ANTIBIOTICS PRODUCED BY *STREPTOMYCES FICELLUS* II. FELDAMYCIN AND NOJIRIMYCIN

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Feldamycin, a new antibacterial agent, and nojirimycin, a previously described antibiotic, have been isolated from cultures of *Streptomyces ficellus*. Feldamycin, $C_{17}H_{25}N_7O_5$, is an amphoteric compound which inhibits a variety of bacteria *in vitro* but is found to be ineffective in the treatment of experimental bacterial infections in mice. Nojirimycin (5-amino-5-deoxy-D-glucose) has been isolated previously from cultures of several species of streptomycetes.

Cultures of *Streptomyces ficellus** grown in a complex medium were found to produce several antibacterial agents. One of these antibiotics, ficellomycin, was described in a recent communication[1]. We now wish to report the production, isolation and chemical characterization of two additional antibiotics produced by *S. ficellus*. The first one, designated feldamycin (U-48266), was found to be a new antibacterial agent while the other has been identified as nojirimycin[2].

Experimental

Assay and Testing Procedures

Antibiotic production and purification was measured by a microbiological disc-plate assay procedure[3] with *Sarcina lutea*, *Staphylococcus aureus* and *Escherichia coli* as the assay organisms.

Thin-Layer Chromatographic Procedures

Thin-layer chromatograms were run on silica gel G using 95% ethanol - water (75: 25, v/v) as the solvent system. The antibiotics present in the fermentation or in preparations obtained during purification were detected by bioautography on *S. lutea* or *E. coli*-seeded trays.

Spectroscopic Methods

Proton magnetic resonance spectra were recorded on a Varian XL-100-15 spectrometer operating at 100 MHz. Solutions (*ca.* 0.4 ml, *ca.* 0.25 M) of the compounds in D_2O or $CDCl_3$ were used.

Carbon magnetic resonance spectra were recorded on a Varian CFT-20 spectrometer operating at 20.0 MHz. PMR and CMR chemical shifts are reported as ppm relative to tetramethylsilane.

Complete high resolution mass spectra were obtained on the CEC-21-110B spectrometer using a photographic plate as detector.

The field desorption mass spectrum was obtained on a Varian-MAT CH-5-DF spectrometer.

Fermentation Procedures

The procedures described by ARGOUDELIS *et al.*[1] were used. Peak titers were obtained after 72~96 hours of incubation.

Isolation of Feldamycin

Florisil Chromatography: The fermentation broth (*ca.* 5,000 liters) was filtered with the aid of diatomaceous earth. The filter cake was discarded. Part of the clear filtrate (2,000 liters) was passed

* Taxonomic studies were done by Miss ALMA DIETZ of The Upjohn Company. The organism is designated *Streptomyces ficellus* UC® 5438.

over a florisil column prepared from 80 kg of acid-washed florisil* at a rate of 5 liters/minute. The spent broth was discarded. The column was washed with 150 liters of water at a rate of 5 liters/minute. The aqueous wash was concentrated *in vacuo* to a volume of *ca.* 40 liters and this concentrate was freeze-dried to give 515 g of crude preparation of feldamycin (Prep A). The column was then eluted with 900 liters of 50% aqueous acetone. The following fractions were obtained: Fraction 1, 35 liters; fraction 2, 40 liters; fraction 3, 225 liters; and fraction 4, 225 liters. Fractions 1 and 2 contained mainly feldamycin and traces of ficellomycin. Fractions 3 and 4 contained ficellomycin. Concentration of fractions 1 and 2 yielded preparations B (103 g) and C (240 g), respectively. Preparations A, B and C were used for the Amberlite XAD-4 chromatography described below.

Amberlite XAD-4 Chromatography: Crude feldamycin, 270 g, obtained as described above, was dissolved in 9 liters of a 10% aqueous solution of sodium chloride. The solution was adjusted to pH 10.0 with 5 N aqueous sodium hydroxide and passed over a column containing 9 liters of Amberlite XAD-4 (Rohm and Haas Co., Philadelphia, Pa., U. S. A.) at a flow rate of 10 ml/minute. The spent solution was found bioinactive and was discarded. The column was eluted with water. Fractions containing feldamycin were combined (16 liters). This solution was chromatographed over IRC-50 as described below.

