APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1994, p. 31–38
0099-2240/94/$04.00+0

Vol. 60, No. 1

Structural and Immunological Characterization of a Biosurfactant Produced by *Bacillus licheniformis* JF-2

SUNG-CHYR LIN,[1] MARK A. MINTON,[2] MUKUL M. SHARMA,[3] AND GEORGE GEORGIOU[1]*

Departments of Chemical Engineering,[1] Chemistry,[2] and Petroleum Engineering,[3] The University of Texas at Austin, Austin, Texas 78712

Received 30 August 1993/Accepted 28 October 1993

***Bacillus licheniformis* JF-2 produces a very active biosurfactant under both aerobic and anaerobic conditions. We purified the surface-active compound to homogeneity by reverse-phase C_{18} high-performance liquid chromatography and showed that it is a lipopeptide with a molecular weight of 1,035. Amino acid analysis, fast atom mass and infrared spectroscopy, and, finally, ^{1}H, ^{13}C, and two-dimensional nuclear magnetic resonance demonstrated that the biosurfactant consists of a heterogeneous C_{15} fatty acid tail linked to a peptide moiety very similar to that of surfactin, a lipopeptide produced by *Bacillus subtilis*. Polyclonal antibodies were raised against surfactin and shown to exhibit identical reactivity towards purified JF-2 lipopeptide in competition enzyme-linked immunosorbent assays, thus providing further evidence for the structural similarity of these two compounds. Under optimal conditions, the *B. licheniformis* JF-2 biosurfactant exhibits a critical micelle concentration of 10 mg/liter and reduces the interfacial tension against decane to 6×10^{-3} dyne/cm, which is one of the lowest interfacial tensions ever reported for a microbial surfactant.**

Microbial compounds which exhibit pronounced surface activity are classified as biosurfactants. Microbial biosurfactants include a wide variety of surface- and interfacially active compounds, such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids (6). Biosurfactants consist of distinct hydrophilic and hydrophobic moieties. The former can be either ionic or nonionic and consist of mono-, di-, or polysaccharides; carboxylic acids; amino acids; or peptides. The hydrophobic moieties are usually saturated, unsaturated, or hydroxylated fatty acids. Biosurfactants are easily biodegradable and thus are particularly suited for environmental applications such as bioremediation and the dispersion of oil spills (8, 20–22).

Among the many classes of biosurfactants, lipopeptides are particularly interesting because of their high surface activities and therapeutic potential. For example, surfactin, a well-studied lipopeptide antibiotic produced by *Bacillus subtilis*, is not only a very effective biosurfactant (5) but is also an inhibitor of fibrin clotting (1, 3) and cyclic AMP phosphodiesterase (10).

Bacillus licheniformis JF-2, isolated from oil-field injection brine (13), has been shown to be able to grow and produce a very effective biosurfactant under both aerobic and anaerobic conditions at a very wide range of temperatures and in the presence of high concentrations of salts (12, 17). We have studied the effects of various environmental parameters on the production of the biosurfactant, the formation of fermentation end products, and the growth of *B. licheniformis* JF-2 in batch cultures (17, 18). Although it has been speculated that the JF-2 biosurfactant is similar to surfactin from *B. subtilis* (7, 19), the chemical structure of the JF-2 biosurfactant had not been characterized. Recently, two other *Bacillus* isolates, *B. licheniformis* 86 (9) and another *B. licheniformis* strain isolated by Jenny et al. (14), have been shown to produce lipopeptides with peptide moieties containing C-terminal amino acid residues different from those of surfactin.

In this study, we purified the surface-active compound from *B. licheniformis* JF-2 to apparent homogeneity. The structure of this compound was characterized by amino acid analysis and various spectroscopic techniques. As part of the characterization studies, we raised polyclonal antibodies against surfactin and showed that they exhibit the same reactivity for the JF-2 surfactant. This result together with amino acid analysis and two-dimensional nuclear magnetic resonance (NMR) data indicated that the peptide sequence of the JF-2 surfactant is identical to that of surfactin. However, the two compounds differ with respect to the composition of their fatty acid tails. In addition to the chemical characterization of the JF-2 lipopeptide, we studied its interfacial properties and found that under optimal conditions the JF-2 lipopeptide is one of the most effective bacterial lipopeptide surfactants known.

