ANTIBIOTICS PRODUCED BY MUTANTS OF *STREPTOMYCES CAELESTIS*

II. N-DEMETHYLCELESTICETIN AND N-DEMETHYL-7-O-DEMETHYLCELESTICETIN

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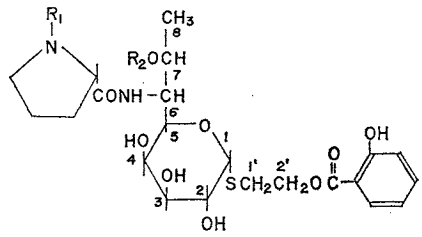
N-Demethylcelesticetin and N-demethyl-7-O-demethylcelesticetin are antibacterial agents produced by *Streptomyces caelestis* strain 22218a, a mutant of *S. caelestis*. As in the case of celesticetin, both antibiotics are active mainly against Gram-positive organisms.

Streptomyces caelistis is known to produce celesticetin (I, Fig. 1)[1,2] desalicetin and celesticetins B, C and D[3]. In a previous communication in this series we reported the production of 7-O-demethylcelesticetin (II, Fig. 1) by a mutant of *S. caelestis*[4]. The present paper describes the production, isolation and properties of two celesticetin-related antibiotics produced by another mutant of *S. caelestis* designated strain 22218a*.

Fig. 1.



Celesticetin (I) : $R_1=CH_3$; $R_2=CH_3$
7-O-Demethylcelesticetin (II) : $R_1=CH_3$; $R_2=H$
N-Demethylcelesticetin (III) : $R_1=H$; $R_2=CH_3$
N-Demethyl-7-O-demethylcelesticetin (IV) : $R_1=H$; $R_2=H$

Experimental

Spectroscopic Methods

Nuclear magnetic resonance spectra were calibrated downfield from internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Spectra were observed with a Varian A-60 spectrometer on solutions (*ca* 0.4 ml, *ca* 0.25 M) on the compounds in deuterium oxide or d_6-dimethylsulfoxide. Infrared spectra were obtained in mineral oil suspension.

Thin-Layer Chromatographic and Assay Procedures

Thin-layer chromatograms were run on silica gel G using chloroform-methanol (6 : 1 v/v) (solvent A), methyl ethyl ketone - acetone - water (186 : 52 : 20 v/v) (solvent B), or 2-pentanone - methyl ethyl ketone - methanol - water (2 : 2 : 1 : 1) (solvent C) as solvent systems. The antibiotics present in the fermentation were detected by bioautography on *Sarcina lutea* seeded agar.

Antibiotic production was measured by a microbiological disc-plate assay procedure[5] using *Sarcina lutea* as the test organism.

* Taxonomic studies on *S. caelestis* strain 22218a were carried out by Miss ALMA DIETZ of The Upjohn Company.

Production of *Streptomyces caelestis* strain 22218a

The mutagenic conditions and techniques employed to isolate *S. caelestis* strain 22218a were in general those described by COATS and ROESER[6] with the only exception that 3 mg/ml of N-methyl-N'-nitro-N-nitrosoguanidine in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-maleic acid buffer (pH 9.0) was used. Auxotrophic mutants were isolated and tested for antibiotic production.

Fermentation Procedures

Seed cultures of *S. caelestis* strain 22218a were prepared in a medium consisting of glucose monohydrate, 25 g/liter and Pharmamedia, 25 g/liter (Trader's Oil Mill Co., Fort Worth, Texas, U.S.A.); seed medium presterilization pH 7.2. The cultures were incubated at 28°C for 72 hours on a rotary shaker (250 rpm). Fermentation medium consisting of Wilson's liquid peptone, 15 g/liter; glucose monohydrate, 15 g/liter; and yeast extract 2.5 g/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

1. Filtration and Adsorption on Amberlite XAD-2:

Fermentation broth (4,400 liters) was filtered with the aid of diatomaceous earth. The filter cake was washed with water and the aqueous wash was combined with the clear filtrate. This solution was passed over a column prepared from 500 liters of Amberlite XAD-2 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) packed in water. The spent beer was bio-inactive and was discarded. The column was washed with 75 liters of water and then eluted with 1,100 liters of 95 % aqueous methanol. The methanolic eluate was concentrated to dryness to give 1,275 g of material containing practically all of the bioactivities present in the fermentation broth.