Chromatography over Amberlite IRC-50 (H^+): The column was prepared from 2 liters of Amberlite IRC-50 in the hydrogen form. The solution containing feldamycin (16 liters), obtained as described above, was passed through the column at a rate of 20 ml/minute. The spent solution was found bioinactive and was discarded. The column was washed successively with 5 liters of water and 5 liters of 0.25 N aqueous ammonium hydroxide. Both fractions were found bioinactive and were also discarded. The column was then eluted with 1 N aqueous ammonium hydroxide. Fractions containing feldamycin (by tlc) were combined and freeze-dried to yield 17.93 g of purified feldamycin.

Silica Gel Chromatography: A column was prepared from 1.8 kg of silica gel (Merck-Darmstadt Art 7034) packed in the solvent system consisting of 95% aqueous ethanol - water (75: 25, v/v). Purified feldamycin, 14.0 g, obtained as described above, was dissolved in 50 ml of water. The solution was mixed with 100 g of silica gel and 150 ml of 95% aqueous ethanol. The mixture was concentrated to dryness and the obtained powder was added to the column. The column was then eluted with the 95% ethanol - water solvent system at a flow rate of 10 ml/minute. Fractions containing feldamycin were combined and this solution (*ca.* 10 liters) was chromatographed over Amberlite IRC-50 as described below.

Amberlite IRC-50 Chromatography: The column was prepared from 1 liter of Amberlite IRC-50 in the hydrogen form. The solution containing feldamycin (10 liters), obtained as described above, was passed through the column. The spent solution was found bioinactive and was discarded. The column was washed with 4 liters of 0.25 N aqueous ammonium hydroxide. Both fractions, found bioinactive, were discarded. Feldamycin was then eluted with 1 N aqueous ammonium hydroxide. Fractions containing the antibiotic were freeze-dried to give 5.3 g of pure feldamycin. Characterization of the antibiotic is described later.

Preparation of Feldamycin Hydrochloride

Feldamycin, 600 mg, was dissolved in 1 ml of water, 1 ml of methanol and 4 ml of 1 N methanolic hydrogen chloride. This solution was added to 400 ml of acetone under stirring. The precipitated feldamycin hydrochloride was isolated by filtration and dried; yield 700 mg. Characterization of feldamycin hydrochloride is described in Results and Discussion.

Isolation of Nojirimycin

Dowex-50 Chromatography: Thirty liters of clear broth (obtained as described above) was adjusted to pH 3.5 with 2 N aqueous hydrochloric acid. The acidic solution was allowed to stand at room temperature for 2 hours. Precipitated material was separated by filtration and the filtrate was passed over a column containing 2.5 liters of Dowex-50 (X-8) in the hydrogen cycle. The spent was found bioinactive and was discarded. The column was washed with 10 liters of water and then eluted with 0.5 N

* Acid-washed florisil is prepared by stirring commercial florisil with 3 N aqueous sulfuric acid. The mixture is let to stand at room temperature for 20 hours, then is washed with deionized water until pH is *ca.* 5.

aqueous ammonium hydroxide. Fractions containing nojirimycin were combined and freeze-dried to yield 60.4 g of crude nojirimycin.

Dowex-1 Chromatography: Sixty g of crude nojirimycin was dissolved in 1 liter of water. This solution was passed over a column containing 2.5 liters of Dowex-1 (x–4) in the hydroxide form. The column was eluted with water. Fractions containing nojirimycin (by tlc) were combined and the solution was freeze-dried to give 4.2 g of highly purified nojirimycin.

Preparation of "Nojirimycin-Bisulfite Adduct" **3**: The method described by **S.** INOUYE *et al.*[4] was used. Compound **3**, isolated crystalline in 90% yield, was transformed to nojirimycin (**1**) by treatment with Dowex-1, x–4, in the hydroxide form.

Acetylation of Nojirimycin. Isolation of Acetate **2**: Treatment of nojirimycin with acetic anhydride and pyridine [procedure similar to that described by ISHIDA[2]] yielded compound **2** which was isolated crystalline by silica gel chromatography using ethyl acetate - Skellysolve B - methanol (30: 70: 1, v/v) as the solvent system.

Results and Discussion

Cultures of *Streptomyces ficellus* contain three major bioactive compounds (Fig. 1). Ficellomycin (comp. I) has been described previously[1]. Feldamycin (comp. II) is highly active against *Sarcina lutea* and relatively inactive against *Staphylococcus aureus*, *Penicillium oxalicum* and several Gram-negative organisms (Table 1). Nojirimycin (comp. III) is active against *S. lutea, S. aureus* and *E. coli*. Since both ficellomycin and nojirimycin are active against *S. aureus* the production and purification of feldamycin was followed by assaying against *S. lutea* and *S. aureus* and by tlc. Bioactivity against *S. lutea* combined with lack of activity against *S. aureus* were good criteria for the exclusive presence of feldamycin in a given preparation. Fermentation studies have shown that the production of the three antibiotics by *S. ficellus*, under the fermentation conditions given in the Experimental, begins at *ca.* 24 hours after incubation and peaks simultaneously at *ca.* 72~96 hours after incubation.

