RANCINAMYCINS, METABOLITES PRODUCED BY *STREPTOMYCES LINCOLNENSIS* IN SULFUR-DEPLETED MEDIA

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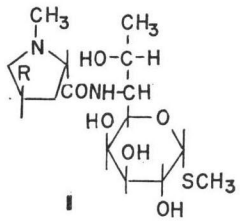
Rancinamycins I, II, III and IV are secondary metabolites produced by *Streptomyces lincolnensis* in a sulfur-depleted culture medium. Rancinamycins I and II, the main components of the mixture, show broad spectrum antibiotic activity *in vitro*. Subcutaneously injected or orally administered antibiotic afforded no protection for experimentally infected mice against lethal challenges of *Staphylococcus aureus*. Radioactive tracer studies failed to demonstrate that the rancinamycins were precursors in the biosynthesis of lincomycin.

Streptomyces lincolnensis was first reported to produce lincomycin (**1**) by MASON *et al.*[1] in 1962. Subsequently, a number of other lincomycin-related antibiotics were found to be produced by this culture[2,3,4,5].

All of the antibiotics reported to be produced by *S. lincolnensis* are produced in either complex or defined media containing inorganic sulfate as the usual source of sulfur. The sulfur of methionine can also be utilized efficiently[6].



I

R = $CH_2CH_2CH_3$

When *S. lincolnensis* is grown in a chemically defined medium in the absence of sulfur-containing organic or inorganic compounds a group of new metabolites is produced. The isolation, purification, characterization and biological properties of these compounds, designated rancinamycins I, II, III and IV, are the subjects of the present communication.

Experimental

Spectroscopic Procedures

PMR spectra were recorded on a Varian A-60 spectrometer. All preparations were run in either D_2O or d_6-dimethylsulfoxide (d_6-DMSO) using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (SDSS) or tetra methylsilane (TMS) as internal reference.

Gas Chromatography-Mass Spectroscopy (GC-MS)

Gas-Chromatographic separation was done on a GC Varian Aerograph series 2700 chromatograph using a 6 ft., 3.8% UCW-98 on 80~100 mesh Diatoport-S column (Hewlett-Packard). The operation was performed isothermally at 185°C. The mass spectra were recorded on a CH7 Massenspectrometer (Varian Mat., West Germany) operating at 70 electron volts.

It was found necessary to prepare the trimethylsilyl ethers of rancinamycins in order to obtain volatile compounds satisfactory for gas chromatographic analysis. This was done by reacting 50 μl of a 1 mg/ml solution of a rancinamycin preparation in dimethylformamide with 50 μl of Regisil® (Regis Chemical Co.). The reaction mixture was allowed to stand at room temperature for 30 minutes.

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Assay Procedures

Antibiotic production was measured by a microbiological disc-plate assay procedure[7] using *Proteus vulgaris* (UC-93), *Proteus rettgeri* (UC-344), *Staphylococcus aureus* (UC-80), and *Sarcina lutea* (UC-130) as the assay organisms.

Paper and Thin-layer Chromatographic Procedures

Fermentation, purification and separation studies were followed by paper chromatography using water-saturated 1-butanol as the solvent system.

Thin-layer chromatograms were run on Analtech silica gel GF plates (Analtech Inc.) using ethyl acetate saturated with water (system I), methyl ethyl ketone - acetone - water (186: 52:10, v/v) (system II) or methyl ethyl ketone - acetone - water (186: 52: 22, v/v) (system III) as the solvent systems.

The antibiotics, separated either by paper or tlc chromatography, were detected by bioautography on *P. vulgaris*-seeded agar trays.

Fermentation Procedures

Seed cultures of *S. lincolnensis* were prepared in a medium consisting of glucose monohydrate, 25 g/liter and Pharmamedia, 25 g/liter (Trader's Oil Mill Co., Forth Worth, Texas, USA); seed presterilization pH 7.2. The cultures were incubated at 28°C for 72 hours on a rotary shaker (250 rpm). A fermentation medium consisting of anhydrous glucose, 30 g/liter; sodium citrate, 1.8 g/liter; dipotassium phosphate, 2.5 g/liter; sodium chloride 0.5 g/liter; ammonium nitrate, 2.0 g/liter; zinc chloride 0.47 mg/liter; ferrous chloride tetrahydrate 0.72 mg/liter; magnesium citrate, 1.2 g/liter and lard oil 10 ml/liter was adjusted to pH 7.2 and inoculated at a rate of 5% (v/v) with the 72-hour seed culture. Fermentations were incubated at 28°C on a rotary shaker and beers were harvested after total fermentation time of 72~96 hours.

