THE ISOLATION AND STRUCTURE ELUCIDATION OF MACROCYCLIC LACTONE ANTIBIOTIC, A26771B

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The isolation, biological properties and structure elucidation of a sixteen-membered macrocyclic lactone antibiotic, designated A26771B (**1**), obtained from *Penicillium turbatum* are discussed.

In addition to the epipolythiopiperazinedione antibiotics[1], *Penicillium turbatum* (WESTLING) produces a sixteen-membered macrocyclic lactone antibiotic, A26771B[2]. A growing list of sixteen-membered macrolide antibiotics belonging to the carbomycin-leucomycin and tylosin-chalcomycin groups has been discovered during the past ten years[3]. Antibiotic A26771B is different in both its chemical structure and biological properties from the known members of this class.

Fermentation

The fermentation conditions for production of A26771B are identical to those described for production of the epipolythiopiperazinedione metabolites A26771A, C and E by *Penicillium turbatum* (NRRL 5630)[2]. The major metabolite is A26771B, which accounts for about 85~95% of the antibiotic activity. The piperazinedione antibiotics A26771A, C and E, are responsible for the remainder of the activity.

Isolation and Physical-Chemical Characterization

The filtered broth from 100 liters of fermentation medium, containing A26771A, A26771C and A26771E, was used as described previously[1]. The mycelium was extracted with 40 liters of ethyl acetate, and the extract was dried over anhydrous sodium sulfate. The extract was then filtered and concentrated under vacuum to about 5 liters. After standing at 4°C for 72 hours, approximately 200 g of crude A26771B settled out of the solution. The crude antibiotic was recovered by filtration, redissolved in 2 liters of ethyl acetate and filtered. The filtrate was allowed to stand at 4°C for crystallization. The first crop yielded 46 g of A26771B, mp 124~125°C. A second crop of crystalline A26771B (54 g, mp 124~125°C) was obtained from the concentrated mother liquor. The two crops were combined (110 g) and recrystallized from acetone-water, mp 124~125°C. The purification of A26771B was monitored by thin-layer chromatography on silica gel plates (Merck, Germany) with chloroform - methanol (98: 2) as the solvent system.

Antibiotic	A26771A	A26771B	A26771C	A26771E
Rf value	0.48	0.05	0.37	0.26

A26771A, B, C and E were detected on TLC plates by spraying with phosphomolybdic acid and heating at 100~110°C. A blue color developed where the spray reagent contacted the compounds. Bioautograms of A26771B were monitored using *Sarcina lutea* as the indicator organism.

The antibiotic A26771B is insoluble in water, but soluble in polar organic solvents such as ethyl and methyl alcohols, acetone and ethyl acetate. It is relatively insoluble in chloroform, diethyl ether and hydrocarbon solvents. The compound has the following properties: UV (EtOH) λ_{max} 220 nm (ε16,000) in neutral and acid solutions, and λ_{max} 365 nm (ε6,000) in basic solution (the shift from 220 to 365 nm is irreversible); IR (KBr) ν_{max} 3000~2500, 1735, 1700, 1300, 1185 cm^{-1}; $[\alpha]_D^{24}$—14° (*c* 0.13, methanol); ^1H NMR ($CDCl_3$) δ10.50 (1H, broad s, exchangeable), 7.27 (1H, d, J=16 Hz), 6.76 (1H, d, J=16 Hz), 5.36 (1H, t, J=5.5 Hz), 5.15 (1H, m), 2.74 (4H, s), 1.30 (3H, d, J=6.5 Hz), 2.0~1.0 (18H, m); ^{13}C NMR ($CDCl_3$) δ 195.2 (s), 177.5 (s), 171.2 (s), 164.7 (s), 135.2 (d), 132.3 (d), 77.7 (d), 72.4 (d), 34.3 (t), 28.7 (two carbon resonances, t), 28.4 (t), 27.8 (t), 27.7 (t), 27.1 (t), 26.9 (t), 26.8 (t), 23.4 (t), 22.1 (t), 19.6 (q); field desorption mass spectrum gives a quasimolecular ion M+1=363.

Anal. Calcd for $C_{20}H_{30}O_7$: C, 62.9; H, 7.9; O, 29.2.
Found: C, 62.9; H, 7.7; O, 29.0.

Reactions of A26771B

Succinic acid from A26771B: To a solution of 0.5 g of A26771B in 50 ml of methanol was added 5 ml of 1% methanolic sodium methoxide. After standing at ambient temperature for 3 hours the reaction mixture was evaporated to dryness. The residue was partitioned between 200 ml of diethyl ether and 25 ml of 1 N sulfuric acid. The ether layer was washed with water and concentrated. Fifty-five mg of a solid, mp 186~188°C, crystallized from the concentrate. The IR and ^1H NMR spectra, TLC movements and mp of this compound and succinic acid were identical.