Table 1. Antibacterial spectrum of feldamycin*

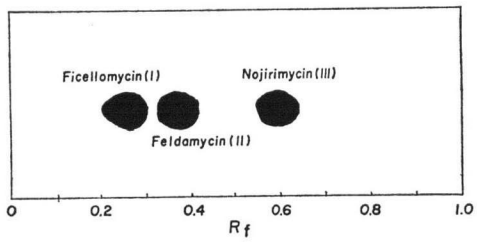
Organism	Zone of inhibition (mm) at indicated concentrations		
	10 mg/ml	5 mg/ml	1 mg/ml
E. coli UC–51	28 (vl)	26 (vl)	22 (vl)
K. pneumoniae UC–57	36 (l)	34 (l)	31 (l)
B. cereus UC–3145	0	0	0
P. oxalicum UC–1268	0	0	0
Ps. aeruginosa UC–95	0	0	0
S. gallinarum UC–265	31 (vl)	29 (vl)	26 (vl)
B. subtilis UC–564	42 (vl)	38 (vl)	25 (vl)
S. lutea UC–130	48	46	40
S. aureus UC–80	19 (l)	0	0
M. avium UC–159	44 (vl)	0	0
P. vulgaris UC–93	38 (vl)	36 (vl)	28 (vl)

* Test method: 0.08 ml aliquots of test solution were applied on 12.5 mm paper discs and the discs were applied on agar trays seeded with the appropriate organisms; vl=very light zone; l=light zone.

Characterization of Feldamycin

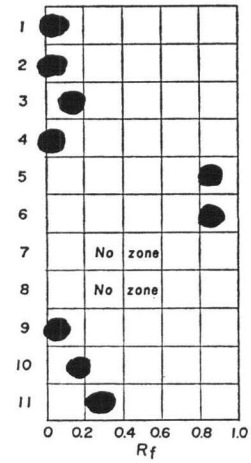
Feldamycin was isolated as an amorphous colorless material by a sequence of chromatographic procedures. Florisil chromatography separated feldamycin and ficellomycin from nojirimycin. Chromatography over Amberlite XAD–4 separated feldamycin from ficellomycin. Chromatography over

Fig. 1. Thin-layer chromatography* of culture filtrates of *Streptomyces ficellus*



* Silica gel G; 95% Ethanol - water (75: 25). Antibiotics I, II, III were detected by bioautography on *S. aureus* (I) or *S. lutea* (II) or *E. coli* (III)-seeded agar.

Fig. 2. Paper chromatographic mobility of feldamycin.



Solvent systems: 1) 1-butanol - water (84: 16); 2) 1-butanol - water (84: 16) and 0.25% *p*-toluenesulfonic acid; 3) 1-butanol - acetic acid - water (2: 1: 1); 4) 2% piperidine (v/v) in 1-butanol - water (84: 16); 5) 1-butanol - water (4: 96); 6) 1-butanol - water (4: 96)+0.25% *p*-toluenesulfonic acid; 7) 0.5 M phosphate buffer pH 7.0; 8) 0.075 M NH_4OH saturated with methyl isobutyl ketone, lower phase; 9) benzene - methanol - water (1: 1: 2); 10) 1-butanol - water (84: 16) and 2% *p*-toluene sulfonic acid; 11) methanol - 15% aqueous sodium chloride (4: 1).

The paper used (Whatman No. 1) is impregnated with 0.1 M Na_2SO_4.

AmberliteIRC-50 yielded feldamycin free of inorganic impurities. Pure feldamycin was obtained by a combination of silica gel and Amberlite IRC-50 chromatographies. Feldamycin, $[\alpha]_D^{25}$, $-6.6°$ (*c* 1, water) is soluble in water and lower alcohols. It is insoluble in ketones, halogenated or saturated hydrocarbon solvents and ethyl acetate or other ester type solvents. The paper chromatographic behavior of feldamycin in several solvent systems is shown in Fig. 2.

