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The Structure of Anticapsin, a New Biologically Active Metabolite of *Streptomyces griseoplanus*

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1. Physical and analytical data obtained on crystalline anticapsin indicated the empirical formula $C_9H_{13}NO_4$. Spectral data (u.v., i.r. and proton magnetic resonance) and formation of L-tyrosine on hydrolysis revealed the functionalities and carbon skeleton of the new epoxy keto amino acid. 2. The optical properties of anticapsin (optical rotatory dispersion and circular dichroism) permitted assignment of absolute configuration to the new metabolite. 3. Treatment of anticapsin with hot methanolic hydrochloric acid followed by acetylation gave $C_{18}H_{19}NO_5$, the α-alkoxycyclohexenone derivative. Analysis of the nuclear-magnetic-resonance and mass spectra of the latter allowed its structure to be determined and confirmed the assigned structure of anticapsin.

Details of the isolation and characterization of anticapsin (Shah, Gorman, Lively, Neuss & Whitney, 1970) and its unique biological properties (Whitney & Funderburk, 1970) have been reported from these laboratories. This metabolite is produced in stirred culture (Boeck & Christy, 1970) by a strain of *Streptomyces griseoplanus*.

Anticapsin at 3μg/ml inhibits the synthesis of the hyaluronic acid capsule of *Streptococcus pyogenes*. With the exception of activity against *Salmonella gallinarum* (minimum inhibitory concentration of 6.25μg/ml), anticapsin had no significant antimicrobial activity at low concentrations. The compound was isolated from the filtered broth by a series of column chromatograms with a variety of adsorbents (Shah *et al.* 1970). Final purification was achieved by adsorption of the partially purified material on cellulose (Schleicher and Schuell no. 286 pulp) and elution with propan-1-ol–water (7:3, v/v). The pure metabolite crystallized on evaporation *in vacuo* of appropriate eluates but could not be recrystallized. Anticapsin gave a well-defined X-ray powder pattern, but did not have a definite melting point (decomp. at 150°C). Elemental analysis showed it to be a $C_9H_{13}NO_4$ compound with pK'_a values of 4.3 and 10.1 (66% dimethylformamide), indicating an α-amino acid with mol. wt. 210 (calc. for $C_9H_{13}NO_4$: 199).

Anticapsin does not give a satisfactory mass spectrum. The empirical formula was substantiated by a high-resolution mass spectrum of *N*-acetylanticapsin methyl ester. This derivative was prepared by esterification of anticapsin with diazomethane, acetylation with acetic anhydride in pyridine and purification by chromatography. The amorphous, rather unstable, compound gave a satisfactory molecular ion at M^+ 255 (Found: 255.1075. Calc. for $C_{12}H_{17}NO_5$: 255.1107). Other fragments indicate loss of $CO \cdot O \cdot CH_3$, $CH_2{:}C{:}O$ and the $CH_2 \cdot CH(NH \cdot CO \cdot CH_3) \cdot CO \cdot O \cdot CH_3$ side chain.

Amino acid analysis revealed the presence of a single characteristic peak; depending on the purity of the metabolite various amounts of other minor unidentified peaks were present. Pure anticapsin appeared as a round purple spot with R_F 0.62 on a cellulose thin-layer plate (Brinkmann Cellulose F) with propan-1-ol–water (7:3, v/v) as solvent system and fresh ninhydrin solution as spraying reagent.

The α-amino acid function accounted for the nitrogen and two of the oxygen atoms of anticapsin; the function of the remaining two oxygen atoms was revealed by its spectral properties. In the i.r. spectrum (mineral oil mull) there is an unconjugated carbonyl band at 1710 cm^{-1}, and in the u.v. spectrum an absorption with $\lambda_{max.}$ 310 nm, $\epsilon = 32.4$ (water).

The n.m.r. spectrum of anticapsin in D_2O shows a complex multiplet centred at δ 2.1 p.p.m. together with two doublets H_A at δ 3.74 p.p.m. and H_B at δ 3.40 p.p.m. with J_{AB} 4.0 Hz (tetramethylsilane as external standard). In addition, the lower-field doublet exhibits a further splitting of less than 1Hz. Decoupling shows this to be due to methine protons at δ 2.23 p.p.m. The chemical shifts of these protons correspond to values characteristic for hydrogen atoms of an αβ-epoxy ketone. The abnormal paramagnetic shift of the H_A may be

attributed to long-range deshielding of the carbonyl group (Bhacca & Williams, 1964). The corresponding protons in 2,3-epoxycyclohexanone (prepared as described by Yang & Finnegan, 1958) are found at δ 3.60 and 3.20 p.p.m. ($CDCl_3$) (tetramethylsilane as internal standard) with J_{AB} 4 Hz. The triplet at δ 3.85 p.p.m. in the spectrum of anticapsin is characteristic of the α-hydrogen of an amino acid, and is coupled to a saturated methylene group at δ 2.1 p.p.m.