Isolation Procedures

Adsorption on Amberlite XAD-4: The whole beer (10 liters) was filtered using diatomaceous earth (Dicalite, 4200, Grefco Inc.). Part of the clear beer (1.5 liters) was passed through a column prepared from 100 ml of Amberlite XAD-4* (Rohm and Haas Co.). The spent, collected in one fraction, was bioinactive and was discarded. The column was then washed with 500 ml of water; the aqueous wash was also bioinactive and was discarded. The column was eluted with methanol-water (95: 5, v/v). Fractions containing rancinamycins were concentrated to an aqueous solution and freeze-dried to yield 2.55 g of crude rancinamycin preparation.

Adsorption on Granulated Activated Carbon: Another portion (1.5 liters) of the clear beer, obtained as described above, was passed through a column containing 80 g of Cal Carbon (Pittsburgh Activated Carbon). The column was washed with750 ml of water; both spent and the aqueous wash were found bioinactive and were discarded. The column was successively eluted with 750 ml of acetone - water (20: 80, v/v), 250 ml of acetone - water (40: 60, v/v) and acetone - water (90: 10, v/v). Fractions collected with acetone - water (90: 10, v/v), containing rancinamycins, were combined, concentrated to an aqueous solution and freeze-dried to yield 1.22 g of crude rancinamycins.

Purification Procedures

Separation of Rancinamycins by Countercurrent Distribution: Three grams of crude rancinamycins, obtained by the carbon adsorption method, was distributed in an all glass countercurrent distribution apparatus (500 tubes, 10 ml/phase) using a solvent system consisting of equal volumes of 1-butanol and water. A total of 467 transfers were completed. The distribution was followed by determination of solids and bioactivity. Results which are presented in Fig. 2 showed the presence of four active components with K values of 0.52, 1.15, 2.40 and 5.95. Tubes under the peak with a K value of 1.15 were found to contain rancinamycin I, while rancinamycin II was found in the fractions under the peak with a K value of 2.40. Concentration of appropriate fractions yielded rancinamycins

* Amberlite XAD-4 was successively washed with the following before use: 2 bed volumes of 50: 50 acetone-water, 2 bed volumes of acetone, 2 bed volumes of methanol, 2 bed volumes of 50:50 methanol - water, and 20 bed volumes of water.

I and II isolated as viscous oils which crystallized (colorless needles) on standing. The small amounts of the bioactive components with K values of 0.52 (rancinamycin III) and 5.95 (rancinamycin IV) were not isolated by this procedure but by the purification sequence described below.

Separation of Rancinamycins by Amberlite XAD-4 Chromatography, Silica Gel Chromatography and Countercurrent Distribution: Ninety grams of crude rancinamycins obtained from a large scale fermentation (by the carbon process) were dissolved in one liter of water (resulting pH was 4.5). This solution was passed over a 2-liter Amberlite XAD-4 column. The spent, collected as one fraction, was bioinactive and was discarded. The column was then washed with water (6 liters) collected as one fraction. The aqueous wash was also bioinactive and was discarded. The column was eluted with methanol-water (50: 50, v/v). Fractions containing rancinamycins were combined, concentrated to an aqueous solution and freeze-dried to give 56 g of material containing all rancinamycins.

Twenty-one grams of this crude rancinamycin was dissolved in 50 ml of water. The solution was mixed with 100 g of silica gel (Merck-Darmstadt, Art 7704). This mixture was dried *in vacuo* and added on the top of a glass column (7 cm internal diameter) containing 1.8 kg of silica gel packed in a solvent consisting of methyl ethyl ketone - acetone - water (186: 52: 3, v/v). Bioactive fractions obtained by elution of the column with the solvent system were combined and concentrated to dryness *in vacuo* to give 7.9 g of slightly colored viscous material containing rancinamycins I to IV.

The purified rancinamycin mixture, obtained as described above, was dissolved in 50 ml of each phase of the solvent system consisting of equal volumes of 1-butanol - water. The solutions were added in the first five tubes of an all-glass countercurrent distribution apparatus (500 tubes, 10 ml/phase). After 500 transfers the distribution was analyzed by thin-layer chromatography using systems I and II and by bioassays. Rancinamycins I, 0.64 g; II, 0.58 g; III, 0.29 g; and IV, 1.79 g were isolated by concentration of the appropriate fractions. Characterization of these materials is described in Discussion and Results.

