Journal of Chromatography, 484 (1989) 381–390
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 938

PROCESS-SCALE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY PURIFICATION OF LL-E19020α, A GROWTH PROMOTING ANTIBIOTIC PRODUCED BY *STREPTOMYCES LYDICUS* SSP. *TANZANIUS*

D. R. WILLIAMS, G. T. CARTER, F. PINHO and D. B. BORDERS
American Cyanamid Company, Medical Research Division, Lederle Laboratories, Pearl River, NY 10965 (U.S.A.)

SUMMARY

LL-E19020α is a novel antibiotic produced by fermentation of the soil microorganism *Streptomyces lydicus* ssp. *tanzanius*. The compound is highly effective in inducing increases in weight gain and feed conversion efficiency in livestock. In order to obtain kilogram quantities of the material for field trials, pilot plant scale fermentations (up to 7500 l) were carried out. The antibiotic was recovered from the fermentation broth by solvent extraction. The resultant crude extract was subjected to reversed-phase (C_{18}) chromatography on a process-scale high-performance liquid chromatography (HPLC) unit. The heart of the instrumentation is the Millipore Kiloprep® chromatograph with the standard 12-l cartridge column. The laboratory housing the chromatograph has been specifically designed for this work. Tanks for mobile phase preparation are mounted on load cells for precise measurement of components. In this explosion-proof laboratory, all solvent handling areas are well ventilated and a separate breathing air system is provided for the operators. For the purification of the LL-E19020 antibiotics, the mobile phase consisted of a gradient of acetonitrile in 0.1 *M* ammonium acetate at pH 4.5. The effluent was monitored by UV absorbance at 325 nm. Fractions were collected across the peaks of interest and these were analyzed by analytical HPLC. The maximum yield of LL-E19020α obtained in a single run was approximately 100 g. The antibiotic was recovered from the mobile phase by extraction with methylene chloride. The methylene chloride phase was concentrated under reduced pressure to yield a gummy residue which was finally freeze-dried from tertiary butanol to yield an off-white solid suitable for blending with various feed components.

INTRODUCTION

Two recently discovered antibiotics designated LL-E19020α and LL-E19020β (Fig. 1)[1] are highly modified versions of the aurodox family[2]. The LL-E19020 antibiotics have an exceptionally narrow antimicrobial spectrum against human pathogens, showing meaningful activity *versus Streptococcus* species and certain anaerobes (minimum inhibitory concentration, MIC 0.1–1 μg/ml). These compounds

0021-9673/89/$03.50

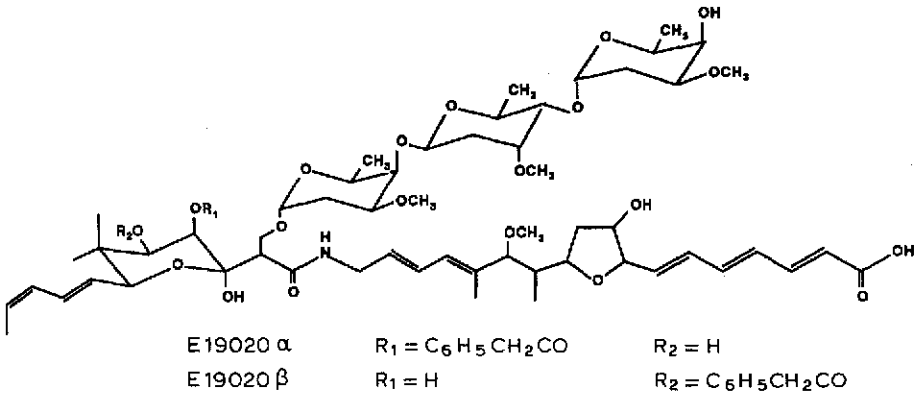

Fig. 1. Structures of the LL-E19020 antibiotics.

are highly effective as growth promoting agents in animals, producing superior performance in chicks during comparison trials with bacitracin and virginiamycin. The unique structural features of these compounds, including the hydroxymethyl-linked trisaccharide and shortened carbon skeleton ending in a carboxylic acid, may explain their greatly enhanced growth promoting activity relative to aurodox. After our initial structure reports[3,4], the structures of phenelfamycins E and F were revised[5] indicating that they were identical to E19020α and β, respectively.

