DIRECTED BIOSYNTHESIS OF NEW CELESTOSAMINIDE-ANTIBIOTICS BY *STREPTOMYCES CAELESTIS*

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Addition of aromatic acids to the culture medium of *Streptomyces caelestis* results in the production of antibiotics different from the celestosaminides normally produced by the organism. The new antibiotic produced when 4-aminosalicylic acid was added to the culture medium was isolated and characterized as desalicetin 2′-(4-aminosalicylate). Although biological evaluation of this antibiotic is not complete, its *in vitro* antibacterial spectrum is identical to that of celesticetin.

Radioactive salicylic acid added to the fermentation medium of *Streptomyces caelestis* was incorporated into the celesticetin molecule (**I**, Fig. 1), without randomization, to form the ester bond at the C-2′ hydroxyl of desalicetin (**II**, Fig. 1)[1]. This finding, combined with the isolation[2,3,4] of several celestosaminide antibiotics from culture filtrates of *S. caelestis* suggested that different acids, added to the fermentation medium, might be utilized by the organism for the production of new celestosaminides. The present communication describes the development of a microbial process for the production of new celesticetin-related antibiotics and the isolation of a new celestosaminide, desalicetin 2′-(4-aminosalicylate) (**III**, Fig. 1).

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) of the compounds in deuterium oxide. Infrared spectra were obtained in mineral oil suspension (Nujol mull).

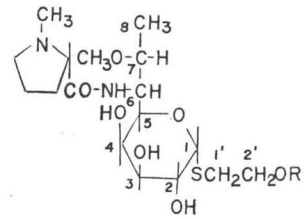
Thin-Layer Chromatographic Procedures

Thin-layer chromatograms were run on Eastman's 6061 silica gel sheets using the following systems: System A, chloroform-methanol (6:1, v/v); system B, ethyl acetate-acetone-water (8:1:5, v/v); system C, 2-pentanone-methyl ethyl ketone-methanol-water (2:2:1:1, v/v). Antibiotics were detected by bioautography on *Sarcina lutea* (UC-130)-seeded agar trays.

Fermentation Procedures

Seed cultures of *S. caelestis* (UC-2011) were prepared in a medium consisting of glucose monohydrate, 10 g/liter; Bacto peptone (Difco Lab., Detroit, Mich.), 10 g/liter and Bacto yeast extract (Difco Laboratories), 2.5

Fig. 1.



Celesticetin R = -C(=O)-(2-OH-C6H4) (I)

Desalicetin R = H (II)

Desalicetin 2′-(4-aminosalicylate) R = -C(=O)-(2-OH-4-NH2-C6H3) (III)

g/liter. The cultures were incubated at 28°C for 96 hours on a rotary shaker (250 rpm). Fermentation medium (100 ml per 500-ml Erlenmeyer flask) consisting of glucose monohydrate 45 g/liter; Buffalo starch (CPC International, Englewood Cliffs, N. J.), 40 g/liter; Black strap molasses (Knappen Molasses Co., Chicago, Illinois), 20 g/liter; calcium carbonate, 8 g/liter; potassium sulfate, 2g/liter; and Wilson's granular Peptone (Wilson Protein Technology, Div. of Wilson Pharm. and Chem. Co., Calumet City, Illinois), 25 g/liter, was adjusted to pH 7.2 and inoculated at a rate of 5% (v/v) with the 96-hour culture. After 24 hours of incubation the precursor acids (Table 1) were added to the culture medium at levels of 0.5~2 g/liter. The cultures were then reincubated and harvested after a total fermentation time of 96~120 hours.

Table 1*. Acids added to the fermentation medium of *Streptomyces caelestis*

1.	4-Aminosalicylic acid
2.	Anthranilic acid
3.	*m*-Aminobenzoic acid
4.	*p*-Aminobenzoic acid
5.	*N*-Methyl anthranilic acid
6.	*p*-Dimethylaminobenzoic acid
7.	*p*-Methylaminobenzoic acid
8.	*p*-Acetamidobenzoic acid

* For details on timing of addition, levels of the acids added and other fermentation conditions *see* experimental.

Fermentation Conditions for Production of Desalicetin 2'-(4-aminosalicylate)

The fermentation procedures used for the production of desalicetin 2'-(4-aminosalicylate) were identical to those described above. 4-Aminosalicylic acid was added to the culture medium at a level of 2 g/liter 24 hours after inoculation. Fermentations were harvested after a total fermentation time of 120 hours.