MATERIALS AND METHODS

Microorganisms and growth conditions. *B. licheniformis* JF-2 (ATCC 3097) was obtained from the American Type Culture Collection (Rockville, Md.). The bacteria were grown aerobically in a mineral salt medium (13), containing 0.1% $(NH_4)_2SO_4$, 0.025% $MgSO_4$, 1% (wt/vol) glucose, 0.5% NaCl in 100 mM phosphate buffer (pH 7.0), and 1.0% (vol/vol) trace metals solution, in 2-liter Erlenmeyer flasks with a working volume of 1 liter at 42°C for 15 h. The trace metals solution contained 0.1% (wt/vol) EDTA, 0.3% $MnSO_4$, 0.001% $FeSO_4$, 0.01% $CaCl_2$, 0.01% $CoCl_2$, 0.01% $ZnSO_4$, 0.001% $CuSO_4$, 0.001% $AlK(SO_4)_2$, 0.001% H_3BO_4, and 0.001% Na_2MoO_4 (4).

Isolation and purification. The cells were removed from the culture by centrifugation at 8,000 × *g* for 15 min. Surface-active compounds were then isolated from the clear broth either by acid precipitation with concentrated HCl at pH 2.0 (7) or by XAD-2 (Sigma, St. Louis, Mo.) adsorption chromatography. For the former, the surfactant-containing

* Corresponding author. Mailing address: Department of Chemical Engineering, The University of Texas at Austin, Austin, Texas 78712. Phone: (512) 471-6975. Fax: (512) 471-7963. Electronic mail address: gg@mail.che.utexas.edu.

precipitate was collected by centrifugation and resuspended in 15 ml of water adjusted to pH 6.0 and subsequently lyophilized. The lyophilized material was then extracted with 5 ml of a mixture of chloroform and methanol (1:2 [vol/vol]). For XAD-2 adsorption chromatography, the fermentation broth supernatant was loaded onto a column (16 by 500 mm) at a flow rate of 1 ml/min. The column was eluted with 1.5 bed volume of methanol following a 1.5 bed volume of water wash. Nonvolatile material in the eluent was concentrated by evaporation in a rotary evaporator at 45°C. Samples obtained by either procedure were designated as the crude biosurfactant preparation. The crude biosurfactant preparation was dissolved in 3 ml of a mixture of chloroform and methanol (1:1) and further separated into five fractions by liquid chromatography on a silica gel (no. 62; Mallinckrodt, Paris, Ky.) column (28 by 500 mm) eluted with 5% methanol in chloroform at a flow rate of 2 ml/min. The fraction containing the surface-active component was identified by interfacial tension measurements, as described below. The active compound was purified to homogeneity by preparative reverse-phase liquid chromatography at room temperature with a Waters high-performance liquid chromatography (HPLC) system (Milford, Mass.) equipped with a Waters C_{18} μBondapak column (19 by 300 mm). The solvent system consisted of mobile phase A (10 mM KH_2PO_4 buffer at pH 6.0) and mobile phase B (20% tetrahydrofuran in acetonitrile [HPLC grade; Fisher Scientific, Fair Lawn, N.J.]). The column was developed with 53% B isocratically at a flow rate of 2 ml/min. Biosurfactant-containing fractions were lyophilized and extracted with methanol to remove salt. For analytical reverse-phase C_{18} HPLC analysis, a μBondapak C_{18} column (7.8 by 300 mm) was used at a flow rate of 0.5 ml/min. The A_{210} of the eluent was monitored.

Characterization. Infrared spectroscopy was performed on a Nicolet 60SXR FT-IR spectrometer. The spectrum was measured in a sample compartment purged for at least half an hour with dry nitrogen before acquiring data, which were measured at a resolution of 4 cm^{-1} and were averaged over 500 scans. Baselines were electronically adjusted to zero absorbance for the measurement of spectral intensities.