The compounds are isomers of molecular weight 1225 with very similar physico-chemical properties (Table I). The only difference between the two is that LL-E19020α has the phenyl acetate ester linked at C-23, whereas LL-E19020β has this ester group at C-24 (see Fig. 1).

Interest in providing relatively pure kilogram quantities of each antibiotic for field trials in a short period of time necessitated an evaluation of resources and possible methods for achieving this goal. Development of a new chemical isolation process at

TABLE 1
CHARACTERIZATION DATA FOR LL-E19020α AND β

	α	β
Molecular formula	$C_{65}H_{95}NO_{21}$	$C_{65}H_{95}NO_{21}$
Molecular weight	1225	1225
Molecular ion $[M+Na]^+$ [a]	1248.6187	1248.6193
	(Calc. 1248.6270)	
$[\alpha]_{26}^{D}$ (methanol)	−8° (1.0%)	−17° (0.46%)
UV absorbance (nm) methanol	233 (ε 49800)	233 (ε 47000)
	290 (ε 36600)	290 (ε 34100)
IR absorbance (cm^{-1}) (KBr)	3420 1617	3430 1543
	2970 1525	2970 1454
	2925 1445	2930 1367
	1717 1365	1712 1265
	1695 1092	1648 1098
	1647 1018	1620 1020

[a] Determined with high-resolution fast atom bombardment mass spectrometry.

this stage of the project was rejected as being too lengthy and labor intensive. The Millipore Kiloprep® had recently been installed and was operational. Laboratory experience suggested that direct scale-up of purification of the crude LL-E19020 mixture by preparative high-performance liquid chromatography (HPLC) should work. The decision to implement preparative HPLC, therefore, was made and proved to be the most efficacious method of meeting our goal.

EXPERIMENTAL

Fermentation

Isolation of LL-E19020α and LL-E19020β (Fig. 2) begins with the fermentation of the soil microorganism *Streptomyces lydicus* ssp. *tanzanius*[6] in a complex medium containing dextrin, a soy product, corn steep liquor and calcium carbonate for pH control. Inoculum build-up proceeded through three stages beginning with a shaker flask (100 ml culture) and culminating in a 300-l stirred fermentor. After inoculation,