Rancinamycin I-2,4-Dinitrophenylhydrazone

One hundred sixty mg of rancinamycin I was dissolved in 10 ml of water. This solution was mixed with 420 ml of a solution prepared by dissolving 1 g of 2,4-dinitrophenylhydrazine in 1 liter of 2 N aqueous hydrochloric acid. The mixture was allowed to stand at room temperature for 24 hours. The crystalline precipitate formed, was separated by filtration and dried, 130 mg.

Anal. Calcd. for $C_{17}H_{20}N_4O_9$: C, 48.15; H, 4.75; N, 13.22; O, 33.96; molecular weight, 424.
Found: C, 47.65; H, 4.25; N. 12.93; O, 33.36; equivalent weight 411.

Rancinamycin II-2,4-Dinitrophenylhydrazone

One hundred mg of rancinamycin II was dissolved in 5 ml of 95% ethanol and this solution was mixed with 300 ml of a solution prepared by dissolving 1 g of 2,4-dinitrophenylhydrazone in 1 liter of 2 N aqueous hydrochloric acid. Crystalline rancinamycin II-2,4-dinitrophenylhydrazone was isolated by filtration and dried; yield 90 mg.

Anal. Calcd. for $C_{18}H_{22}N_4O_9$: C, 49.36; H, 5.06; N, 12.79; O, 32.88; molecular weight, 438.
Found: C, 48.77; H, 4.96; N, 12.79; O, 31.50; equivalent weight 421.

Preparation of the Mixture of 2,4-Dinitrophenylhydrazones of Rancinamycins

Crude rancinamycin, obtained by the carbon adsorption process described earlier, was dissolved in 50 ml of water. This solution was mixed with 2 liters of a solution prepared by dissolving 1 g of 2,4-dinitrophenylhydrazone in 1 liter of 2 N aqueous hydrochloric acid. Formation of an orange precipitate started immediately. The mixture was allowed to stand at room temperature for 24 hours. The precipitated mixture of the 2,4-dinitrophenylhydrazones of rancinamycins I and II was separated by filtration and dried (2.28 g).

Separation of 2,4-Dinitrophenylhydrazones of Rancinamycins I and II. Countercurrent Distribution.

The mixture of 2,4-dinitrophenylhydrazones of rancinamycins I and II was distributed in an all-glass CRAIG countercurrent distribution apparatus (500 tubes, 10 ml/phase) using a solvent system consisting of equal volumes of cyclohexane, ethyl acetate, 95% ethanol and water. After 465 transfers the distribution was analyzed by solid determination. Tubes 180~230, containing rancinamycin I-2,4-

dinitrophenylhydrazone, were concentrated to dryness to give 790 mg of crystalline material. Similarly, tubes 243~280 gave 400 mg of crystalline rancinamycin II-2,4-dinitrophenylhydrazone.

Preparation of Radioactive Rancinamycins

The fermentation conditions were identical to those described earlier. Uniformly labeled ^{14}C-glucose was used as the radioactive precursor. Approximately one per cent of the radioactivity added was incorporated into the crude rancinamycins isolated by the carbon adsorption process discussed earlier. This radioactive rancinamycin mixture (specific activity of 6×10^4 cpm/mg) was added to a lincomycin-producing fermentation of *S. lincolnensis* as described below.

Addition of ^{14}C-Rancinamycins to Fermentation of *S. lincolnensis* Producing Lincomycin

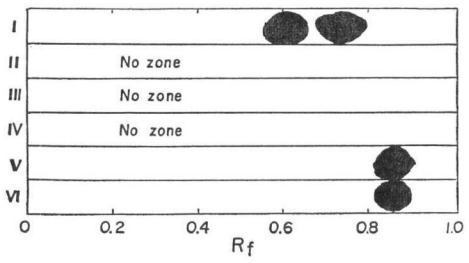
The fermentation conditions were identical to those described by ARGOUDELIS *et al*[6]. Radioactive ^{14}C-rancinamycin obtained as described above was added to the fermentation medium 48 hours after inoculation. The fermentation was followed by bioactivity determinations using both *P. vulgaris* (specific for rancinamycins) and *S. lutea* (specific for lincomycin) as the assay organisms. Results are discussed in the Results and Discussion section.