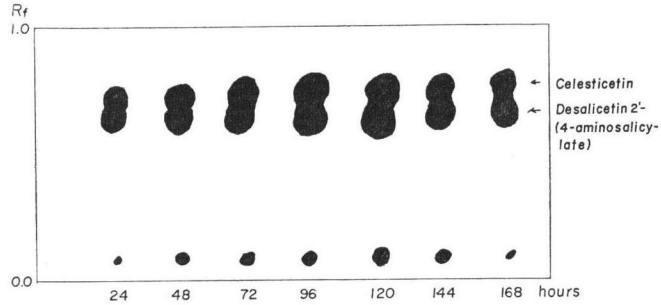
Determination of Antibiotic Production

The total antibiotic production was determined by use of a disc plate assay using *Sarcina lutea* as the assay organism[5]. In order to determine the time of highest production of desalicetin 2'-(4-aminosalicylate) samples of the fermentation were taken at different time intervals and analyzed by tlc (system A). Results of a typical fermentation containing 4-aminosalicylic acid are presented in Fig. 2.

Isolation of Desalicetin 2'-(4-aminosalicylate). Filtration and Adsorption on Amberlite XAD-2

Fermentation broth (*ca* 10 liters), containing desalicetin 2'-(4-aminosalicylate), was filtered with the aid of diatomaceous earth. The filter cake was washed with 2 liters of water and the aqueous wash was combined with the clear filtrate. This solution was passed over a column containing 500 ml 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 2 liters of water and then eluted with 3 liters of 95% aqueous methanol. The methanolic eluate was concentrated to dryness to give 11.7 g of material containing all the bioactivities present in the fermentation broth (see Fig. 2). Further purification of this preparation was

Fig. 2*. Antibiotic production by *S. caelestis* in the presence of 4-aminosalicylic acid



* Tlc on silica gel; system A
** Fermentation hours after addition of 4-aminosalicylic acid

obtained by the counter double current distribution described below.

Counter Double Current Distribution of Material Obtained by Amberlite XAD-2 Chromatography

The material (11.7 g) obtained by Amberlite XAD-2 chromatography (see above) was dissolved in 100 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 four center tubes of an all-glass counter double current distribution apparatus (100 tubes, 25 ml/phase). The distribution was analyzed after 178 transfers by tlc and bioactivity determination using *S. lutea* as the assay organism. Tubes containing desalicetin 2′-(4-aminosalicylate) alone or in mixture with celesticetin were combined and the solution was concentrated to dryness to give 1.5 g of highly bioactive material containing (by tlc) both desalicetin 2′-(4-aminosalicylate) and celesticetin hydrochlorides. Separation of these antibiotics was obtained by silica gel chromatography described below.

Separation of Desalicetin 2′-(4-aminosalicylate) from Celesticetin. Silica Gel Chromatography

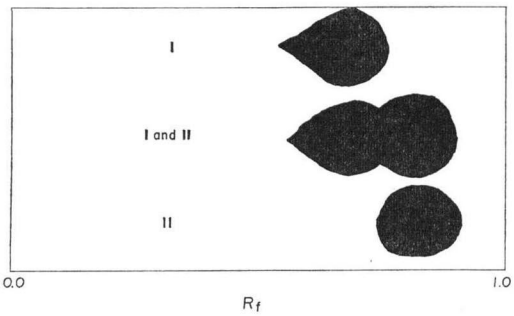
A column was prepared from 500 g of silica gel (Merck-Darmstadt, 7734) packed in the solvent system consisting of chloroform-methanol (6:1, v/v). The mixture of the antibiotics obtained by counter double current distribution (see above), *ca* 1.5 g, was dissolved in 50 ml of the solvent system. This solution was mixed with 30 g of silica gel and the mixture was concentrated to dryness. The powder obtained was added to the top of the column and the column was then eluted with the chloroform-methanol solvent system. Bioactive fractions were analyzed by tlc. Celesticetin was eluted first followed by desalicetin 2′-(4-aminosalicylate). Fractions containing desalicetin 2′-(4-aminosalicylate) were combined and concentrated to dryness. The residue was dissolved in 10 ml of methanol, 10 ml of chloroform and 500 ml of ether. Methanolic hydrogen chloride (1 N, 5 ml) added to the solution resulted in the precipitation of colorless desalicetin 2′-(4-aminosalicylate) hydrochloride which was isolated by filtration; yield 250 mg. Characterization of this material is described later in this paper.