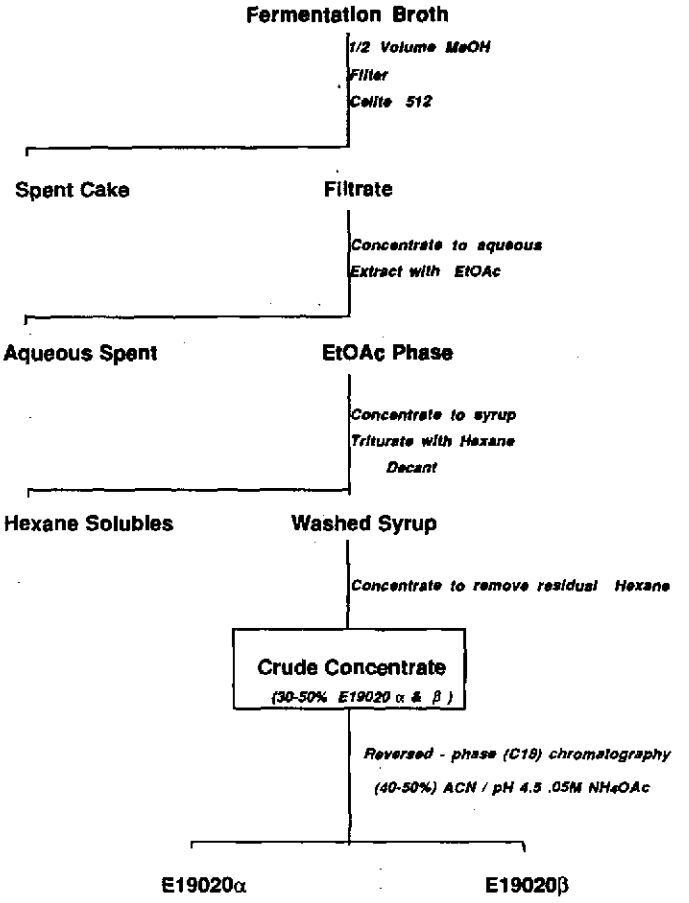

Fig. 2. Isolation and purification of LL-E19020α and β. MeOH = Methanol; EtOAc = ethylacetate; ACN = acetonitrile; NH_4OAc = ammonium acetate.

the large-scale fermentation was carried out at 28–30°C in 1500 and/or 3000-l fermentor(s) for five to six days. Aeration was set at 0.67 VVM (= volume of air per volume of fermentation broth per minute) and agitation was sufficient to maintain dissolved oxygen levels above critical levels at all times. The fermentors were then harvested for refining. Harvest potencies for LL-E19020α averaged approximately 300 μg/ml and approximately 100 μg/ml for LL-E19020β as determined by analytical HPLC.

Fermentation processing

The initial processes in the purification of this fermentation product were recovery of the material from the broth and concentration to a workable volume. In this example, recovery and concentration were achieved through solvent extraction and evaporation as follows. The harvested fermentation mash (3200 l) was mixed with toluene (45 l) for 30 min. Methanol (1600 l) was added and the suspension was stirred for 1 h. Celite 512 filter aid (150 lbs) was then added and mixing was continued for 15 min. The suspension was filtered through a filter press and washed with 320 l of water. The press cake was discarded. The combined filtrate and wash were concentrated under reduced pressure from 5000 to 2950 l. Ethyl acetate (1500 l) was added to the concentrate and the mixture was stirred for 2 h. Separation of the aqueous phase left approximately 900 l of ethyl acetate phase containing the antibiotics, which was concentrated under reduced pressure to 80 l. To this concentrate was added ethyl acetate (190 l) and the mixture was stirred for 1 h. The aqueous phase (20 l) was removed and the ethyl acetate phase concentrated under reduced pressure to 1–2 l. In the lab, this concentrate was allowed to further evaporate in the hood to a thick black syrup. The syrup was washed repeatedly with a equal volumes of hexane, which was decanted and discarded, to remove fats, oils and antifoam agent prior to reversed-phase chromatography. The final preparation, 2 l of thick black syrup, contained 0.385 g/ml E19020α and 0.136 g/ml E19020β. A typical analytical chromatogram of LL-E19020 crude extract is shown in Fig. 3. The ratio of α and β components varies in individual fermentations from about 1:1 to 9:1, depending upon the conditions selected.

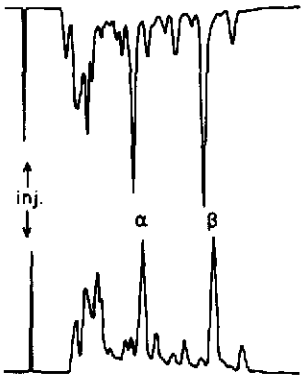

Fig. 3. Analytical HPLC of defatted crude LL-E19020; system A (chart speed 0.25 cm/min).

PROCESS-SCALE CHROMATOGRAPHY

General

Operations in the analytical laboratory which are routine and taken for granted become significant problems to be overcome in process-scale chromatography. The building housing the Millipore Kiloprep is designated as an explosion-proof area (class I, division 1, group D), wich means that all equipment must meet explosion proof standards. Even the hand tools used to open the various 55-gallon solvent drums must be made of non-sparking material. Electrical power is run in sealed conduit, enclosures, and fixtures. Pneumatically operated pumps and agitators are also in use. Instrumentation is either intrinsically safe or is housed in explosion-proof enclosures. All equipment is grounded and portable grounds are available for movable equipment and solvent drums to prevent an explosion due to a static spark. A fire alarm, emergency lighting, and sprinkler system are installed.