For amino acid analysis, the purified biosurfactant was hydrolyzed in 6 M HCl at 105°C for 24 h. The hydrolysate was subjected to an Applied Biosystems 420H Derivatizer/Analyzer with on-line 130A Separation System and 920A Data Analysis Module (Foster City, Calif.). Norleucine was added in the samples to a final concentration of 500 pM as an internal standard.

Fast atom bombardment-mass spectroscopy (FAB-MS) analysis was performed on a Finnigan TSQ 70 mass spectrometer with an NBA matrix. Mass spectra were collected from 100 to 1,200 AMU. Positive ions were detected.

NMR analysis (^1H, ^{13}C, COSY, TOCSY, and ROESY NMR) of JF-2 biosurfactant was performed in a Bruker high-field (11.9-T) NMR spectroscope, with 1,2-dideuteriotetrachloroethane ($CDCl_2CDCl_2$) (Norell, Landisville, N.J.) as solvent at 348 K.

Production of polyclonal antibodies against surfactin. A total of 2 mg of surfactin (Calbiochem, San Diego, Calif.), dissolved in a 400-μl mixture of phosphate-buffered saline (PBS) and dimethylformamide (PBS-dimethylformamide [3:1]), was mixed with 300 μl of Pierce Imject keyhole limpet hemocyanin (KLH)–100 μl of 1 M [1-ethyl-3-(dimethylamino)propyl]-carbodiimide–50 μl of 0.1 M *N*-hydroxyl-sulfosuccinimide (Pierce, Rockford, Ill.) at room temperature. The conjugation reaction was allowed to proceed for 20 min and subsequently quenched by the addition of 3.0 ml of 50 mM glycine (11). The conjugate was dialyzed against PBS overnight and used to immunize rabbits by subcutaneous injection with incomplete Freund's adjuvant (Sigma) on the basis of a standard schedule (2). Blood samples were collected from the ear vein 10 weeks after immunization (2 weeks after the booster injection). Sera were prepared by centrifuging the blood samples at 5,000 × *g* for 10 min.

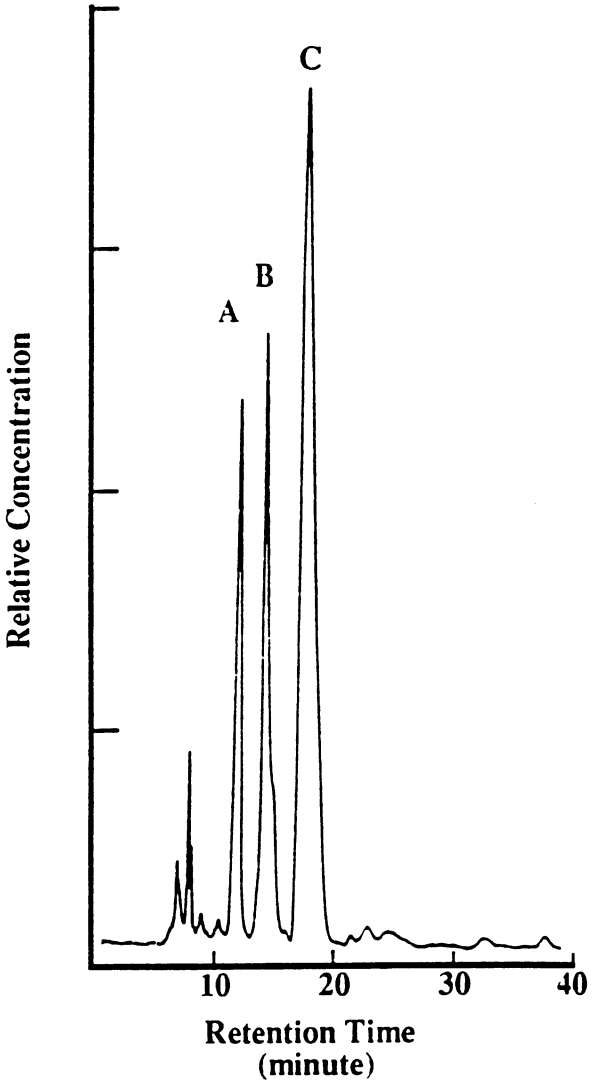

FIG. 1. Analytical reverse-phase C_{18} HPLC chromatogram of surface-active material obtained from silica gel liquid chromatography. Fraction C was identified as the most active component by interfacial tension measurements.

