolved in 60 ml H_2O - CH_3CN (6 : 4) by heating. Factor C precipitated upon refrigeration of the solution. The precipitate was recovered by filtration, washed with Me_2CO, and dried under vacuum to give 428 mg of pure factor C.

Purification of A41030D

A 6.0 g portion of the antibiotic complex was dissolved in 200 ml of H_2O - CH_3CN - NaCl (83 : 17 : 2 g/liter). The solution was chromatographed on the 4-liter Chromatospac Prep-100 reversed-phase column described in the purification of factor A. The column was eluted with the same solvent used for sample dissolution, collecting 480 ml fractions. The eluate was monitored as 254 nm. Selected fractions were analyzed for the presence of factor D by analytical HPLC (Fig. 3). Fractions 35~53 were combined and concentrated to 500 ml.

Concentrates from two similar purifications were combined, diluted to 3 liters with H_2O, and desalted as previously described. The eluate was concentrated and lyophilized to give 2.33 g of a factor D enriched mixture of factors.

A 1.15 g portion of this mixture was dissolved in 25 ml of H_2O - CH_3CN - dibutylamine (79.5 : 20 : 0.5), adjusted to pH 7.8 with phosphoric acid, by addition of tetrabutylammonium hydroxide until dissolution occurred. The sample was applied at 5 ml/minute to a 2.8×59 cm column[4] packed with 25~40 μm LiChroprep RP-8. Elution was performed at 5 ml/minute with the same solvent used for sample dissolution, collecting 25 ml fractions. The eluate was monitored at 254 nm. Selected fractions were analyzed for the presence of factor D by analytical HPLC (Fig. 3). Fractions 104~136 were combined and concentrated to 300 ml.

Concentrates from three similar purifications were combined, dissolved by addition of phosphoric acid to pH 7.7, and desalted as previously described. The eluate was concentrated and lyophilized to give 0.63 g of partially purified factor D. This preparation was dissolved in 15 ml H_2O - CH_3CN - dibutylamine (79.5 : 20 : 0.5), adjusted to pH 7.8 with phosphoric acid, by addition of tetrabutylammonium hydroxide until dissolution occurred. The solution was chromatographed on 25~40 μm LiChroprep RP-8 in a 2.8×59 cm column[4] as previously described. Fractions 100~120 were combined, concentrated to 200 ml, and desalted as previously described. The eluate was concentrated and lyophilized to give 193 mg of partially purified factor D.

A 259 mg portion of two combined partially purified factor D preparations was dissolved in 6 ml H_2O - CH_3CN - dibasic sodium phosphate (82 : 18 : 0.03 M, adjusted to pH 7.8 with phosphoric acid) and adjusted to pH 10 with NaOH. The solution was applied to a 2.8×59 cm column[4] packed with 25~40 μm LiChroprep RP-8 and the column was eluted at 4 ml/minute, collecting 27 ml fractions. The eluate was monitored at 254 nm. Selected fractions were analyzed for the presence of factor D by analytical HPLC (Fig. 3). Fractions 15~42 were combined, concentrated to 500 ml, and desalted as previously described. The eluate was concentrated and lyophilized to give 120 mg of pure factor D.

Purification of A41030E

A 0.3 g portion of the antibiotic complex was dissolved in 30 ml of H_2O - CH_3CN - NaCl (85 : 15 : 2 g/liter) and applied to a 2.8×59 cm column[4] packed with 25~40 μm LiChroprep RP-8. The column was eluted at 12 ml/minute with the same solvent used for sample dissolution, collecting 24 ml fractions. The eluate was monitored at 254 nm. Fractions 55~122 were combined and concentrated to 50 ml.

Concentrates from 13 similar purifications were combined, diluted to 1.5 liters with H_2O and desalted as previously described. The eluate was concentrated and lyophilized to give 1.04 g of a factor E enriched mixture of factors.

A 0.5 g portion of this mixture was dissolved in 10 ml of H_2O - CH_3CN - NaCl (86 : 14 : 2 g/liter). The solution was applied to a 2.8×59 cm column[4] packed with 25~40 μm LiChroprep RP-8. The column was eluted at 5 ml/minute with the same solvent used for sample dissolution, collecting 25 ml fractions. The eluate was monitored at 254 nm. Selected fractions were monitored for the presence of factor E by analytical HPLC (Fig. 3). Fractions 60~72 were combined and concentrated to 50 ml.

Concentrates from three similar purifications were combined, diluted to 1 liter, and desalted as previously described. The eluate was concentrated and lyophilized to give 202.2 mg of partially purified factor E.

This preparation was dissolved in 4 ml of H_2O - CH_3CN - NaCl (86 : 14 : 2 g/liter). The solution was chromatographed at 4 ml/minute on a 2.8×59 cm column[4] packed with 25~40 μm LiChroprep RP-8 as previously described. Fractions 82~100 were combined and concentrated to 50 ml.

Concentrates from three similar purifications were combined and desalted as previously described. The eluate was concentrated and lyophilized to give 242 mg of pure factor E.