The building has an extensive ventilation system from which the airflow inwards is ballanced. The ventilator system to the walk-in hood, the laboratory bench, and the sink area is equipped with high-efficiency particulate air (HEPA) filters to prevent the release of toxic material (if any) to the environment. Portable ventilator trunks are strategically located wherever open solvent containers could be in use. The ventilation system is also monitored and controlled by the fire alarm system.

A breathing air system is available for use, not only in the process chromatography building, but throughout the entire pilot plant complex. An oil-free air compressor linked with an air purification and alarm system provides breathable air to conveniently located 3M filter regulator panels (W-2806). The operator plugs in his or her air hose and is supplied cooled or heated (as needed) breathable air to the 3M white cap helmet.

Other services provided to the building include compressed air to operate the pumps and agitators and nitrogen to inert the process tanks when pumping flamable solvents. The process tanks are vented to the atmosphere and use the appropriate flame arresters. Nitrogen is also used to inert the detector enclosure on the chromatograph. High-pressure steam is used to operate the two-stage steam ejector system (Kinema) which provides vacuum for routine use and to operate two Buchi 20-l rotary evaporators. The steam condensate is removed by an automatically pumped condensate system. For the storage of process samples and product, *etc.*, a roof-mounted refrigeration unit is used to provide cooling ($5 \pm 2°C$) for an explosion-proof chill room. A Millipore Super-Q system coupled with the Millipore Continental Water system de-ionized water system delivers high-quality de-ionized water for chromatographic buffer system preparation. The de-ionized water is piped to the process tanks in the process chromatography building and also the main pilot plant.

System preparation

There are three process tanks (640 l each) associated with the chromatograph. They are identical with the exception that the "prep tank" has an agitator. All tanks are mounted on load cells and therefore can only have flexible couplings to the tanks. The load cells effectively weigh each tank and shows each reading on individual explosion-proof light-emitting diode (LED) displays. It is therefore important to make the necessary corrections for the density of solvents different from water. The aqueous

buffer system (0.1 *M* ammonium acetate at pH 4.5) is prepared in the prep tank as follows: 519 l of de-ionized water are drawn in and 4 kg of reagent-grade ammonium acetate are dissolved with mixing. The pH is adjusted to 4.5 with HPLC-grade acetic acid. The buffer system is then pumped through a Millipore Rogard® cartridge filter to tank A, which feeds one inlet of the Kiloprep unit. ACS reagent-grade acetonitrile is then pumped either directly or through the filter system into the other tank, tank B, which feeds a separate inlet to the chromatograph. The valving, piping, pumping, and filtration system have been designed so that it is possible to transfer and/or filter from any tank to any tank. In addition, the dual Rogard cartridge filter assembly is set up in parallel so one cartridge can be changed while the other is in use.

Operation of the process chromatograph

The A and B tank inlets to the unit are each connected to recycle pumps and back-pressure valves on the unit, which continuously recycle the solvent or buffer system to its respective tank. This provides a constant-pressure feed for the solvent/buffer delivery system regardless of the tank volumes. As the A and B tanks are recycling their respective contents, a microprocessor-controlled system of check and solenoid valves sample the two liquid streams to give the desired composition of acetonitrile–0.1 *M* ammonium acetate of pH 4.5 (45:55), initially. This mixture is pumped using the Bran & Lubbe pump (variable from 0 to 5 l/min) through the 3-l guard column and the standard Millipore 12-l cartridge, each packed with 55–105-μm μBondapak C_{18}. A system of valves and tubing allows flow to be directed to waste or to fraction collection bottles. Also, a small portion of the flow (30 ml/min) is diverted to the detector to aid in fraction collection. The column was equillibrated for 0.5 h before the charge (400 ml of defatted syrup described above dissolved in 700 ml methanol) was injected. The initial mobile phase consisted of acetonitrile–0.1 *M* ammonium acetate of pH 4.5 (45:55) flowing at 1.6 l/min. Following elution of the LL-E19020α, a linear gradient was run, increasing the acetonitrile content from 45 to 65% in 60 min. During this gradient elution, the flow-rate was maintained at 2.0 l/min. The chromatographic effluent was monitored with a UV absorbance detector set at 325 nm at 2.0 a.u.f.s. deflection. Throughout the chromatography, fractions were collected at 10-min intervals as noted on the chart in Fig. 4. These fractions were assayed by the analytical HPLC method described below. Based upon these measurements for the run shown in Fig. 4, fractions 8–11 were combined as LL-E19020α and 17–20 were combined as LL-E19020β.