ELISA. Microtiter plates were coated by an overnight incubation at 37°C with a surfactin-ovalbumin conjugate which was prepared in the same procedure as the surfactin-KLH conjugate used for immunization. The plate was then blocked with 3% bovine serum albumin (BSA) in PBS by incubation at 37°C for 3 h. For competition enzyme-linked immunosorbent assays (ELISAs), 50 μl of diluted rabbit surfactin-specific serum (1:1,000 in 3% BSA in PBS) and 100 μl of sample were preincubated at 37°C for 2 h. Subsequently, the mixture was transferred to microtiter plates precoated with the surfactin-ovalbumin conjugate and incu-

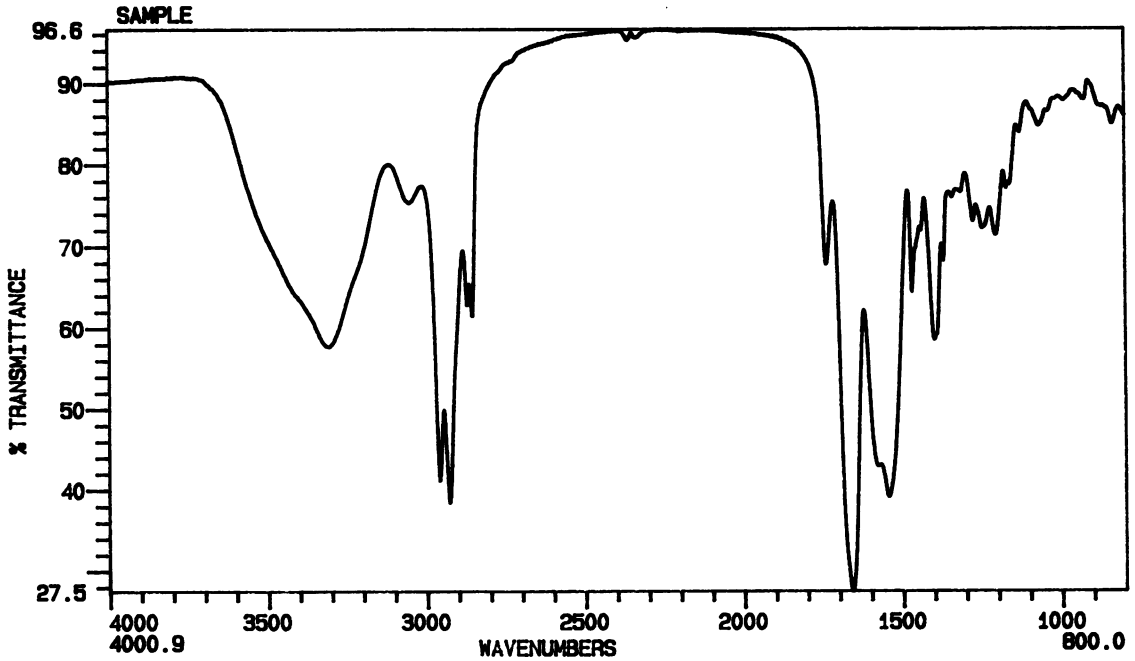

FIG. 2. The infrared spectrum of the JF-2 biosurfactant.

bated for an additional 2 h. A 3% BSA solution in PBS and surfactin at a concentration of 25 mg/liter also in PBS were used as negative and positive controls, respectively. Plates were washed with deionized water 10 times; washing was followed by the addition of 100 μl of diluted goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate diluted 1:1,000 in 3% BSA in PBS (Bio-Rad, Richmond, Calif.). The plates were incubated for 2 h at 37°C and washed again as described above. A total of 100 μl of substrate, 1-Step ABTS (Pierce), was then added to each well. The enzyme reaction was allowed to proceed for 10 min and then stopped by the addition of 50 μl of 1% sodium dodecyl sulfate (SDS). The A_{405} of the solution in each well was measured with a Dynatech MR300 microtiter plate reader (Chantilly, Va.).