2. Purification by Counter Double Current Distribution. Isolation of N-Demethyl-7-O-demethylcelesticetin Hydrochloride:

Fifty grams of the material obtained as described above was dissolved in 200 ml of each phase of the solvent system consisting of equal volumes of 1-butanol - water. The solution was adjusted to pH 3.5 using 2 N aqueous hydrochloric acid. This solution was then added in eight center tubes of an all glass counter double current distribution apparatus (100 tubes, 25 ml per phase). The distribution was analyzed after 112 transfers by determination of bioactivity of selected fractions against *S. lutea* and by tlc. Fractions containing bioactive material(s) were combined and concentrated to dryness to yield 2.13 g of a mixture of N-demethyl-7-O-demethylcelesticetin and N-demethylcelesticetin hydrochlorides.

Following the above procedure five additional counter double current distribution runs were carried out each time using 50 g of the same starting material. The purified mixtures of the antibiotics obtained in these distributions were then combined and triturated with 150 ml of absolute methanol. Crystalline material insoluble in methanol was separated by filtration. Recrystallization from water-acetone yielded 700 mg of colorless needles identified as N-demethyl-7-O-demethylcelesticetin hydrochloride (*see* Discussion and Results). *Anal.* Calcd. for $C_{22}H_{32}N_2O_9S \cdot HCl \cdot H_2O$: C 47.65, H 6.31, N 5.06, O 29.06, Cl 6.31, S 5.77. Found: C 46.78; H 6.01, N 5.32, Cl 6.13, S 5.34.

The filtrate from the trituration with methanol was mixed with 2 liters of ether. The resulting precipitate (12.7 g) contained both N-demethylcelesticetin and N-demethyl-7-O-demethylcelesticetin as their hydrochloride salts. Separation of the two antibiotics was obtained by countercurrent distribution as described below.

3. Separation of N-Demethylcelesticetin and N-Demethyl-7-O-Demethylcelesticetin Hydrochlorides. Countercurrent Distribution:

The solvent system used consisted of equal volumes of 1-butanol-water. The starting material, 12.7 g, obtained as described above, was dissolved in 50 ml of each phase and added in 5 tubes of an all-glass countercurrent distribution apparatus (500 tubes, 10 ml/

phase). The distribution was analyzed after 1,500 transfers by testing for antibiotic activity and by tlc. Fractions 280~330 yielded 2.2 g of N-demethyl-7-O-demethylcelesticetin by concentration to dryness and crystallization from methanol. Fractions 340~400 contained N-demethylcelesticetin, small amounts of N-demethyl-7-O-demethylcelesticetin and bioinactive material(s). These fractions were then combined and the solution was concentrated to dryness; yield 1.5 g. This material was further purified by silica gel chromatography.

4. Isolation of N-Demethylcelesticetin Hydrochloride. Silica Gel Chromatography:

A column was prepared from 250 g of silica gel (Merck-Darmstadt, 7734) packed in the solvent system consisting of chloroform - methanol (6 : 1 v/v). The starting material, 1.3 g, obtained by the countercurrent distribution discussed above was dissolved in 20 ml of the solvent system. This solution was then mixed with 20 g of silica gel and the mixture concentrated to dryness. The obtained powder was added on the top of the column and the column eluted with the above solvent system. Fractions (20 ml each) were analyzed by UV and bioactivity determinations. Fractions 160~240 containing N-demethylcelesticetin were combined and the solution was concentrated to dryness. The residue was dissolved in 10 ml of 1 N methanolic hydrogen chloride and this solution was mixed with ether. N-Demethylcelesticetin hydrochloride, which precipitated as a colorless amorphous material, was isolated by filtration and dried; yield 130 mg. (For characterization of this material *see* Discussion and Results).