Results and Discussion

Production of Rancinamycins

Fermentations of *Streptomyces lincolnensis* var. *lincolnensis* grown in a synthetic medium deprived of sulfur-containing compounds produce several metabolites, designated rancinamycins, which display bioactivity against *Proteus vulgaris, Proteus rettgeri* and *Staphylococcus aureus*. Addition of inorganic sulfates or sulfur-containing organic compounds to the fermentation medium results in the production of lincomycin instead of rancinamycins. Paper chromatographic analysis of the fermentation broth indicated the presence of two bioactive components with Rf values of 0.60 and 0.70 in water-saturated 1-butanol. Purified preparations however contained two additional bioactive components. The paper chromatographic behavior of a rancinamycin-producing fermentation of *S. lincolnensis* is shown in Fig. 1.

Fig. 1. Paper chromatographic analysis of fermentation of *S. lincolnensis* producing rancinamycins
Solvent systems:
I 1-butanol, water (84: 16), 16 hours.
II 1-butanol, water (84: 16) plus 0.25% *p*-toluenesulfonic acid, 16 hours.
III 1-butanol, acetic acid, water (2:1:1), 16 hours.
IV 2% piperidine (v/v) in 1-butanol, water (84: 16), 16 hours.
V 1-butanol, water (4: 96), 5 hours.
VI 1-butanol, water (4: 96) plus 0.25% *p*-toluenesulfonic acid, 5 hours.
Bioactivities were detected by bioautography on *P. vulgaris*-seeded agar trays.



Antibiotic production in fermentations was followed by bioassays using *P. vulgaris* and *P. rettgeri* as test organisms. *Sarcina lutea*, an organism highly sensitive to lincomycin was also used throughout this study to detect the presence of lincomycin, which, as discussed above, could be produced by *S. lincolnensis*. Assay results combined with paper and thin-layer chromatographic analysis of the fermentation broth showed exclusive production of rancinamycins under the fermentation conditions used. Titers of rancinamycins in shake flask fermentations reached a maximum after 72 hours. Tank fermentations however gave maximum antibiotic production after 120 hours. Large variations of the rancinamycins (I to IV) produced were observed between fermentations and were reflected in the rancinamycin content of crude

Fig. 2. Countercurrent distribution of rancinamycins

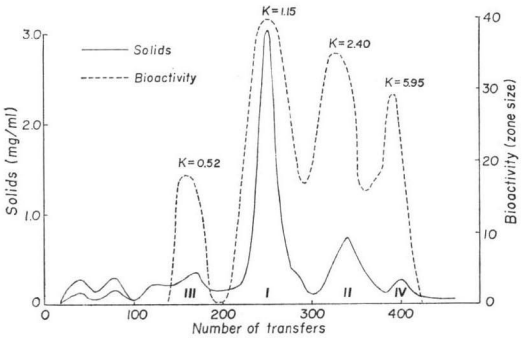
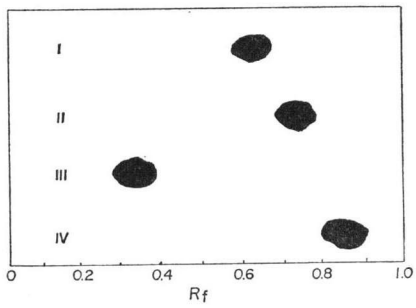


Fig. 3. Thin-layer chromatogram* of rancinamycins I, II, III and IV

*Silica gel; methyl ethyl ketone - acetone - water (186: 52: 10, v/v). Antibiotics were detected by bioautography on *P. vulgaris*-seeded agar trays.



preparations.

Isolation and Purification. Designation of Rancinamycins I, II, III and IV.

The bioactive components were recovered from the fermentation broth by filtration followed by adsorption on either granulated activated carbon or Amberlite XAD-4. Purification was achieved by chromatography on Amberlite XAD-4 and silica gel. Separation of rancinamycins was obtained by countercurrent distribution using 1-butanol - water (1: 1, v/v) as the solvent system (Fig. 2). The bioactive components in the tubes under the peaks with K values of 1.15 and 2.40 were defined as rancinamycins I and II, respectively. Rancinamycins I and II had Rf values of 0.68 and 0.78 in the papergram system discussed earlier. Rancinamycins III and IV were defined in the bioactive materials in the tubes under the peaks with K values of 0.52 and 5.95, respectively.

Rancinamycins I, II and III were isolated as colorless viscous materials which crystallized on standing (colorless needles). Crystalline rancinamycins I, II and III behaved as single entities in several paper chromatographic and tlc systems (Fig. 3). However gas chromatography-mass spectroscopy (see below) indicated that each of rancinamycins I, II and III was a mixture of isomeric compounds which could not be separated by either CCD or the chromatographic techniques (paper or tlc) used. Rancinamycin IV on the other hand, isolated as colorless crystalline (needles) solid, was found to contain one material only.

