7-METHOXY-3'-DEACETYLCEPHALOSPORIN C FROM *STREPTOMYCES* HPL Y-22996. ISOLATION AND CHEMICAL CHARACTERIZATION

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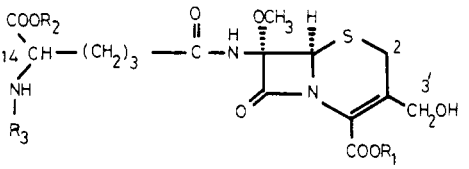
Abstract.—*Streptomyces* HPL Y-22996 produces a complex of β-lactam antibiotics. The major component was isolated and identified as 7-methoxy-3'-deacetylcephalosporin C by high resolution mass spectrometry.

During the course of our screening for broad spectrum β-lactam antibiotics possessing β-lactamase stability, a *Streptomyces* species numbered HPL Y-22996 was isolated. Details of the morphology, physiology and biochemical properties of the culture will be reported elsewhere (1). This culture was found to produce a complex of β-lactam antibiotics belonging to the Cephamycin family. The major component of this complex has now been identified as 7-methoxy-3'-deacetylcephalosporin C (7-methoxy DCPC) (**1**). While there are two earlier reports on the isolation of this compound (2,3), this is the first time that a detailed description of the isolation procedure and structure determination by high resolution mass spectrometry are reported.



1 $R_1 = Na$, $R_2 = H$, $R_3 = H$

2 $R_1 = R_2 = CH_3$, $R_3 = -C(=O)-CH_2-O-C_6H_5$

EXPERIMENTAL[1]

Fermentation of streptomyces y-22996.—Ten liters of fermentation fluid were routinely collected for the isolation of 7-methoxy-3'-deacetylcephalosporin C (7-methoxy DCPC). One well sporulated slant was used to inoculate five 500 ml Erlenmeyer flasks each containing 50 ml of seed medium. The seed was grown at 30° for 48 hours on a rotary shaker at 220 rpm. Five ml of the seed was inoculated into every 100 ml of production medium distributed into 500 ml Erlenmeyer flasks. The production cycle was 72 hours at 30° and 220 rpm. At the end of the fermentation, the mycelium was separated by centrifugation. The clear fluid had a pH of 6.8 and contained 150–250 micrograms of 7-methoxy DCPC per ml as estimated by bioassay.

The seed medium contained glycerol 1.5%, sucrose 1.5%, soya-bean meal 1.5%, yeast extract 1.0%, tryptone 0.5%, $MgSO_4.7H_2O$ 0.1%, K_2HPO_4 0.02%. The pH of the medium was adjusted to 6.8. The production medium contained starch (4.5%), soya-bean meal (2.0%), cornsteep liquor (1%), glycerol (1%), tryptone (0.5%), calcium carbonate (0.2%), sodium chloride (0.3%), ferrous sulphate (0.01%), cobalt chloride (0.001%). The pH of the medium was adjusted to 6.8.

Biological assay.—The potency of the culture fluid was estimated by bioassay by the standard agar diffusion method (4) with *E. coli* TEM as the test organism. A standard curve

[1]Uv spectra were obtained on a Carl-Zeiss UV-VIS Specord Spectrophotometer. Ir spectra were obtained on a Perkin-Elmer Spectrophotometer, model 521, using KBr pellets. Nmr spectra were recorded on a Varian T-60 Spectrometer with tetramethylsilane as internal standard. Mass spectra were obtained on an AEI MS-902S spectrometer equipped with an on-line data system DS-50SH. Melting points are uncorrected.

of the zone diameter of inhibition was plotted against the log concentration of pure 7-methoxy DCPC.

ISOLATION OF 7-METHOXY-3'-DEACETYLCEPHALOSPORIN C.—The culture filtrate (10 liters) was adjusted to pH 7 and passed through a one liter bed of Amberlite IRA-68 (OAc^-) resin. The activity was eluted with 1M sodium chloride (2 liters) in Tris buffer (pH 7.2). The pH of the pooled active eluates was adjusted to 2.5 and this was applied on a column of Amberlite XAD-4. The resin was washed with deionized water, and the antibiotics were eluted with 10% aqueous isopropanol. The XAD-4 resin eluate containing the antibiotics was concentrated to a small volume and submitted to further chromatography on DEAE-Sephadex A-25. Elution with a linear gradient of sodium chloride (0–0.3 M) in Tris buffer (pH 7.2) separated 7-methoxy-3'-deacetyl-cephalosporin C from at least three other minor constituents. The active fractions were pooled and desalted on a column of Amberlite XAD-2 (200 ml) with water as the eluant. Salt free fractions were concentrated to a small volume and subjected to gel filtration on Biogel P-2 with water as the eluant. The active eluates were freeze-dried and chromatographed over silica gel with methanol-ethyl acetate (7:3) as the eluant. Finally, chromatography on cellulose with acetonitrile-water (4:1) gave the pure compound (105 mg) as a white hygroscopic solid, mp 160–170°C (with decomp); λ max (in phosphate buffer pH 6.5) (ϵ): 268 nm (145), 242 (129); ν max: 1760 cm^{-1} (β-lactam); nmr (D_2O): δ 5.13 (s, 1H, C_6-*H*), 3.53 (s, 3H, OCH_3), 2.6 (m, 2H, NH-C-CH_2), 1.83 (m, 4H, CH_2-CH_2-CH_2-C), the geminally coupled 2-methylene protons (S-CH_2) overlapped with C_{14}-*H* triplet in the region δ 3.33–4.0, 3'-methylene group (-CH_2OH) merged with the HDO signal.