Discussion and Results

Antibiotics Produced by *Streptomyces caelestis* and Its Mutants

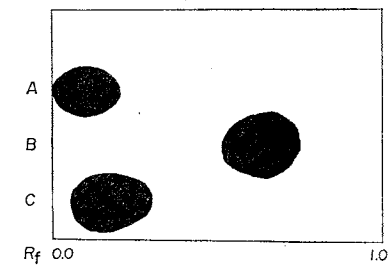
The major activity produced by *S. caelestis* is celesticetin[1]. Recent studies have shown the presence of several antibacterial agents in fermentations of this organism. In addition to celesticetin and desalicetin, celesticetin B (desalicetin 2'-isobutyrate), celesticetin C (desalicetin 2'-anthranilate) and celesticetin D (desalicetin 2'-acetate) have been isolated[3]. Furthermore *S. caelestis* strain 22227a has been reported recently[4] to produce mainly 7-O-demethylcelesticetin and a minor bioactive component tentatively identified as 7-O-demethyldesalicetin.

Culture filtrates of *S. caelestis* strain 22218a, the organism used in the present studies, examined by tlc* were found to contain one zone of bioactivity, Rf^A, 0.05; Rf^B, 0.10; Rf^C, 0.65. However, extraction of the filtrate revealed the presence of three antibiotics.

The major antibiotic component of the fermentation has been identified as N-demethyl-7-O-demethylcelesticetin (IV). One of the minor components was found to be N-demethylcelesticetin (III) while the nature of the second of the minor components is still unknown. As shown in Figs. 2, 3 and 4, N-demethyl-7-O-demethylcelesticetin and N-deme-

Fig. 2. Thin-layer chromatographic comparison of N-demethyl-7-O-demethylcelesticetin (A, 10 mcg), celesticetin (B, 5 mcg) and N-demethylcelesticetin (C, 5 mcg).

Solvent: Chloroform-methanol (6 : 1 v/v).
Support: Eastman's Chromagram 6061 silica gel sheets.
Antibiotics were detected by bioautography on *Sarcina lutea* seeded agar.



* Silica gel G: Rf^A, Rf^B, Rf^C, refer to Rf values in solvent A, solvent B and solvent C, respectively. Bioactive compounds were detected by bioautography on *Sarcina lutea*-seeded trays.

Fig. 3. Thin-layer chromatographic comparison of N-demethyl-7-O-demethylcelesticetin (A, 10 mcg), celesticetin (B, 5 mcg) and N-demethylcelesticetin (C, 5 mcg).

Solvent: Methyl ethyl ketone-acetone-water (186 : 52 : 20 v/v).
Support: Eastman's chromagram 6061 silica gel sheets.
Antibiotics were detected by bioautography on *Sarcina lutea* seeded agar.

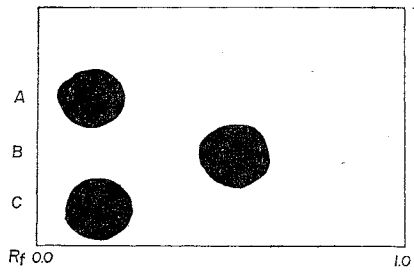
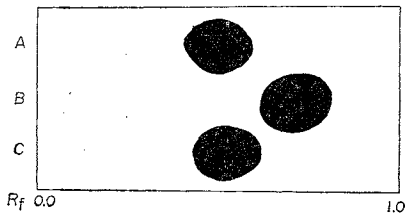


Fig. 4. Thin-layer chromatographic comparison of N-demethyl-7-O-demethylcelesticetin (A, 10 mcg), celesticetin (B, 5 mcg) and N-demethylcelesticetin (C, 5 mcg).

Solvent: 2-Pentanone-methyl ethyl ketone-methanol-water (2 : 2 : 1 : 1 v/v).
Support: Eastman's chromagram 6061 silica gel sheets.
Antibiotics were detected by bioautography on *Sarcina lutea* seeded agar.