Table 1. Physical and chemical properties of rancinamycins.

	Rancinamycin I	Rancinamycin II
Molecular formula	$C_{11}H_{16}O_6$[a,c]	$C_{12}H_{18}O_6$[b,c]
Molecular weight		
Found	244.0919[a]	258[b]
Calculated	244.0946	—
Molecular weight of TMS-[d]derivative		
Found	460.2105[a]	474.2282[a]
Calculated	460.2138	474.2288
Molecular formula of TMS-derivative	$C_{11}H_{18}O_6 \cdot [Si(CH_3)_3]_3$[a]	$C_{12}H_{18}O_6 \cdot [Si(CH_3)_3]_3$[a]
$[\alpha]_D^{25}$ (c 1.0, water)	+96°	+69°
UV [λ_{max} (ε); 95% ethanol]	220 nm (9.5×10^3)	220 nm (7.74×10^3)
Titration	Neutral	Neutral
IR (Neat)	3400; 1735; 1700; 1250; 1195~1150; 1100~1050 cm^{-1}	3405; 1726; 1706; 1266; 1110 cm^{-1}

[a] By high resolution mass spectroscopy.
[b] By mass spectroscopy.
[c] By mass spectrometry and data of its 2,4-dinitrophenylderivative (see Table 2).
[d] TMS=trimethylsilyl.

Table 2. Physical properties of rancinamycin I- and II-2,4-dinitrophenylhydrazones.

	Rancinamycin I-2,4-DNPH	Rancinamycin II-2,4- DNPH
Molecular formula	$C_{17}H_{20}N_4O_9$	$C_{18}H_{22}N_4O_9$
Molecular weight		
Calculated	424	438
Found	411 (equivalent weight)	421 (equivalent weight)
Melting point	146~149°	162~163°
$[\alpha]_D^{25}$ (*c* 1.0, 95% ethanol)	−24°	−17°
IR (Nujol)	3410; 1735; 1712; 1613; 1600; 1513; 1331; 1313; 1268; 1107; 840; 762 cm^{-1}	3405; 1726; 1706; 1613; 1600; 1514; 1329; 1310; 1266; 1110; 840
UV [λ max (ε)] in 95% ethanol	208 (1.8×10^4), 235 (1.37×10^4) (sh), 248(1.35×10) (sh), 283(7.84×10^3) (sh), 370 (2.76×10^4)	208 (1.78×10^4), 235 (1.35×10^4) (sh), 248 (1.33×10^4) (sh), 283(7.79×10^4(sh), 370 (2.68×10^4)
Calculated molecular formula for rancinamycins	$C_{11}H_{16}O_6$	$C_{12}H_{18}O_6$

Fig. 4. Infrared spectra of rancinamycin I (upper) and rancinamycin II (lower).

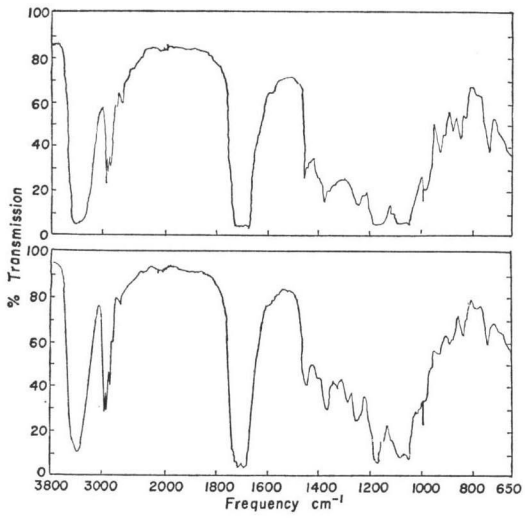
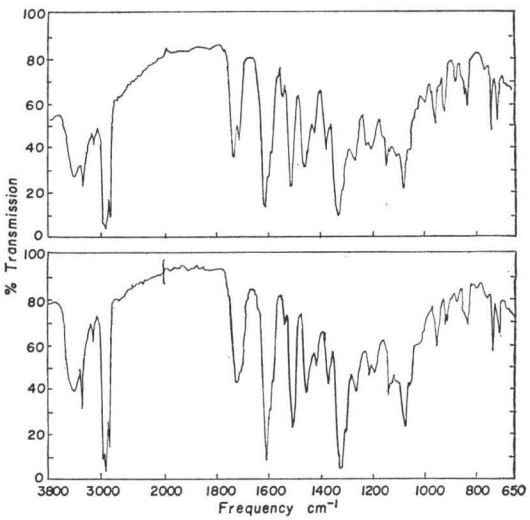