Purification of A41030F

A 9.0 g portion of the antibiotic complex was dissolved in 200 ml H_2O - CH_3CN - NaCl (83 : 17 : 2 g/liter). The solution was chromatographed on the 4-liter Chromatospac Prep-100 reversed-phase column described in the purification of factor A. The column was eluted at 60 ml/minute with the same solvent used for sample dissolution, collecting 480 ml fractions. The eluate was monitored at 254 nm. Selected fractions were monitored for the presence of factor F by analytical HPLC (Fig. 3). Fractions 26~36 were combined and concentrated to 500 ml.

Concentrates from three similar purifications were combined and desalted as previously described. The eluate was concentrated and lyophilized to give 2.6 g of a factor F enriched mixture of factors.

A 500 mg portion of this preparation was dissolved in 10 ml of H_2O - CH_3CN - NaCl (84 : 16 : 2 g/liter) by adjustment to pH 7.0 with NaOH. The solution was applied to a 4.7×45 cm column[4] packed with 25~40 μm LiChroprep RP-18. The column was eluted at 6 ml/minute with the same solvent used for sample dissolution, collecting 24-ml fractions. The eluate was monitored at 254 nm. Selected fractions were analyzed for the presence of factor F by analytical HPLC (Fig. 3). Fractions 80~105 were combined and concentrated to 300 ml.

Concentrates from two similar purifications were combined and desalted as previously described. The eluate was concentrated and lyophilized to give 299 mg of pure factor F.

Purification of A41030G

An 8 g portion of the antibiotic complex was dissolved in 200 ml of H_2O - CH_3CN - NaCl (84 : 16 : 2 g/liter). The solution was chromatographed on the 4-liter Chromatospac Prep-100 reversed-phase column described in the purification of factor A. The column was eluted at 60 ml/minute with the same solvent used for sample dissolution, collecting 480 ml fractions. The eluate was monitored at 254 nm. Selected fractions were analyzed for the presence of factor G by analytical HPLC (Fig. 3). Fractions 22~35 were combined and concentrated to 500 ml.

Concentrates from three similar purifications were combined, adjusted to pH 8.5 with NaOH, and desalted as previously described. The eluate was concentrated and lyophilized to give 2.85 g of a factor G enriched mixture of factors.

A 0.5 g portion of this material was dissolved in 10 ml of H_2O - CH_3CN - dibutylamine (79.5 : 20 :

0.5), adjusted to pH 7.8 with phosphoric acid, by addition of dibutylamine until dissolution had been accomplished. The solution was applied to a 2.8×59 cm column[4] packed with 25~40 μm LiChroprep RP-8. The column was eluted at 4 ml/minute with the same solvent used for sample dissolution, collecting 10-ml fractions. The eluate was monitored at 254 nm. Selected fractions were analyzed for the presence of factor G by analytical HPLC (Fig. 3). Fractions 54~74 were combined with fractions from two similar purifications and desalted as previously described. The eluate was concentrated and lyophilized to give 960 mg of pure factor G.

Discussion

The A41030 complex of antibiotic factors are members of the glycopeptide class of antibiotics. The peptide nucleus is most closely related to that of actaplanin and teichoplanin. A41030F and G, which were inseparable by HPLC and TLC, were differentiated by fast atom bombardment mass spectrometry. A41030A, B, D and E are unique among the reported glycopeptides, in that they contain no sugar moieties.

Preparative low pressure liquid chromatography systems as described here have been used successfully in our laboratories for several years for the rapid and convenient purification of compounds needed for chemical analysis and biological evaluation. The systems have proven to be easy to use, efficient, reusable, time saving, reproducible, and economical.

References

1) BOECK, L. D.; F. P. MERTZ & G. M. CLEM: A41030, a complex of novel glycopeptide antibiotics produced by a strain of *Streptomyces virginiae*. Taxonomy and fermentation studies. J. Antibiotics 38: 1~8, 1985
2) HUNT, A. H.; J. L. OCCOLOWITZ, M. DEBONO, R. M. MOLLOY & G. M. MACIAK: A47934 and A41030 factors—new glycopeptides and glycopeptide aglycones: Structure determination. Program and Abstracts of the 23rd Intersci. Conf. Antimicrob. Agents Chemother., No. 441, p. 164, Las Vegas, Oct. 24~26, 1983
3) HUNT, A. H.; D. E. DORMAN, M. DEBONO & R. M. MOLLOY: Structure of Antibiotic A41030A. J. Org. Chem. 50: 2031~2035, 1985
4) Liquid Chromatography Catalog, 1983 Edition, Ace Glass Incorporated, Vineland, N.J., 08360 U.S.A.
5) MICHEL, K. H. & R. F. MILLER (Eli Lilly): Refillable columns for chromatography at elevated pressure. US 4,131,547, Dec. 26, 1978
6) EM Science, A Division of EM Industries, Inc., Cherry Hill, N.J., 08034-0395, U.S.A.
7) ABBOTT, B. J. & D. S. FUKUDA (Eli Lilly): A30912B Nucleus. U.S. 4,299,763, Nov. 10, 1981