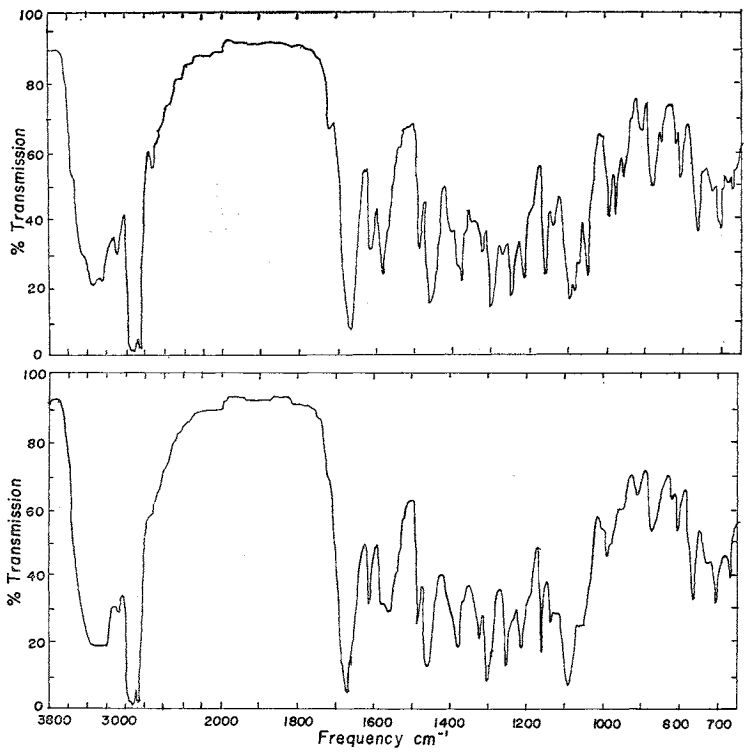


thylcelesticetin have very similar Rf values and cannot be separated easily by tlc.

Characterization of N-Demethyl-7-O-demethylcelesticetin Hydrochloride

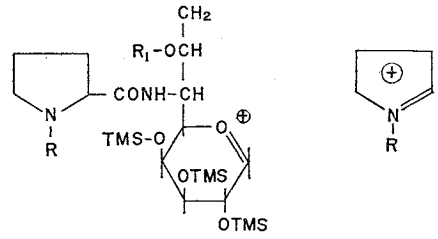
The major bioactive component of fermentations of *S. caelestis* strain 22218a was isolated as the crystalline hydrochloride, $C_{22}H_{32}N_2O_9S \cdot HCl \cdot H_2O$; $[\alpha]_D^{25}$ +122° (*c* 1, water). The molecular weight of the free base was found by mass spectrometry to be 500 (Calcd., 500).

Fig. 5. Infrared spectra (nujol mull) of N-demethyl-7-O-demethylcelesticetin (upper) and N-demethylcelesticetin (lower) hydrochlorides.



The ir spectrum (Fig. 5) which showed main absorption bands at 3540~3260 cm^{-1} and at 1667, 1612 and 1582 cm^{-1} is similar to the ir spectrum of celesticetin hydrochloride. The uv spectrum is identical to that of celesticetin showing maxima at 238 (a=17.66) and at 303 (a=7.20) nm in methanol. The maxima shift to 241 (a=11.42) and 332 (a=8.49) nm in alkaline methanol indicating the presence of the salicyloyl moiety[1] in this antibiotic. This conclusion is in agreement with the nmr data. The nmr spectrum (in d_6-DMSO) of this material (Fig. 7) is identical to that of celesticetin hydrochloride (Fig. 7) in the region from δ, 6.7 to δ, 7.9 (4 aromatic hydrogens assigned to the salicyloyl moiety of III). Furthermore the nmr spectrum showed the presence of absorptions due to $-\underset{\underset{O}{|}}{C}HCH_3$ [δ, 1.10 (3H, d)], hygric acid moiety (δ, 1.95), $-SCH_2CH_2O-$ (δ, 2.95) and anomeric hydrogen [δ, 5.5, 1H, d (J=6.0 Hz)]. All these absorptions are also present in the nmr spectrum of celesticetin hydrochloride. The nmr spectra of the new compound and of celesticetin differ in only two areas. The sharp singlets at δ, 2.9 ($N-CH_3$) and 3.25 (OCH_3) of the spectrum of celesticetin are not present in the spectrum of the new antibiotic. These data combined with the molecular formula and the ir and uv data suggest the N-demethyl-7-O-demethylcelesticetin structure (Fig. 1, IV) for this antibiotic.