Fraction processing

These antibiotics were recovered from the mobile phase by first concentrating the respective fractions to half their original volume under reduced pressure by using the 20-l Buchi rotary evaporators. The antibiotic was then partitioned into one half volume of reagent-grade methylene chloride. The methylene chloride extracts of the individual antibiotics were evaporated and the residues redissolved in warm *tert.*-butyl alcohol. The solvent was removed by lyophilization to yield light tan to light yellow solids. Analytical results for three separate finished batches of the α component, representing 20 chromatographic runs, are presented in Table II. Typical overall recoveries of LL-E19020α through processing and chromatography are summarized in Table III. Analytical HPLC chromatograms of typical LL-E19020α and β material obtained in this manner are shown in Fig. 5.

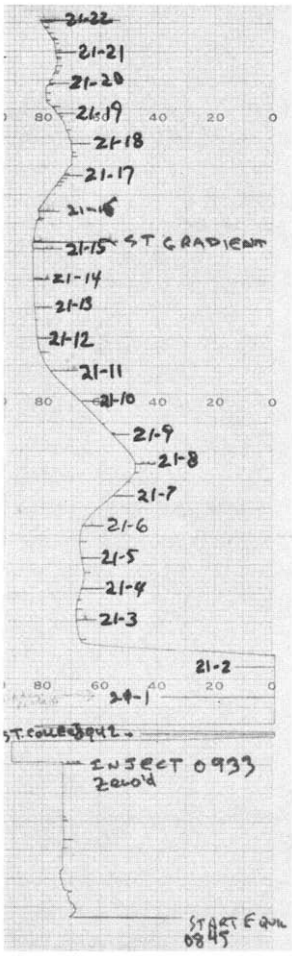

Fig. 4. Typical chromatogram from Kiloprep run. Fractions (21-1 to 22) were collected at 10-min intervals.

Analytical HPLC system

Analytical chromatography was performed with a C_{18} column (μBondapak 10 μm, 100 × 8 mm I.D.) eluted with either mobile phase A [acetonitrile–0.1 *M* ammonium acetate of pH 4.5 (60:40)] at 0.5 ml/min, or B [acetonitrile–0.1 *M* ammonium acetate of pH 4.5 (45:55)] at 1.5 ml/min. Detection was by UV absorbance at 254 nm (lower trace in the figures) and 280 nm (upper trace in the figures). Retention times for α and β in system A were 6.0 and 9.9 min, respectively, and in B 8.0 and 19.3 min, respectively.

RESULTS AND DISCUSSION

The solvent extraction process outlined in the flow-chart of Fig. 2 results in recovery of approximately 80% of the antibiotics as a syrupy concentrate (Table III).

TABLE II
ANALYTICAL FINDINGS FOR LL-E19020α

	Batch 1	*Batch 2*	*Batch 3*
Description	Tan powder	Light yellow powder	Light yellow powder
Batch size (g)	400	356	800
Purity (%), as is	86.3	79.3	84.0
anhydrous	87.4	81.4	85.0
Identification confirmed by UV, IR, ^{1}H-NMR, ^{13}C-NMR, Mass spectrometry	yes	yes	yes
Composition (%)			
LL-E19020α	86.3	79.3	85.0
Impurities	8.2	16.0	11.7
Volatiles	2.8	2.5	1.4
Water (Karl Fischer)	1.09	2.09	0.96
Ash	0.1	0.08	0.02
$[\alpha]_{26}^{D}$ (1% in methanol)	−10°		−8°