To quantify the binding affinities of the serum towards surfactin and the JF-2 biosurfactant, the percent inhibition of antibody-conjugate binding by free antigens is defined as follows (23): percent inhibition = $\{1 - [(A_{Exp} - A_{Neg})/(A_{Pos} - A_{Neg})]\} \times 100$, where A_{Exp} is the A_{405} of the sample, A_{Pos}

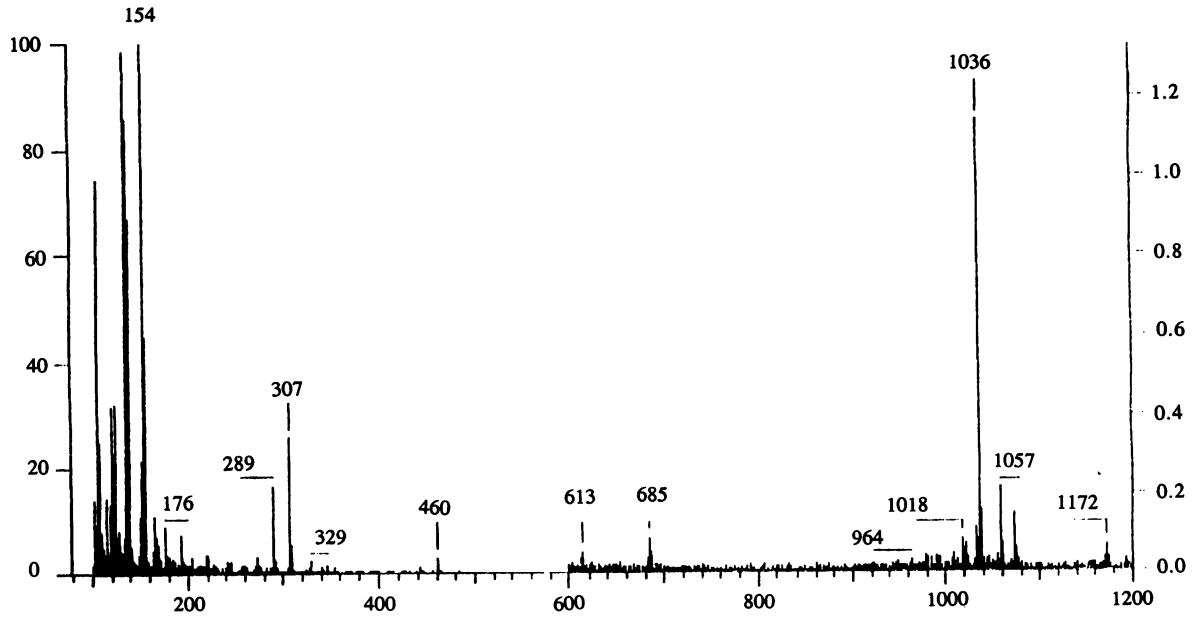

FIG. 3. The FAB-MS spectrum of the JF-2 biosurfactant.