Fig. 6.



Va, *m/e* 663: R = TMS ; R_1 = TMS VIa, *m/e* 142 : R = TMS
Vb, *m/e* 591 : R = H ; R_1 = TMS VIb, *m/e* 70 : R = H
Vc, *m/e* 605 : R = TMS ; R_1 = OCH_3
Vd, *m/e* 533 : R = H ; R_1 = OCH_3

Table 1. Molecular rotations of celesticetin-like antibiotics

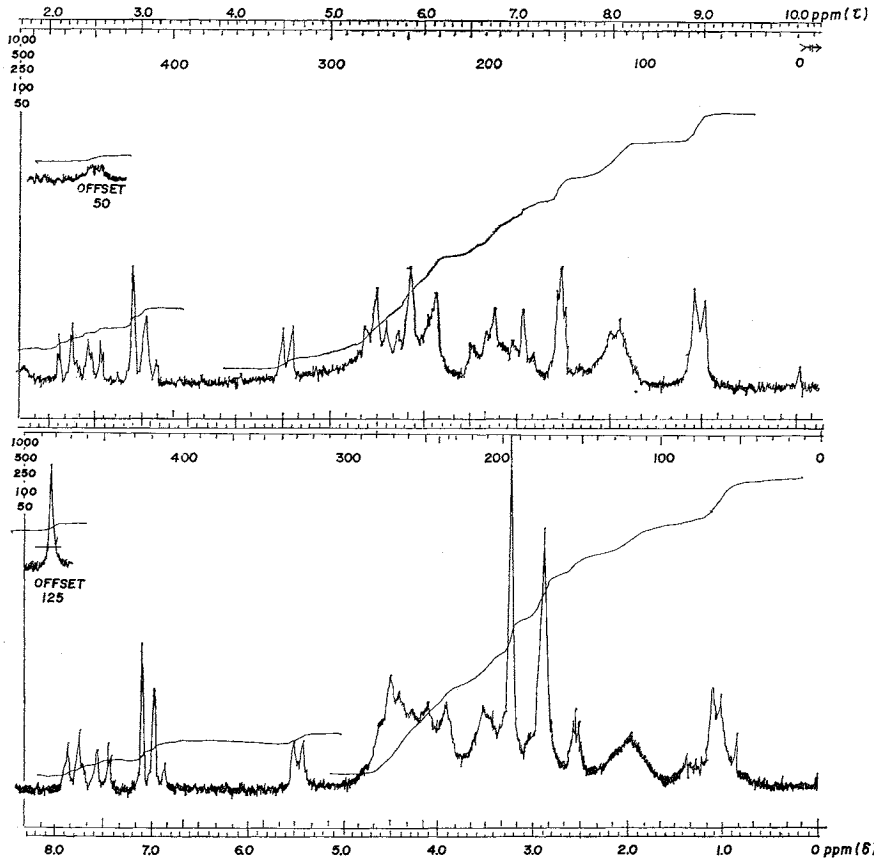
Antibiotic	$[M]_D$
Celesticetin	+654
Celesticetin B	+697
Celesticetin C	+648
N-Demethyl-7-O-demethylcelesticetin	+610
N-Demethylcelesticetin	+577

The mass spectrum of the trimethylsilyl (TMS) derivative of **IV** agrees with the proposed structure. The molecular ion appears at *m/e* 932 (Calcd. for $C_{22}H_{32}N_2O_9S \cdot 6$ TMS, 932). Other ion peaks appear at M^+-15 and at 860 (M^+-72). The latter ion is due to the penta-TMS derivative of **IV**. Ion peaks at 663 and 591 mass units are assigned to fragments **Va** and **Vb** (Fig. 6) respectively. Finally major ion peaks at 142 and 70 mass units are assigned to fragments **VIa** and **VIb** shown in Fig. 6.