Trituration of this viscous material with hexane removes the non-polar by-products of the fermentation as well as any silicone oil antifoam agent added during the fermentation process. This step was included to prolong the useful lifetime of the

TABLE III
TYPICAL YIELDS OF LL-E19020α AND β

	Yield			
	Grams			%
	α	β	*Total*	
Fermentation				
6000 l Broth (3 fermentors)	1020	889	1909	100
Crude refining				
Filtration step (9531 l methanol filtrate and wash)	1005	717	1722	90.2
Solvent extraction steps (3.64 l of syrup)	929	606	1535	80.4
Process-scale chromatography (1 run)				
Charge derived from similar runs (after defatting)	62	40.3	102.3	
Chromatographic fraction processing and lyophilization				
Weight solids (approx. 80% pure)	(39.3)	(36.3)	(75.7)	
weight × 80% purity	31.5	29.6	60.5	
Overall chromatographic yield (Chromatography to LL-E19020)	50.8%	72.1%	59.2%	
Estimated overall yield (fermentation to LL-E19020)			47.6%	

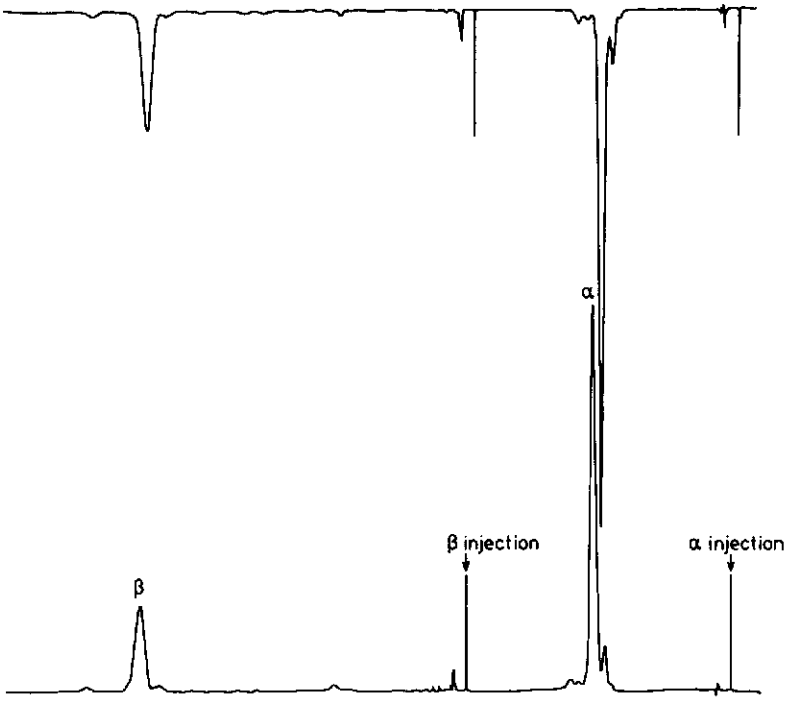

Fig. 5. Analytical HPLC of lyophilized LL-E19020α and LL-E19020β; system B (α retention time 80 min; β retention time 19.3 min).

reversed-phase columns. The defatted crude material contained between 30 and 50% (w/w) antibiotic (α + β), depending on the productivity of a particular fermentation, medium constituents used and by-products produced. The ratio of α to β was also higly dependent on the fermentation conditions employed and was found to vary between about 1:1 and 9:1.