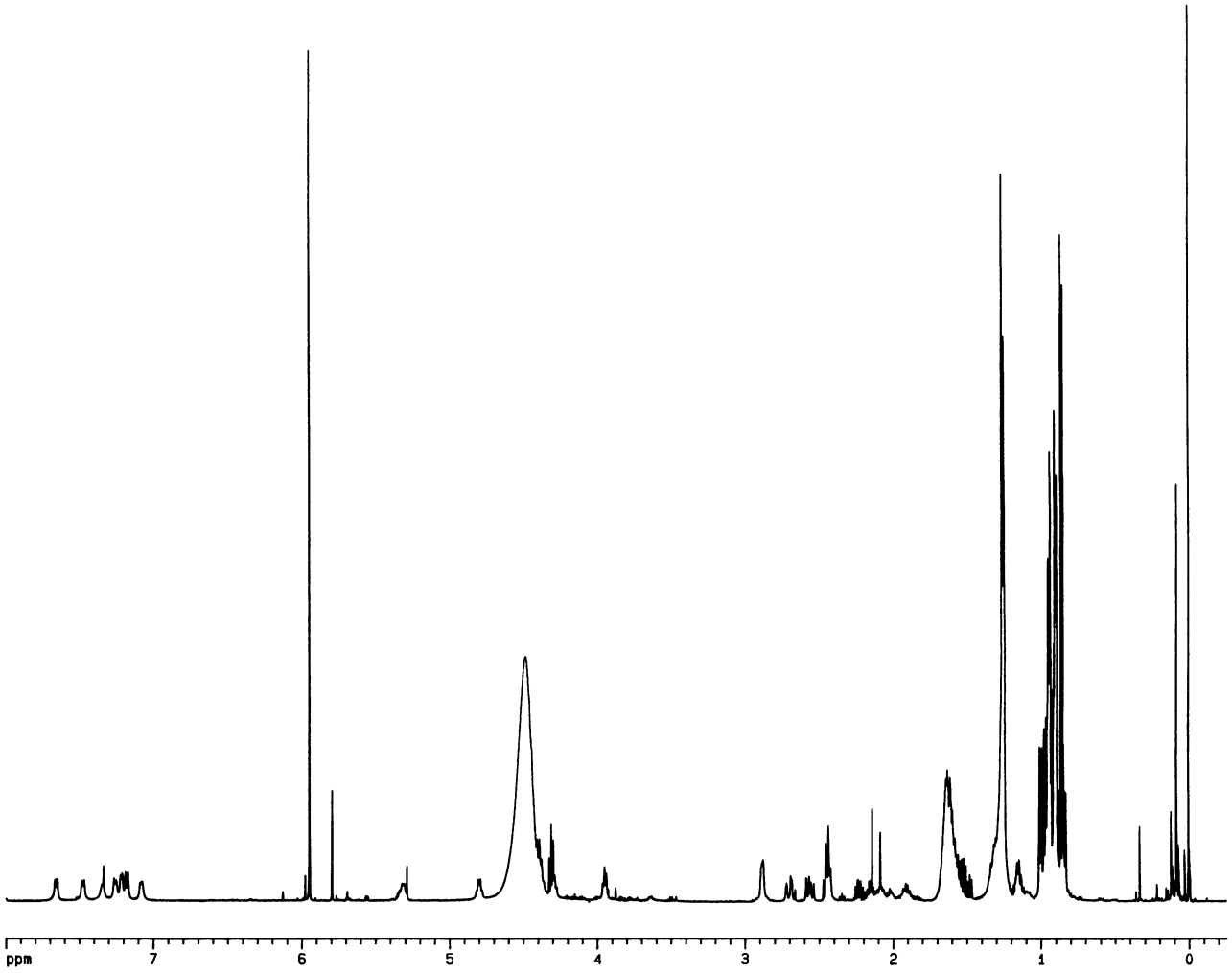

FIG. 4. Proton NMR spectrum of the JF-2 biosurfactant.

is the A_{405} of the positive control (colorless), and A_{Neg} is the A_{405} of the negative control (green).

Interfacial properties. Interfacial tension measurements against decane were performed in a spinning drop interfacial tensiometer (Model 300; University of Texas, Austin). The critical micelle concentration (CMC) was determined by measuring the interfacial tension of the biosurfactant solution following serial dilution. All interfacial tensions were measured against decane.