The physical and spectral data discussed support structure **IV** (exclusive of stereochemistry) as the structure for the major activity produced by *S. caelestis* strain 22218a. It is reasonable to assume on the basis of common biogenesis that all asymmetric centers of N-demethyl-7-O-demethylcelesticetin have the same stereochemistry as those of celesticetin. This assumption is supported by comparison of the molecular rotation of N-demethyl-7-O-demethylcelesticetin to the molecular rotation of other celestosaminides (Table 1).

Furthermore the assignment of an α-anomeric linkage of the thioglycosidic group

Fig. 7. NMR spectra of N-demethyl-7-O-demethylcelesticetin (upper) and celesticetin (lower) hydrochlorides (In D_6-DMSO).



is supported by the observed coupling constant (J=6.0) of the doublet at δ, 5.5 which is due to the anomeric hydrogen.

Isolation and Characterization of N-Demethylcelesticetin Hydrochloride

The second of the antibiotics produced by *S. caelestis* strain 22218a has been identified as N-demethylcelesticetin. This material, separated from N-demethyl-7-O-demethylcelesticetin by silica gel chromatography, was isolated as the colorless amorphous hydrochloride salt, $[\alpha]_D^{25}$, +112.5° (*c* 1, water). Because of scarcity of material available for chemical and biological characterization, elemental analysis was restricted to the demonstration of the presence of C, H, N, S and Cl in the molecule. High resolution mass spectrum of the trimethylsilyl (TMS) derivative of the antibiotic showed molecular ion at 874.3967 mass units; the theoretical molecular weight for $C_{23}H_{29}N_2O_9S$. $[(CH_3)_3Si]_5$ is 874.3958. These results indicate a molecular formula of $C_{23}H_{34}N_2O_9S$ for this material which differs from the formulas of either celesticetin or N-demethyl-7-O-demethylcelesticetin by CH_2 (14 mass units).

The ir spectrum of the hydrochloride salt of III (Fig. 5) is similar to the ir spectra of celesticetin and N-demethyl-7-O-demethylcelesticetin (Fig. 5) hydrochlorides indicating the presence of the amide (-CONH-) carbonyl and hydrogen bonded ester

Fig. 8. NMR spectrum of N-demethylcelesticetin hydrochloride. (In D_2O)

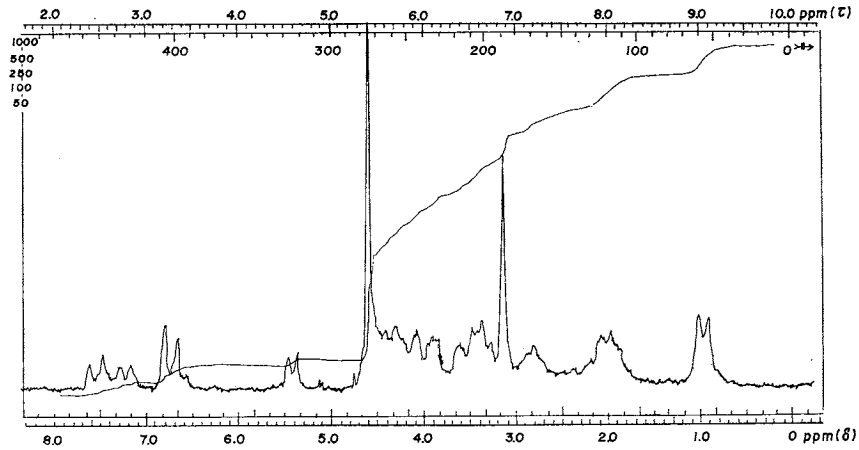


Table 2. *In vitro* antibacterial spectrum* of celesticetin, N-demethylcelesticetin and N-demethyl-7-O-demethylcelesticetin hydrochlorides