Methyl ester of A26771B: Reaction of A26771B with diazomethane does not give the methyl ester, but a complex mixture of products is obtained. However, the desired methyl ester was obtained as follows: To a solution of 5 g of A26771B in 200 ml of absolute methanol at 0°C was added 50 ml of 3% methanolic hydrogen chloride. After 1-hour stirring at 0°C, 2 g of the methyl ester crystallized, mp 85~87°C. Elemental analysis, ^1H NMR, IR, UV and mass spectra were consistent for the mono methyl ester of A26771B.

Biological Properties

The antimicrobial activity of A26771B is summarized in Table 1. Compound A26771B exhibits moderate activity against gram-positive bacteria, mycoplasma and fungi. The antibiotic is inactive against *Proteus* sp., *Salmonella typhosa, Salmonella typhimurium, Klebsiella enterobacter* group, *E. coli* and *Pseudomonas aeruginosa* at 100 μg/ml in the agar dilution test. The compound showed no activity when administered subcutaneously against *Staphylococcus aureus, Streptococcus pyogenes* and *Streptococcus pneumoniae* infections in mice. The toxicity was measured in mice and the i.p. LD_{50} of A26771B was 62 mg/kg. Compound A26771B

Table 1. Antibacterial spectrum of A26771B

Organism	MIC (μg/ml)
Staphylococcus aureus[a]	<1.56
Streptococcus faecalis[a]	25.0
Vibrio coli[b]	50.0
Mycoplasma gallisepticum[b]	3.12
Mycoplasma granularum[b]	50.0
Mycoplasma synoviae[b]	12.5
Mycoplasma hyosynoviae[b]	12.5
Mycoplasma hyopneumoniae[b]	12.5
Erwinia amylovora[a]	<0.78
Pasteurella multocida[b]	6.25
Xanthomonas phaseoli[a]	50.0
Candida tropicalis[a]	100.0
Trichophyton mentagrophytes	6.25
Botrytis cinerea	12.5
Ceratocystis ulmi	6.25
Verticillium albo-atrum	50.0

[a]agar dilution. [b]broth dilution.

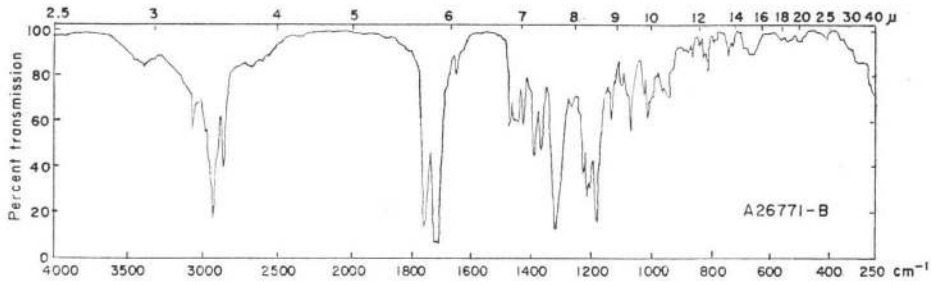
also inhibits potassium dependent ATPase in rat liver mitochondria.

Structure Elucidation

A "macrolide antibiotic" is conceived as a compound that contains a large lactone ring which has a few double bonds but no nitrogen atoms; it has one or more sugars which can be amino sugars, non-nitrogen sugars or both[4]. A cursory examination of the ^{1}H and ^{13}C NMR spectra of A26771B suggests the presence of a macrocyclic lactone moiety, but clearly rules out a sugar residue. The biological properties of A26771B are different from those expected of a macrolide antibiotic. Since the chemical structure and biological activity of A26771B seemed to be unique for a macrolide antibiotic, its structure elucidation was undertaken.

Antibiotic A26771B is a $C_{29}H_{36}O_7$ acidic compound, which forms a mono methyl ester with methanolic hydrogen chloride. The bands at 3000~2500, 1700 and 1300 cm^{-1} in the infrared spectrum confirm the presence of a carboxyl group in A26771B (Fig. 1). Bands at 1735 and 1185 cm^{-1} in the

Fig. 1. Infrared spectrum of A26771B in KBr.



infrared spectrum and the four proton singlet at δ2.74 and one exchangeable proton at δ10.5 in the ^{1}H NMR spectrum suggest a hemi-succinate residue in A26771B. This is further confirmed by the isolation of succinic acid by sodium methoxide hydrolysis of A26771B.

In the ^{1}H NMR spectrum of A-26771B (Fig. 2), there are two signals at δ5.36 and 5.15 which are assigned to protons on carbons linked to the oxygen of succinyl and lactone residues. Double irradiation at *ca.* δ1.90 collapses the triplet at δ5.36 to a singlet.