Anal. Calc. for $C_{15}H_{20}N_3O_8SNa$: C 42.35; H 4.71; N 9.85; S 7.53. Found: C 42.12; H 4.65; N 9.68 and S 7.42%.

N-PHENOXYACETYL-7-METHOXY-3'-DEACETYLCEPHALOSPORIN C DIMETHYL ESTER (**2**).—*N*-acylation of 7-methoxy-3'-deacetylcephalosporin C (42 mg, 0.1 mmole) was accomplished under Schotten-Baumann conditions with phenoxyacetyl chloride (42 mg, 0.3 mmole) at 0° for 2 hours in saturated sodium bicarbonate solution (2 ml). After completion of the reaction, the mixture was adjusted to pH 7.5 with Dowex 50-X8(H^+) and extracted with ether to remove unreacted phenoxyacetyl chloride. The aqueous portion was freeze-dried, and the resulting crude solid was methylated (HMPA/CH_3I) without isolation of the *N*-acyl derivative. After two hours at room temperature, the mixture was extracted with chloroform and concentrated. The resulting gum was chromatographed over silica gel. Elution with benzene-ethyl acetate (9:1) afforded the desired compound as a white solid (38.5 mg, 70% yield), mp 55–60°C. ν max: 1780 (β-lactam), 1720 (ester), 1675 cm^{-1} (C=C-CO_2CH_3); nmr: δ 7.5–6.8 (m, 5H, -O-C_6H_5) 5.06 (s, 1H, C_6-*H*), 4.93 (s, 2H, -CH_2OH), 4.50 (s, 2H, -CH_2-O-C_6H_5), 3.86 (s, 3H, CO_2CH_3), 3.73 (s, 3H, CO_2CH_3), 3.53 (s, 3H, OCH_3), 2.38 (m, 2H, NH-C-CH_2), 1.83 (m, 4H, -CH_2-CH_2-CH_2-C), doublet of S-CH_2 hidden; high resolution mass spectrometry: (compound was silylated with *N*-methyl-*N*-trimethylsilyl trifluoro acetamide (MSTFA), 2 hours at 60°). Calc. for $C_{31}H_{47}O_{10}N_3SSi_2$ ($C_{25}H_{31}O_{10}N_3S$ X 2 $SiMe_3$): $M^+ = m/z$ 709.2520. Found: m/z 709.2549.

RESULTS AND DISCUSSION

Preliminary tests indicated that the culture filtrate of *Streptomyces* HPL Y-22996 contained an antibiotic which showed resistance to hydrolysis by various β-lactamases (1). DEAE-Sephadex A-25 chromatography of a crude powder led to the separation of at least four components. Characteristic features of the nmr spectrum of the major component were the appearance of a three-proton singlet due to methoxy group at δ 3.53 and the β-lactam proton as a singlet at δ 5.13. In addition, the spectrum showed the α-aminoadipyl protons as a multiplet between δ 2.66–1.66. Except for the absence of the three-proton acetyl signal at δ 2.10, the above nmr data was consistent with the structure of 7-methoxy cephalosporin C (5). These findings suggested that the antibiotic might be 7-methoxy-3'-deacetylcephalosporin C. This was confirmed by high resolution mass spectrometric measurements. For this purpose, *N*-phenoxyacetyl-7-methoxy-3'-deacetyl-cephalosporin C dimethyl ester (**2**) was synthesized. But it gave a very poor mass spectrum. However, after trimethyl silylation (MSTFA, 2 hours at 60°), an excellent spectrum (fig. 1) was obtained which contained all the necessary information including the molecular ion peak with the correct elemental composition $M^+ = m/z$ 709.2549 ($C_{31}H_{47}N_3O_{10}SSi_2$, Calc. 709.2520). The most characteristic features of the mass spectrum (fig. 2) were the appearance of two key fragments at m/z 450.1819 ($C_{21}H_{30}N_2O_7Si$, Calc. 450.1822) and m/z 260.0777 ($C_{10}H_{18}NO_3SSi$, Calc. 260.0776) arising from a typical cleavage of the β-lactam ring (5,6).