Test organism		Minimum inhibitory concentration (mcg/ml)		
		Celesticetin HCl	N-Demethylcelesticetin HCl	N-Demethyl-7-O-demethyl-celesticein HCl
S. aureus	UC 80	2.0	15.6	15.6
St. hemolyticus	UC 152	<0.125	<0.125	7.8
St. faecalis	UC 3235	4.0	7.8	31.2
E. coli	UC 51	>500	>500	>900
P. vulgaris	UC 93	>500	>500	>500
K. pneumoniae	UC 57	31.2	250	>500
Ps. aeruginosa	UC 95	>500	>500	>500
D. pneumoniae	UC 41	0.125	0.5	—

* Test method: Two fold dilution endpoints in brain-heart infusion broth; incubate at 37℃ for 20 hours.

grouping. In addition the ir spectra indicates the presence of aromatic hydrogens (absorption band at 1612 cm^{-1}). The uv spectrum showed maxima at 238 (a=15.5) and 304 (a=7.2) nm in methanol. The maxima shift to 241 and 332 nm in alkaline methanol. This behavior, identical to that of celesticetin or N-demethyl-7-O-demethylcelesticetin, indicates the presence of the salicyloyl moiety in the antibiotic. The nmr spectrum (Fig. 8) is identical to that of celesticetin hydrochloride in the aromatic hydrogen region (δ, 6.7 to 7.9, 4 hydrogens) also indicating the presence of the salicyloyl moiety. Furthermore, the nmr spectrum shows the presence of absorptions due to $-\underset{\underset{O}{|}}{C}HCH_3$ [δ, 1.0 (3H, d)], hygric acid moiety (δ, 1.98), $-SCH_2CH_2O-$ (δ, 2.85) anomeric hydrogen [δ, 5.5, 1H, d(J=6.0 Hz)] and $-OCH_3$ (δ, 3.15, s, 3H). All these adsorptions are also present in the spectrum of celesticetin hydrochloride. The nmr spectrum of the new antibiotic is characterized by the lack of the absorption at δ, 2.95 (s, 3H) present in the spectrum of celesticetin and assigned to the $-NCH_3$ group. These data combined with the molecular formula and the ir and uv spectral data suggest the N-demethylcelesticetin structure (Fig. 1, III) for this antibiotic.

The mass spectrum of the trimethylsilyl (TMS) derivative of III agrees with the proposed structure. In addition to the molecular ion appearing at 874.3967 mass units

the spectrum showed ion peak at 859.3724 corresponding to M^+-CH_3 (theoretical for $C_{23}H_{29}N_2O_9S$. $[(CH_3)_3Si]_5-CH_3$, 859.3723). Another ion peak appeared at 802 mass units and is assigned to the tetra-TMS derivative of **III**. Ion peaks at 605 and 533 mass units are assigned to ion fragments **Vc** and **Vd** (Fig. 6) respectively. Finally, major peaks at 142 and 70 mass units are assigned to fragments **VIa** and **VIb** (Fig. 6).

The physical and spectral data support structure III (exclusive of stereochemistry) for the minor component produced by *S. caelestis* strain 22218a. N-Demethylcelesticetin has significant *in vitro* antibacterial activity (Table 2). This fact* combined with biosynthetic and molecular rotation arguments (*see* above) suggest stereochemistry at C-1, C-2, C-3 and C-4 as shown in **III**. The assignment of the α-anomeric linkage of the thioglycosidic group is supported by the observed coupling constant of the doublet at δ, 5.5 which is due to the anomeric hydrogen.

Bioactivity of N-Demethylcelesticetin and N-Demethyl-7-O-demethylcelesticetin

The *in vitro* antibacterial spectra of N-demethylcelesticetin, N-demethyl-7-O-demethylcelesticetin and celesticetin hydrochlorides are presented in Table 2. All three antibiotics are active against Gram-positive organisms. Of the two antibiotics produced by *S. caelestis* strain 22218a, N-demethylcelesticetin is the more active. *In vivo* evaluation of these antibiotics is incomplete.

Acknowledgements

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* Dr. B. BANNISTER of The Upjohn Company has shown[7] that alterations of the stereochemistry at C-2, C-3, or C-4 of the aminosugar present in lincomycin result in complete loss of activity. This aminosugar has identical stereochemistry with the aminosugar present in celesticetin (*see* Ref. 2 and 4).