Double irradiation at δ1.60 and at the center of the methyl doublet at δ 1.30 simplifies the complex multiplet at δ5.15. However, triple irradiation at δ

Fig. 2. ^{1}H NMR spectrum of A26771B in $CDCl_3$.

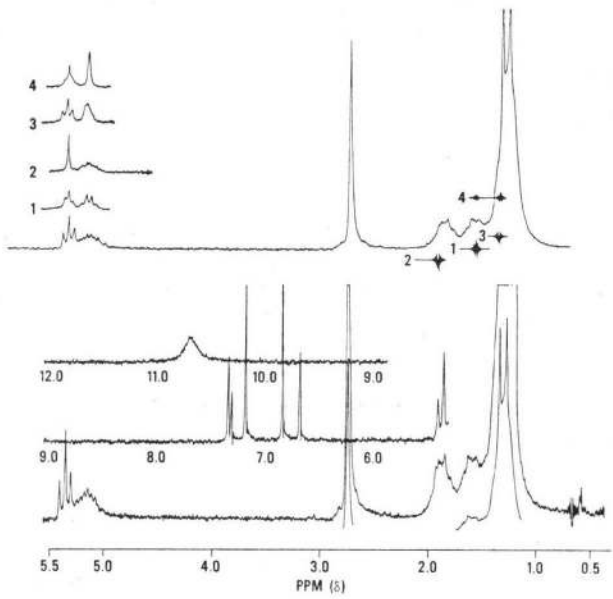
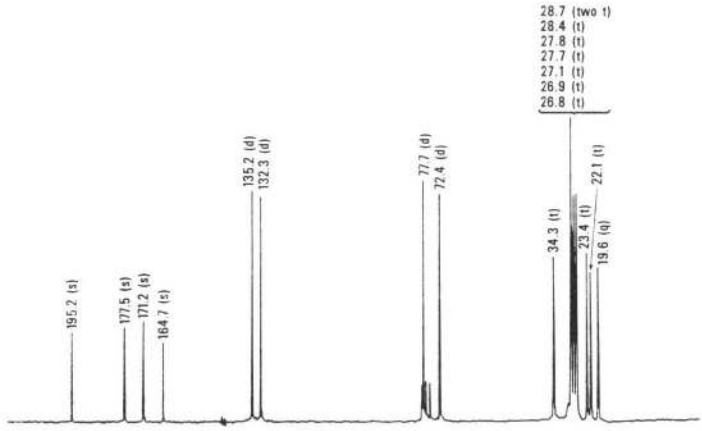
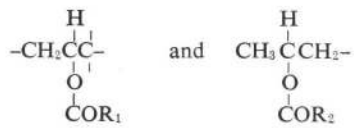


Fig. 3. Proton decoupled ^{13}C NMR spectrum of A26771B in $CDCl_3$.



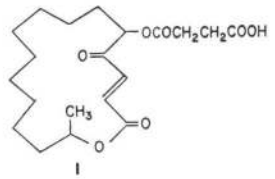
1.60 and 1.30 collapses the complex multiplet at δ5.15 to a singlet. These decoupling experiments suggest the following moieties:



where R_1 and R_2 are hemi-succinate and lactone residues.

The two doublets at δ7.27 and 6.76 with J = 16 Hz suggest a *trans* double bond, and the absence of any other long range coupling suggests that the *trans* double bond is flanked by carbons that do not carry a hydrogen atom.

Fig. 4.



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Examination of the proton coupled and proton decoupled ^{13}C NMR spectra of A26771B reveals singlets at δ195.2, 177.5, 171.2 and 164.7 suggesting the presence of four carbonyl groups. The doublets at δ135.2 and 132.2 could arise from two double bond carbons, and the doublets at δ77.7 and 72.4 reveal the presence of two methine hydroxy carbons (H–C–O). Of the four carbonyl resonances, the signal at δ195.2 is consistent for an α,β-unsaturated ketone and the other three carbonyl resonances could arise due to the presence of carboxylic acid, ester or lactone carbonyl moieties. The quartet at δ19.6 suggests an aliphatic CH_3 group. The remaining eleven carbon resonances are triplets and are probably due to CH_2 groups. The ultraviolet spectrum is compatible with that of a –C(=O)–C=C–COO group[5], but this group is destroyed in basic solution. On the basis of the above data, structure **1** can be written for A26771B (Fig. 4).

The structure of A26771B was confirmed by the preparation of a number of derivatives of A26771B in the course of a chemical modification effort. The preparation of A26771B derivatives and their biological properties will be described in a subsequent publication.

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