The preparative HPLC procedure represents a nearly direct scale-up from the analytical system developed to monitor titers of the antibiotics during the fermentation. Attempts were made to replace acetonitrile with methanol as the organic modifier in the mobile phase to reduce the cost of the process, however no alternative system was found to produce equivalent chromatographic results. Buffering the mobile phase at pH 4.5 was essential for good resolution and also enhanced the stability of the material. At significantly lower pH's decomposition is evident and at pH greater than 8 equilibration of the α and β forms occurs as well as decomposition. Loadings for the preparative separation were determined empirically by gradual increases in the amount of crude extract charged onto the column and monitoring the effect of each successive increment on the quality of the separation achieved. In order to obtain material consistently with 85% purity, no more than about 500 g of the crude syrup (containing up to 150 g of α and a lower variable amount of β) could be used. Owing to solubility constraints, the crude had to be dissolved in methanol. To obtain the required resolution, no more than 1200 ml of solution could be used for an injection. Flow-rates were maintained between 1 and 2 l/min primarily to minimize

back pressure. Within this range, variations in the flow-rate had little effect on the chromatographic resolution.

The analytical profiles of the 3 batches of LL-E19020α presented in Table II show that 80–85% material was routinely obtained. Overall recovery from broth to final product approached 50% (Table III). The most common volatile impurities found in the batches were acetic acid from the mobile phase and *tert.*-butanol from incomplete lyophilization. The impurities listed in Table II ranged from 8.2 to 16.0% between batches. These are evidently related components, since their UV spectra show very similar chromophores to LL-E19020α and β. The identities of these minor components are still under investigation.

In general, preparative HPLC provides a method of obtaining relatively pure material in a timely fashion. Once the operating parameters were defined there was very little fine-tuning required from run to run. The availability of a chromatograph with this capacity, which conformed so closely with the analytical separation, made it possible to achieve the production goals for these experimental compounds within a short and predictable timeframe.

In terms of chromatographic hardware, we are continuing to upgrade the preparative HPLC operation for increased control and safety. The electrically operated transfer pumps have been replaced with safer air-operated pumps. The determination of mobile phase concentration will be facilitated with the installation of a mass flow meter. Currently the difference in weight *versus* time of the load cell readings corrected for density is used. The mass flow meter should give "instantaneous" readings. It is hoped to install a new UV detector with a variable-path flow cell so that "on stream" detection will alleviate some of the need for analytical HPLC support from the laboratory.

ACKNOWLEDGEMENTS

The skillful assistance of Ms. J. A. Nietsche and Mr. J. L. Baker for providing chromatographic support is gratefully acknowledged. Mr. J. D. Korshalla and Mr. K. A. Cote are thaked for providing additional chromatographic support. They also provided microbiological testing and maintained the fermentation processes. The staff of both the Fermentation and Refining Pilot Plants are thanked for their assistance. In addition, Drs. J. C. James and L. Gehrlein are acknowledged for providing analytical services on the lyophilized LL-E19020α.

REFERENCES

1 G. T. Carter, D. W. Phillipson, J. J. Goodman, T. S. Dunne and D. B. Borders, *J. Antibiot.*, 41 (1988) 1511–1514.
2 H. Maehr, M. Leach, T. H. Williams and J. F. Blount, *Can. J. Chem.*, 58 (1980) 501–526.
3 G. T. Carter, in A. L. Demain, G. A. Somkuti, J. C. Hunter-Cevera and H. W. Rossmoore (Editors), *Novel Microbial Products for Medicine and Agriculture*, Society for Industrial Microbiology, Amsterdam 1989, Ch. 26, pp. 233–237.
4 G. T. Carter, D. W. Phillipson, T. S. Dunne, D. R. Williams, F. Pinho, J. A. Nietsche, J. J. Goodman, M. J. Torrey and D. B. Borders, *presented at the 28th Interscience Conference on Antimicrobial Agents and Chemotherapy, Los Angeles, CA, 1988*, abstract 312.
5 J. E. Hochlowski, M. H. Buytendorp, D. N. Whittern, A. M. Buko, R. H. Chen and J. B. McAlpine, *J. Antibiot.*, 41 (1988) 1300–1315.
6 W. M. Maiese, M. P. Lechevalier, H. A. Lechevalier, J. D. Korshalla, J. J. Goodman, M. J. Wildey, N. A. Kuck, S. D. Conner and M. Greenstein, *J. Antibiot.*, in press.