Results and Discussion

As mentioned earlier *S. caelestis* produces a series of celestosaminide antibiotics differing from celesticetin in the acid moiety esterified to the primary hydroxyl (C-2′) of desalicetin. This finding strongly suggested that the enzymatic system responsible for the ester formation is not specific for salicylic acid and that other acids could be used by the enzyme(s) if they could become available at the site of the enzymatic reaction. The observation that exogenous salicylic acid is incorporated into celesticetin[1] selectively without randomization, also suggested that other acids might be utilized by *S. caelestis* for the production of new celestosaminides.

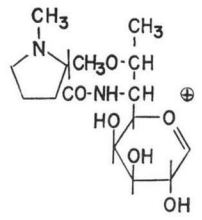
The acid-precursors used in the present study (Table 1) were added to cultures of *S. caelestis* 24 hours after inoculation. The production of antibiotics including celesticetin or desalicetin was followed by tlc and by determination of the antibacterial spectrum of the antibiotic mixture present in the fermentation broth. Since the effect of 4-aminosalicylic acid on antibiotic production by *S. caelestis* was studied in detail this work will be discussed in this paper. Our observations with the other aromatic acids listed in Table 1 will be described in subsequent communications. We would like to report at present that in all cases a new antibiotic with antibacterial spectrum similar to that of celesticetin was detected 24 hours after the addition of the acid-precursors to the culture medium. This indicates that the presence of a hydroxyl or an amino group, ortho to the carboxyl of the aromatic acid is not required for the esterification reaction.

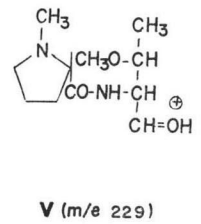
Fig. 3*. Thin-layer chromatogram of desalicetin 2′-(4-aminosalicylate) (I) and celesticetin (II)

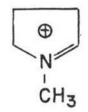


* Eastman's 6061 silica gel; system A (*see* experimental); Detection by bioautography on *S. lutea*-seeded agar tray

Fig. 4.







The sequence of antibiotic production* when 4-aminosalicylic acid was used as precursor is presented in Fig. 2. The peak of production of desalicetin 2′-(4-aminosalicylate) was found by both tlc and bioactivity determination to be 72~96 hours after the addition of the precursor to the culture medium.

Celesticetin and desalicetin 2′-(4-aminosalicylate) the two major antibiotics present in culture filtrates of *S. caelestis* (see Fig. 2) were isolated by a process involving adsorption on Amberlite XAD-2 and elution with 95% aqueous methanol. Partial separation of the two antibiotics was obtained by counter double current distribution. Desalicetin 2′-(4-aminosalicylate) was separated from celesticetin by silica gel chromatography and isolated as the colorless amorphous hydrochloride salt.

The paper chromatographic pattern of desalicetin 2′-(4-aminosalicylate) in several systems is almost identical to that of celesticetin. The tlc mobility of this antibiotic relative to celesticetin is presented in Fig. 3. Desalicetin 2′-(4-aminosalicylate) hydrochloride $[\alpha]_D^{25}+111°$ (*c* 1, water), is very soluble in water, soluble in methanol and ethanol and relatively insoluble in less polar organic solvents. The high resolution mass spectrum of desalicetin 2′-(4-aminosalicylate) demonstrated a molecular formula of $C_{24}H_{37}N_3O_9S$; molecular weight, 543.2255 (calculated molecular weight, 543.2249), in agreement with the postulated structure **III** (Fig. 1). The molecular formula of **III** differs from that of celesticetin (**II**, Fig. 1) by a $-NH_2$ group suggesting that salicylic acid has been replaced by 4-aminosalicylic acid in **III**. This conclusion is supported by spectral data. The UV spectrum of **III** showed maxima at 208 (ε=12,500), 240 (ε=4,550), 288 (ε=7,300) and 306 (ε=9,200) nm expected for a compound containing 4-aminosalicylic acid[8]. The IR spectrum of desalicetin 2′-(4-aminosalicylate) is almost identical to the IR spectrum of celesticetin

Table 2. Molecular rotations of celesticetin-like antibiotics

	$[M]_D$
Celesticetin (**II**)	+654
Celesticetin B*	+697
Celesticetin C*	+648
Desalicetin 2′-(4-aminosalicytate) (**III**)	+603

* For structures of celesticetins B and C *see* Ref. 3.