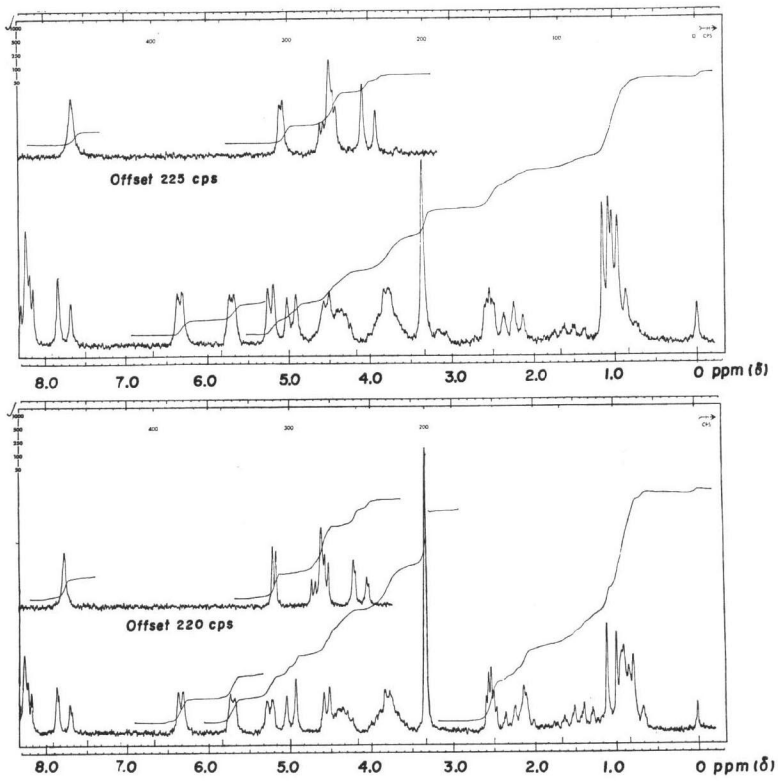
Fig. 5. Infrared spectra of rancinamycin I-2,4-DNPH (upper) and rancinamycin II-2,4-DNPH (lower) (Nujol mull).



Rancinamycins-2,4-Dinitrophenylhydrazone (-2,4-DNPH)

Rancinamycins I, II and III are hygroscopic materials which do not give satisfactory analytical data. Therefore, use was made of the property of all rancinamycins to give crystalline 2,4-dinitrophenylhydrazone derivatives which could be purified and characterized. Two procedures were used for the preparation of these derivatives. In the first method rancinamycins I, II, III and IV were separated by CCD (as described earlier) and the individual rancinamycins were reacted with solution of 2,4-dinitrophenylhydrazine in aqueous hydrochloric acid. In the second procedure crude rancinamycin was transformed to the mixture of rancinamycins-2,4-DNPH. Rancinamycins I and II-2,4-DNPH were then separated by CCD using cyclohexane - ethyl acetate - 95% ethanol - water (1: 1: 1: 1, v/v). The dinitrophenylhydrazones obtained by either method had identical properties and like the rancinamycins were mixtures of isomeric compounds.

Fig. 6. Nuclear magnetic resonance spectra of rancinamycin I-2,4-DNPH and rancinamycin II-2,4-DNPH (60 MHz, in d_6-dimethylsulfoxide).



Characterization of Rancinamycins

Rancinamycins I, II and III are hygroscopic materials soluble in water, lower alcohols, ethyl acetate and acetone. They are less soluble in chlorinated hydrocarbon solvents and insoluble in ether and saturated hydrocarbons. Rancinamycin IV has limited solubility in water but it is soluble in alcohols, ethyl acetate, acetone, chloroform and ether.

The properties of rancinamycins I and II and rancinamycins I-and II-2,4-dinitrophenylhydrazones are listed in Tables 1 and 2, respectively. The data presented indicate that the two antibiotics are closely related and that rancinamycin II ($C_{12}H_{18}O_6$)

Table 3. Gas chromatographic-mass spectroscopic analysis*.