These observations together with the empirical formula of the parent ion

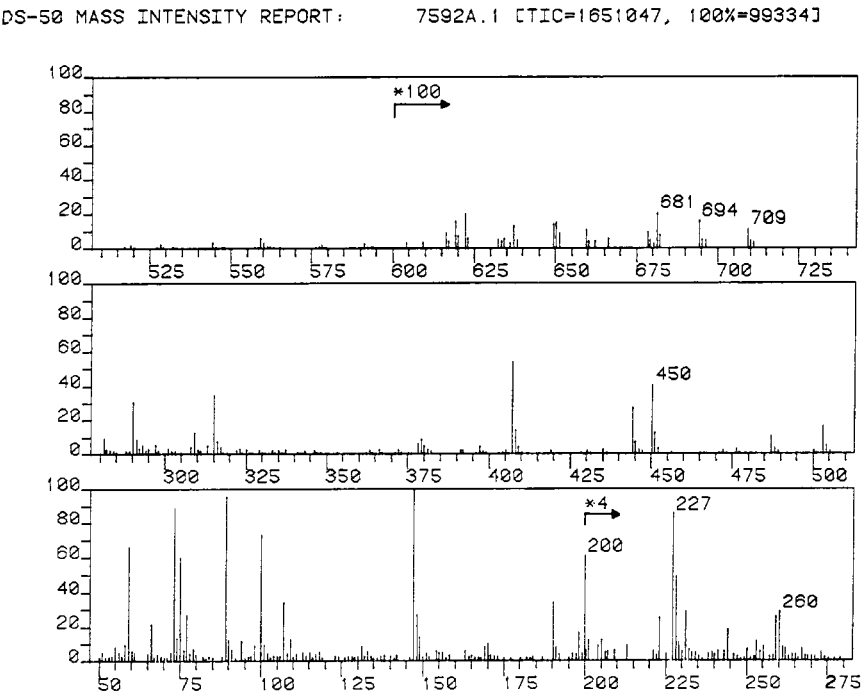


FIG. 1. Mass spectrum of *N*-phenoxyacetyl-7-methoxy-3'-deacetylcephalosporin C dimethyl ester

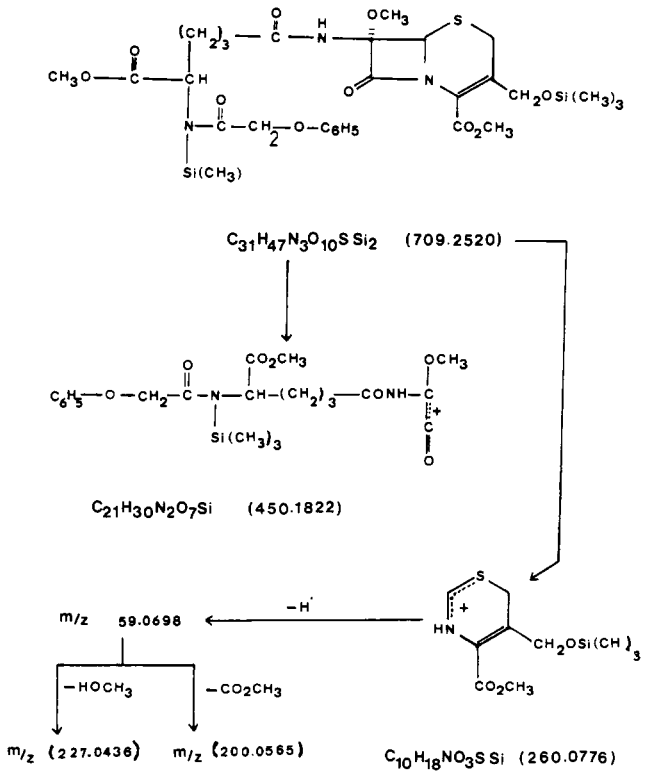


FIG 2. Mass spectral fragmentation scheme of *N*-phenoxyacetyl-7-methoxy-3'-deacetylcephalosporin C dimethyl ester.

clearly point to the correctness of structure (**1**). Finally, the antibiotic was found to be identical in all respects (ir, nmr, chromatographic mobility) with an authentic specimen of 7-methoxy DCPC kindly supplied by Dr. Harada.

It should be mentioned here that the title compound has also been obtained by enzymatic hydrolysis of cephamycin A or B (7) and also by a 5-step chemical transformation of Cephamycin C (8).

Further work on the isolation and structure elucidation of three other components present in the culture filtrate is under progress and will be reported subsequently.

ACKNOWLEDGMENTS

We sincerely thank Dr. K. Harada, Director, Scientific Information Section, Meiji Seika Kaisha Ltd., Japan, for a sample of 7-methoxy DCPC. We also thank Mr. R. Bhatt and R. R. Vidwans for their technical assistance, and Mr. M. Girg for the mass spectroscopic measurements.

Received 30 June 1981

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