* Desdanine, a broad spectrum antibacterial agent[6,7] was detected in fermentations of *S. caelestis* supplemented with 4-aminosalicylic acid.

Table 3. *In vitro* antibacterial spectrum* of desalicetin 2′-(4-aminosalicylate) and celesticetin hydrochlorides

Test organism	Mininum inhibitory concentration (mcg/ml)	
	Celesticetin HCl	Desalicetin 2′-(4-aminosalicylate) HCl
Staphylococcus aureus UC 76	1.0	2.0
Streptococcus hemolyticus UC 152	<0.5	<0.5
Streptococcus faecalis UC 3235	2.0	2.0
Escherichia coli UC 51	>500	>500
Proteus vulgaris UC 93	>500	>500
Klebsiella pneumoniae UC 57	500	500
Mycobacterium avium UC 159	2.0	1.0
Pseudomonas aeruginosa UC 95	>500	>500
Bacillus subtilis UC 564	1.0	4.0
Diplococus pneumoniae UC 41	1.0	<0.5
Sarcina lutea UC 130	1.0	<0.5

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

and specifically in the ester carbonyl and amide carbonyl regions (1675, 1650 sh, 1625 sh, 1565 cm^{-1}). This indicates that the hydrogen bonded ester grouping and the primary (-CONH-) amide which are present in celesticetin are also present in the new antibiotic (**III**). The nmr spectrum of desalicetin 2′-(4-aminosalicylate) is identical to that of celesticetin in all regions except the aromatic hydrogens region (δ 6.0~8.0). This suggests that hygric acid, $-NCH_3$, $-OCH_3$, and the same sugar moiety are present in both antibiotics. The aromatic hydrogen region shows the presence of three hydrogens as expected for desalicetin 2′-(4-aminosalicylate). The mass spectrum of **III** (in addition to the molecular ion discussed already) shows major peaks at 331, 229 and 84 mass units. These ion peaks are also present in the spectrum of celesticetin.

The data discussed support structure **III** (exclusive of streochemistry) as the structure for the new activity produced by *S. caelestis* in a medium supplemented with 4-aminosalicylic acid. We assume, on the basis of common biogenesis, that all asymmetric centers of **III** have the same stereochemistry as those of celesticetin. This assumption is supported by comparison of the molecular rotation of **III** to the molecular rotation of other celestosaminides (Table 2).

Furthermore, the assignment of an α-anomeric linkage of the thioglycosidic group is supported by the observed coupling constant (J=6.0) of the doublet at δ 5.5 which is due to the anomeric hydrogen.

Bioactivity of Desalicetin 2′-(4-aminosalicylate)

The *in vitro* antibacterial spectra of desalicetin 2′-(4-aminosalicylate) and celesticetin hydrochloride are presented in Table 3. Both antibiotics have the same antibacterial spectrum and are equally active against the organisms listed. *In vivo* evaluation of desalicetin 2′-(4-aminosalicylate) is in progress.

Acknowledgement

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References

1) ARGOUDELIS, A.D. & J.H. COATS: Incorporation of exogenous salicylic acid into celesticetin. J. Antibiotics 27: 674~676, 1974
2) HOEKSEMA, H.: Celesticetin. V. The structure of celesticetin. J. Am. Chem. Soc. 90: 755~757, 1968
3) ARGOUDELIS, A. D. & T. F. BRODASKY: Studies with *Streptomyces caelestis*. I. New celesticetins. J. Antibiotics 25: 194~196, 1972
4) ARGOUDELIS, A. D. & T. F. BRODASKY: Studies with *Streptomyces caelestis*. III. Antibiotics containing lincosamine or celestosamine. J. Antibiotics 27: 642~645, 1974
5) HANKA, L.J.; M.R. BURCH & W.T. SOKOLSKI: Psicofuranine. IV. Microbiological assay. Antibiot. & Chemoth. 9: 432~435, 1959
6) MEYER, C. E. & D. J. MASON: New antibiotics produced by *Streptomyces caelestis*. Antimicr. Agents & Chemoth. -1965:850~854, 1966
7) ARGOUDELIS, A. D.; H. HOEKSEMA & H. A. WHALEY: Identity of desdanine, pyracrimycin and cyclamidomycin. J. Antibiotics 25:432~433, 1972
8) Sadtler Catalog of UV Spectrum: No. 3162