	Compound	Retention time (min.)	Relative abundance (%)	M^+	Number of TMS groups
1. Rancinamycin I-TMS**	Ia-TMS	19.2	36	460	3
	Ib-TMS	23.5	51	460	3
	Ic-TMS	26.5	1	460	3
	Id-TMS	28.5	3	460	3
	Ie-TMS	33.3	8	460	3
2. Rancinamycin II-TMS	IIa-TMS	27.5	67	474	3
	IIb-TMS	31.0	7	474	3
	IIc-TMS	33.3	3	474	3
	IId-TMS	36.5	4	474	3
	IIe-TMS	40.5	19	474	3
3. Rancinamycin III-TMS	IIIa-TMS	11.5	15	462	4
	IIIb-TMS	13	10	462	4
	IIIc-TMS	14.5	15	462	4
	IIId-TMS	17	60	462	4

* For conditions see Experimental.
** TMS=trimethylsilyl.

Table 4. Antibacterial spectra of rancinamycins I, II, III and IV*.

	Zone size (mm)			
	I	II	III	IV
Klebsiella pneumoniae (UC-57)	24	20	16.5	24
Escherichia coli (UC-51)	22	18.5	15.5	25.5
Proteus vulgaris (UC-93)	33	30.5	28.5	26
Salmonella schottmuelleri (UC-126)	24	20	16.5	24
Salmonella gallinarum (UC-265)	25	21.5	21.5	22.5
Pseudomonas aeruginosa (UC-95)	0	0	0	16.5
Streptococcus pyogenes (UC-152)	21	20	20.5	0
Sarcina lutea (UC-130)	22	22	16.5	18
Penicillium oxalicum (UC-1268)	0	0	0	0
Bacillus subtilis (UC-564)	17	16	trace	22.5
Mycobacterium avium (UC-159)	0	0	0	29

* The concentration of the solutions used was 10 mg/ml; agar diffusion test.

Table 5. Characteristics of a lincomycin-producing fermentation of *S. lincolnensis* containing rancinamycins as precursors.

Time (hours)	pH	Assay(*P. vulgaris*; bu/ml)	Lincomycin (mcg/ml)	Radioactivity* (cpm/ml)
0	6.1	12	4.3	61,880
12	5.1	8.9	4.3	67,890
36	7.8	0	51.2	65,430
60	7.9	0	128	63,045
84	8.3	0	285	64,940
108	8.6	0	193	66,125

* Radioactivity present in the fermentation broth.

is a higher homolog of rancinamycin I ($C_{11}H_{16}O_6$) differing from the latter by a $-CH_2-$ group. The IR spectra of rancinamycins I and II (Fig. 4) and the IR and PMR spectra of rancinamycins I-and II-2,4-dinitrophenylhydrazones (Figs. 5 and 6) also indicate the close structural similarity between these two antibiotics.

Gas chromatographic-mass spectroscopic (GC-MS) analysis of the trimethylsilyl (TMS) derivative of rancinamycin I showed that rancinamycin I is a mixture of five compounds designated rancinamycins Ia, Ib, Ic, Id and Ie in order of increasing retention time (Table 3) of their TMS derivatives. All five compounds form TMS-derivatives of identical molecular weight (460) and identical fragmentation pattern[8] in their mass spectra. Furthermore, all five TMS-derivatives contain three TMS groups indicating the presence of three hydroxyl groups in each of the components. This suggests that the antibiotics, themselves, have identical molecular formulas ($C_{11}H_{16}O_6$) and isomeric structures. Rancinamycins Ia and Ib were the main components comprising *ca.* 87% of rancinamycin I. Rancinamycins Ic, Id and Ie, being present in small amounts, are not expected to effect the spectra (CMR, PMR, IR and UV) of rancinamycin I which are discussed in detail in a subsequent paper[8].

Similarly, rancinamycin II was found to be a mixture of five components (rancinamycins IIa, IIb, IIc, IId and IIe) (Table 3). All components had identical molecular weight (474) and identical fragmentation patterns in the mass spectra of their TMS-derivatives[8], indicative of the isomeric character of the rancinamycin II components. The formation of tri-TMS derivatives indicates that rancinamycins II also contains three hydroxyl groups. Rancinamycin IIa and IIe are the main components comprising *ca.* 86% of rancinamycin II. The characterization data recorded on Tables 1 and 2 for rancinamycins I, II and their 2,4-dinitrophenylhydrazones, with the exception of the specific rotations and the melting points, are not effected by the finding that each antibiotic is a mixture of isomeric components.