Feldamycin is an amphoteric compound isolated in its zwitterionic form. The IR spectrum (Fig. 3, upper) shows broad absorptions at 3130~3370 (OH, -NH) and at 1665 (shoulder), 1622 and 1570 cm^{-1} (shoulder) assigned to the presence of amide, COO^- NH^+ zwitterion and the imidazole system (see Fig. 6) in the molecule of feldamycin[5]. The antibiotic forms a colorless hydrochloride salt, $[\alpha]_D^{25}$ $+12°$ (*c* 1, water) which exhibits biological properties identical to those of feldamycin. The IR spectrum of feldamycin hydrochloride (Fig. 3, lower), distinctly different from that of feldamycin, could be used for differentiation of the two salt

Fig. 3. Infrared spectrum of feldamycin (Nujol mull) and feldamycin hydrochloride (Nujol mull)

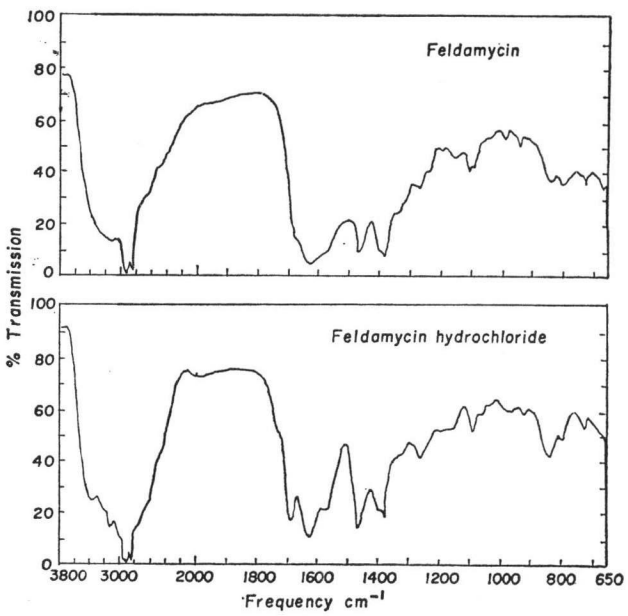
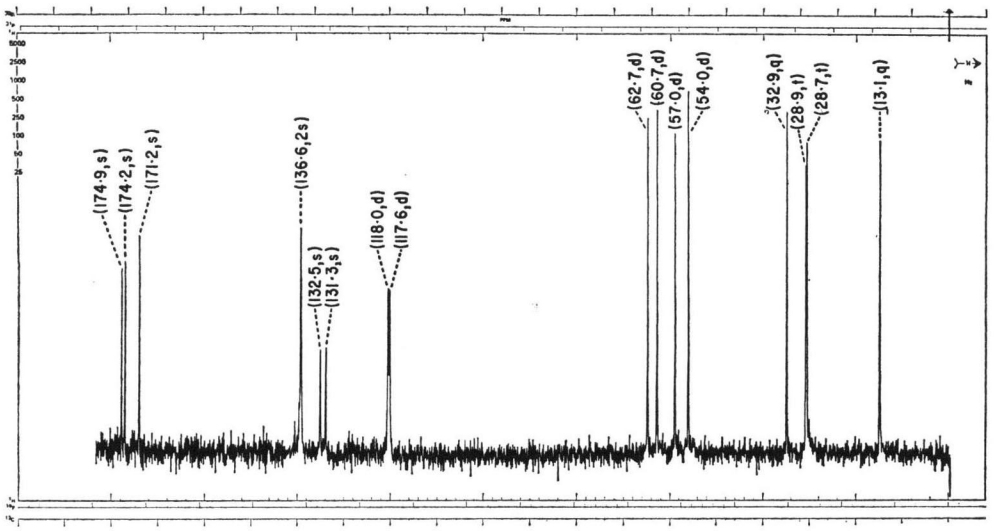
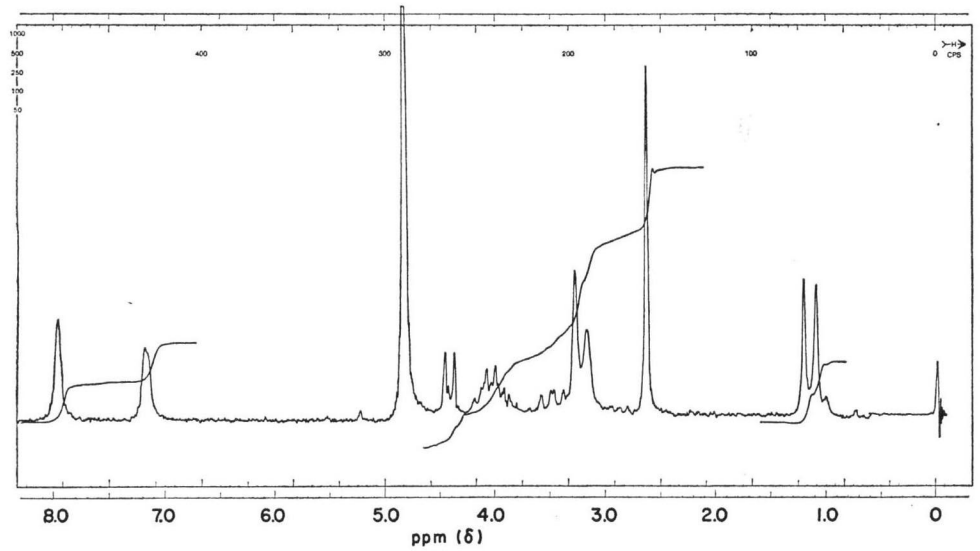