RESULTS

Isolation and purification. Interfacial tension measurements indicated that the biosurfactant can be effectively isolated from the cell-free culture supernatant by either acid precipitation or XAD-2 adsorption chromatography. The interfacial tension increased from 0.085 dyne/cm for the cell-free culture to more than 25 dyne/cm for the reneutralized acid precipitation supernatant of the culture. When acid precipitation was used as the first purification step, 250 mg of acid precipitate was obtained per liter of culture after lyophilization. Subsequently, 110 mg of water-soluble material remained after organic extraction. The crude biosurfactant preparation was further separated into five fractions by silica gel chromatography. Only one fraction, which was eluted after 1.8 bed volumes, contained surface active material. Analytical reverse-phase C_{18} HPLC analysis showed the presence of three major peaks in this material (Fig. 1). Preparative reverse-phase C_{18} HPLC was used to isolate three fractions corresponding to the material in each of the three peaks. Interfacial tension measurements indicated that fraction C contained the most surface active compound. At a concentration of 50 mg/liter in fresh medium with 5% NaCl and adjusted to pH 6.0, the material obtained from fractions A, B, and C gave interfacial tensions against decane of 1.317, 1.873, and 0.060 mN/cm, respectively. Material from fraction C was extracted with an equal volume mixture of chloroform and methanol to remove the salt from the HPLC mobile phase. Approximately 25 mg of the highly active compound per liter of culture was obtained. Since the concentration of the surfactant in the fermentation broth is 34 mg/liter, the overall yield is approximately 70%. The purified material gave a single peak in analytical reverse-phase C_{18} HPLC (data not shown). Lack of anomalous peaks in proton and carbon NMRs, as discussed below, provides further confirmation of the purity of the biosurfactant. The same yield and final purity were obtained with either acid precipitation or XAD-2 chromatography as the first separa-