Since small amounts of rancinamycin III were isolated, this material was not completely characterized. High resolution mass spectrum of rancinamycin III-TMS showed molecular formula of $C_7H_6O_5 \cdot [Si(CH_3)_3]_4$; (Molecular weight: calcd. 462.21093, found, 462.20788). Therefore the mole-

cular formula of rancinamycin III is $C_7H_{10}O_5$. Rancinamycin III, like rancinamycins I and II, was found to be a mixture of four components (rancinamycins IIIa, IIIb, IIIc and IIId) (Table 3). The molecular weight (462) and fragmentation pattern of the TMS-derivatives of these components was identical. The formation of tetra-TMS derivative shows the presence of four hydroxyl groups. Rancinamycin III forms a 2,4-dinitrophenylhydrazone which exhibits UV spectrum identical to that of rancinamycins I and II (Table 2) indicating the presence of the same chromophoric system in rancinamycins I, II and III.

Rancinamycin IV, $C_7H_6O_3$ (molecular weight: found 138.0307; calcd. 138.0317) was isolated as colorless crystalline material. Gas chromatographic mass spectroscopic analysis of the trimethylsilyl derivative of rancinamycin IV indicated the presence of one component with retention time of 4.5 minutes. IR and PMR spectra indicated that rancinamycin IV is 3,4-dihydroxybenzaldehyde[8].

Biological Properties of Rancinamycins

The antibacterial spectrum of rancinamycins I, II, III and IV is presented in Table 4. The antibacterial activity of rancinamycins I, II and III is destroyed by cysteine, by reagents reacting with carbonyl groups such as hydrogen sulfite and by reduction with sodium borohydride. In addition, the antibiotics were unstable in acidic or basic solutions. Rancinamycins I and II were inactive both orally and subcutaneously against *Staphylococcus aureus* infected mice.

Rancinamycins as Precursors in Lincomycin Biosynthesis

The possibility that rancinamycins might be intermediates in the biosynthesis of lincomycin was suggested by the finding that these compounds are related to shikimic acid[8] and that aromatic acids (phenylalanine, tyrosine) are involved in the biosynthesis of the amino acid moiety of lincomycin[9]. To test this hypothesis uniformly labeled ^{14}C-rancinamycin complex was prepared by the use of uniformly labeled ^{14}C-glucose. About 1.0% of the radioactivity added to the fermentation medium was incorporated into rancinamycins. The radioactive rancinamycin complex was then added into a lincomycin producing fermentation. As shown in Table 6 the rancinamycin bioactivity (*P. vulgaris*) was rapidly lost. The radioactivity level in the fermentation supernatant liquid, however, remained constant throughout the fermentation. Lincomycin, isolated as the crystalline hydrochloride, was radioinactive indicating that rancinamycins were not utilized for biosynthesis of lincomycin under the conditions used. The rapid loss of *P. vulgaris* activity was due, probably, to the instability of the rancinamycins to the acidic conditions early in the fermentation.

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References

1) MASON, D. J.; A. DIETZ & C. DEBOER: Lincomycin, a new antibiotic. I. Discovery and biological properties. Antimicr. Agents & Chemoth. -1962: 554~559, 1963
2) ARGOUDELIS, A. D.; J. A. FOX & T. E. EBLE: U-21,699: A new lincomycin-related antibiotic. Biochemistry 4:698~703, 1965
3) ARGOUDELIS, A. D. & D J. MASON: Studies on the biosynthesis of lincomycin. I. Antibiotic U-11,921, an S-ethyl homolog of lincomycin. Biochemistry 4: 704~709, 1965
4) ARGOUDELIS, A. D.; J. A. FOX & D. J. MASON: Studies on the biosynthesis of lincomycin. II. Antibiotic U-11,973, N-demethyl-lincomycin. Biochemistry 4:710~713, 1965

5) ARGOUDELIS, A. D.; T. E. EBLE & D. J. MASON: Studies on the biosynthesis of lincomycin. V. Effect of ethionine on fermentations of *S. lincolnensis*. J. Antibiotics 23:1~8, 1970
6) ARGOUDELIS, A. D.; T. E. EBLE, J. A. FOX & D. J. MASON: Studies on the biosynthesis of lincomycin. IV. The origin of methyl groups. Biochemistry 8:3408~3411, 1969
7) HANKA, L. J.; M. R. BURCH & W. T. SOKOLSKI: Psicofuranine. VI. Microbiological assay. Antibiot. & Chemoth. 9:432~435, 1959
8) ARGOUDELIS, A. D.; R. W. SPRAGUE & S. A. MIZSAK: Rancinamycins I, II, III and IV. Structural studies. J. Antibiotics 29: 787~796, 1976
9) WITZ, D. F.; E. J. HESSLER & T. L. MILLER: Bioconversion of tyrosine into the propylhygric acid moiety of lincomycin. Biochemistry 10:1128~1132, 1971