Fig. 4. Carbon-13 magnetic resonance spectrum of feldamycin*



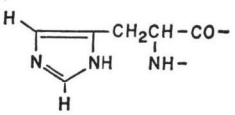
* Multiplicities in the off-resonance decoupled spectra: s=singlet; d=doublet; t=triplet; q=quartet.

Fig. 5. Proton magnetic resonance spectrum of feldamycin



forms (zwitterion *vs.* external) of the antibiotic. Specifically the IR spectrum of feldamycin hydrochloride has a characteristic absorption at 1695 cm^{-1} assigned to –COOH group(s).

Fig. 6.



Feldamycin forms a mixture of trimethylsilyl (TMS)-derivatives containing four, five or six TMS groups. The tetratrimethylsilyl-feldamycin, the major component of the mixture, has a molecular composition of $C_{17}H_{21}N_7O_5 \cdot 4Si(CH_3)_3$, molecular weight, calcd. 695.3498; found (HRMS), 695.3490. The molecular formula of feldamycin, therefore, is $C_{17}H_{25}N_7O_5$, molecular weight 407. The field desorption mass spectrum showed a molecular ion at *m/e* 407 in agreement with

Fig. 7. Mass spectrum of tetra-TMS feldamycin

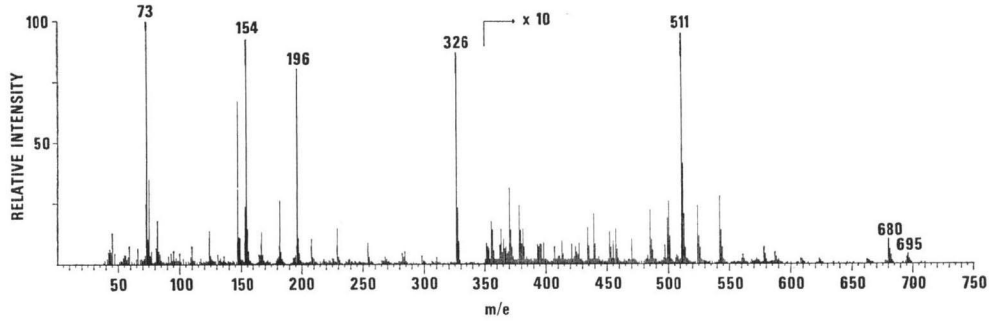
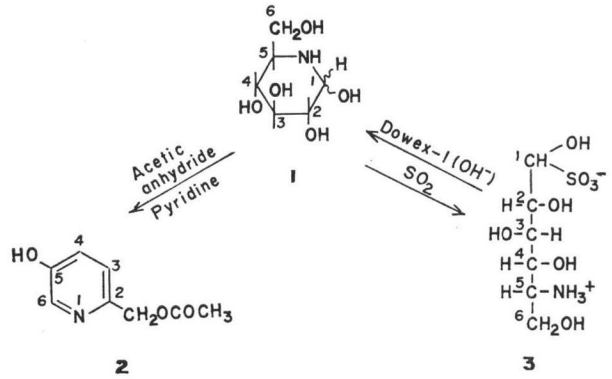


Fig. 8.



the data obtained by high resolution mass spectrometry of the tetra-TMS-feldamycin.