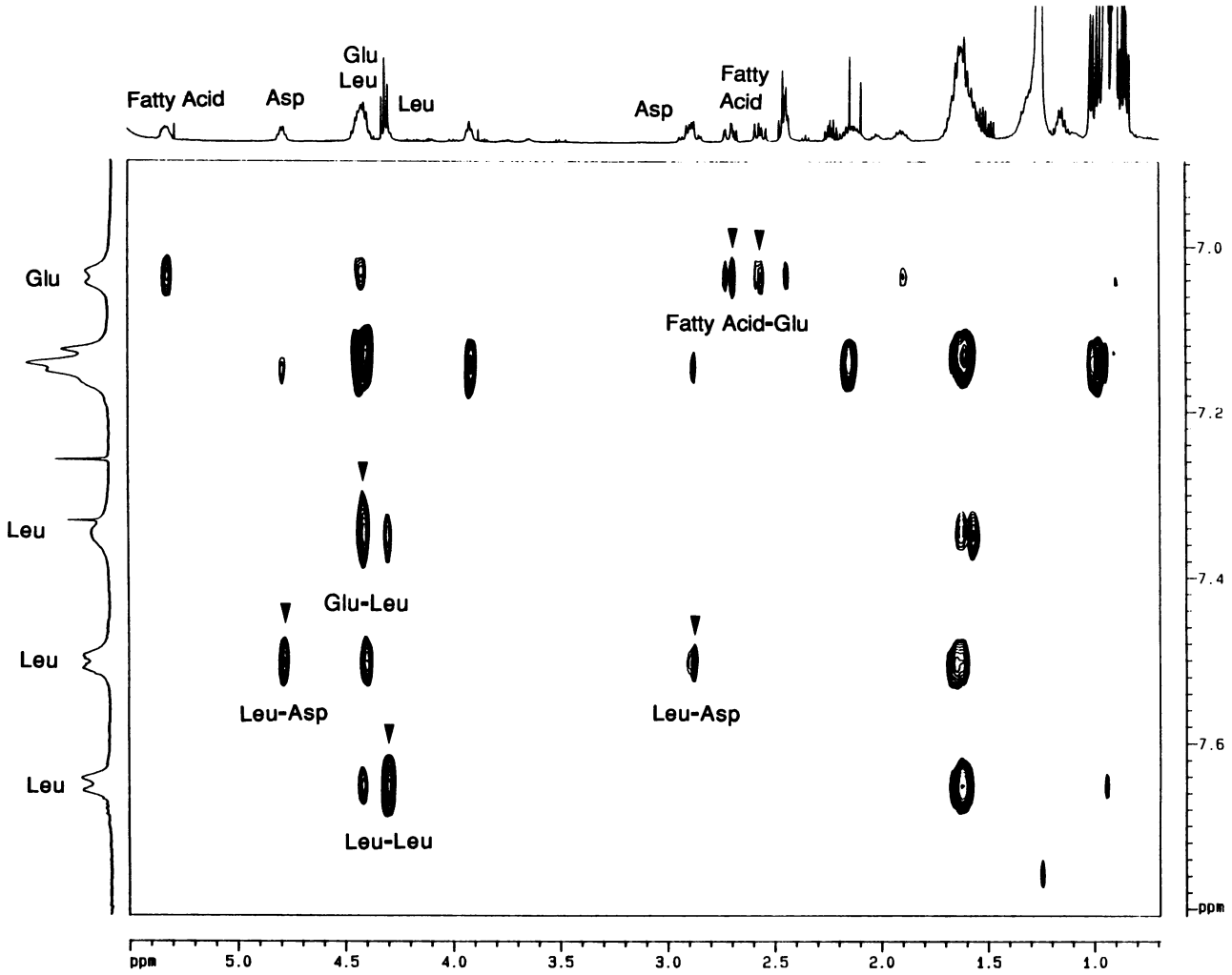

FIG. 5. The ROESY spectrum of the JF-2 biosurfactant obtained in $CDCl_2CDCl_2$ at 348 K. Signals corresponding to nongerminal proton-proton relationships are indicated by filled arrowheads and labeled.

tion step. XAD-2 chromatography is likely to be better suited for the continuous removal of the surfactant from the fermentation broth and for scale-up purposes.

Chemical structure of the surfactant. Figure 2 shows the infrared spectrum of the JF-2 biosurfactant. Bands characteristic of peptides (wave number 3,430:NH, wave number 1,655:CO, and wave number 1,534:CN) and aliphatic chains (wave number 3,000 to 2,800, CH_2 and CH_3) were observed, indicating that this compound is a lipopeptide. Also observed was a band corresponding to an ester carbonyl group (wave number 1,730:CO) (17). Amino acid analysis indicated the presence of four different amino acid residues in the peptide moiety of the biosurfactant. The composition was determined to be glutamic acid:aspartic acid:valine:leucine = 1:1:1:4. The FAB-MS spectrum (Fig. 3) indicated that the biosurfactant has a molecular mass of 1,035 Da.

Proton NMR (Fig. 4) showed seven NH signals (δ 7.0 to 7.7) and seven corresponding CH signals (δ 3.9 to 4.9) for the α-amino acids of the peptide. These were readily correlated with one another as well as with the signals of the corresponding alkyl residues via two-dimensional COSY and TOCSY spectra (data not shown). Since there were no signals for free $CONH_2$, eliminating asparagine and glutamine as possibilities, the identities of the amino acids were confirmed as aspartic acid, glutamic acid, leucine, and valine. An additional low-field signal at δ 5.3 consistent with CHO of the alcohol moiety of an ester (or lactone) was also observed.

Attempts to obtain the sequence of the amino acids by two-dimensional NOE (ROESY) NMR were moderately successful (Fig. 5). Germinal as well as more distant proton-proton relationships were observed in the ROSEY spectrum. These germinal proton-proton relationships, which can be considered as noises in peptide sequencing, were also observed in the TOCSY spectrum (data not shown) and, therefore, can be eliminated from the ROESY spectrum, leaving signals resulting from distant proton-proton relationships. These remaining proton-proton relationships yield the following partial sequences that are the same as those found in surfactin (15): fatty acid-Glu-Leu, Asp-Leu, and Leu-Leu.

On the bases of the composition of the peptide moiety and the molecular weight of the molecule, the lipid chain was determined to be a C_{15} fatty acid amidated to the N-terminal amine of the peptide. It was obvious that a mixture of

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.