The UV spectrum of feldamycin showed end-absorption only. The CMR* spectrum (Fig. 4) showed the presence of one C–CH_3 at δ 13.1 (q) and of one N–CH_3 at δ 32.9 (q). This is in agreement with PMR* absorptions (Fig. 5) at δ 1.15 (d) and δ 2.6 (s) assigned to –$CHCH_3$ and N–CH_3 groups, respectively. Furthermore the PMR spectrum of feldamycin shows the presence of two histidine-like moieties (Fig. 6) [δ 7.95 (s, 2H); 7.15 (s, 2H); 4.0 (m, 2H); and 3.2 (m, 4H)] in the antibiotic.

Detailed analysis of both PMR and CMR spectra and the mass spectrum of the tetra-TMS derivative of feldamycin (Fig. 7) will be presented in a subsequent communication related to the structure of feldamycin.

Biological Properties of Feldamycin

The *in vitro* antibacterial spectrum of feldamycin is presented in Table 1. The antibiotic was ineffective in the treatment of experimental *S. aureus* or *S. hemolyticus* infections in mice. Feldamycin was found inactive against a variety of fungi and viruses *in vitro*. The antibiotic showed low inhibitory activity against L–1210 mouse leukemia cells (ID_{50}, 38 mcg/ml) grown in culture. Preliminary studies indicate that feldamycin interacts with DNA as evidenced by an increase in the thermal stability of DNA. The antibiotic also shows moderate inhibition of bacterial RNA-polymerase.

* Multiplicity in the pmr and off resonance-cmr spectra: q=quartet; d=doublet; s=singlet; m=multiplet.

Table 2. C-13 Nuclear magnetic resonance spectra of:

Nojirimycin[a] **1** (Fig. 8)			Acetate **2**[b] (Fig. 8)		"Bisulfite adduct"[a] **3** (Fig. 8)	
Chem. shift (δ)[c]			Chem. shift (δ)[e]	Assignment	Chem. shift (δ)[d]	Assignment
α-Anomer	β-Anomer	Assignment				
79.0 (d)	82.6 (d)	C-1	153.38 (s)	C-2	76.58 (d)	C-1
72.6 (d)	76.0 (d)	C-2	123.09 (d)	C-3	71.10 (d)	C-2, C-3 or C-4
74.1 (d)	76.6 (d)	C-3	122.61 (d)	C-4	70.03 (d)	
72.0 (d)	71.5 (d)	C-4	145.94 (s)	C-5	67.88 (d)	
53.6 (d)	57.1 (d)	C-5	137.45 (d)	C-6	61.16 (d)	C-5
62.3 (t)	61.6 (t)	C-6	66.28 (t)	$-CH_2-$	58.12 (t)	C-6
			170.06 (s)	$-\overset{\overset{O}{\|\|}}{C}-$		
			20.55 (q)	$-CH_3$		

[a] D_2O used as solvent.
[b] $CDCl_3$ used as solvent.
[c] Relative to TMS using acetone as an internal standard.
[d] Relative to TMS using dioxane as an internal standard.
[e] Relative to TMS.
[f] The calculated chemical shifts for C-2 to C-6 are 155.0, 124.9, 122.4, 150.0 and 136.6 respectively (see Ref. 8).
Multiplicities in the off-resonance decoupled spectra: s=singlet, d=doublet; t=triplet; q=quartet.

Isolation and Identification of Nojirimycin

Of the three antibiotics produced by *S. ficellus* only compound **III** (Fig. 1) was active against *E. coli*. Therefore, isolation of this compound was followed by assaying against *E. coli* and by tlc. Compound **III** was recovered from clear filtrates by adsorption on Dowex-50 (H^+) and elution with aqueous ammonia. Purification was achieved by chromatography over Dowex-1 (OH^-).

Compound **III** was identified as nojirimycin (**1**, Fig. 8) by comparison of its NMR, IR and mass spectra to those reported for nojirimycin[2,4,6].

Furthermore reaction of compound III with acetic anhydride and pyridine yielded **2** while treatment with sulfur dioxide produced the "bisulfide adduct" **3** (Fig. 8). Both **2** and **3** have been obtained previously from nojirimycin[2,4]. ^{13}C-NMR spectra of **1**, **2** and **3** obtained in the present study and not reported earlier are presented in Table 2.

As shown from the CMR spectra, nojirimycin was isolated as a mixture of α- and β-anomers. Assignment of the chemical shifts was done by off-resonance studies and by comparison to the CMR spectra of α- and β-glucose[7]. Assignments of the carbons of acetate **2** are based on off-resonance studies and comparison of the observed chemical shifts to those calculated for substituted pyridines[8].

Acknowledgements

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