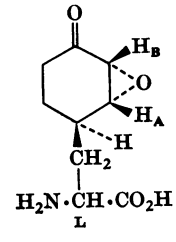
These data allowed us to write the partial structure of anticapsin as (I).

The dihedral angle between H_A and the adjacent single hydrogen must be close to 90° to explain the small coupling constant observed in the n.m.r. spectrum (Bhacca & Williams, 1964).

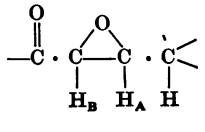
The presence of an epoxide ring adjacent to a carbonyl group is further substantiated by the optical properties of anticapsin. The metabolite is optically active, $[\alpha]_D^{25}+125°$ (*c* 1 in water). The circular-dichroism spectrum showed two positive Cotton effects at 202 nm ($\Delta\epsilon+4.7$) and at 310 nm ($\Delta\epsilon+1.8$). The optical-rotatory-dispersion spectrum displayed the following extrema: $\Phi_{280}-1130$ and $\Phi_{320}+2016$. These data are indicative of an $\alpha\beta$-epoxy ketone with the oxirane ring in the α-configuration. This conclusion was reached on the basis of the correlations established by Djerassi, Klyne, Norin, Ohloff & Klein (1965).

Acid hydrolysis of anticapsin gave, in addition to ammonia, a mixture of L-tyrosine and *m*-tyrosine in the ratio 10:1, as identified by amino acid analysis. L-Tyrosine was obtained by chromatography of the hydrolysis mixture and identified by amino acid analysis, t.l.c. and paper chromatography, n.m.r. spectrum and optical rotatory dispersion. This evidence, together with the previous data, prompted us to suggest structure (II) for anticapsin. The proposal of this structure made us aware of the report on the chemistry of bacilysin from *Bacillus subtilis* by Rogers, Lomakina & Abraham (1965), who suggested provisional structures (III) or (IV) for this metabolite. This prompted us to contact Professor E. P. Abraham, and we agreed to publish our respective findings simultaneously. The direct comparison of anticapsin with the epoxy keto amino acid from bacilysin confirmed that the two substances were in fact identical (Walker & Abraham, 1970). We gratefully acknowledge the sample of bacilysin from the Oxford laboratory as well as the advance information from Professor Abraham pertaining to his unpublished results.

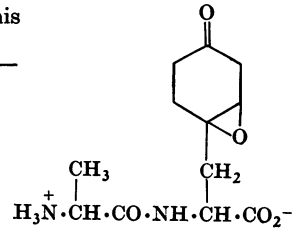
Treatment of anticapsin with hot methanolic hydrochloric acid followed by acetylation with acetic anhydride in pyridine gave a mixture of several compounds. Of these, one (V) (see Scheme 1) was



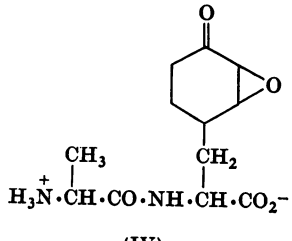
(II)



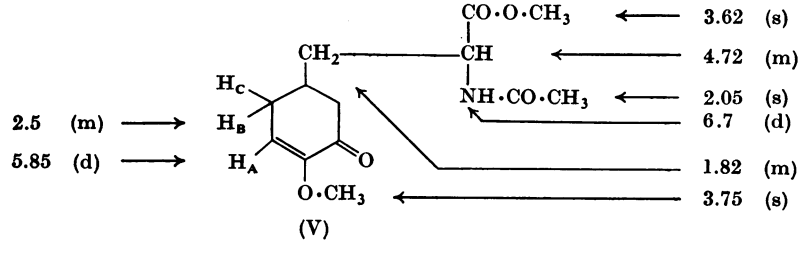
(I)



(III)



(IV)



(V)

Scheme 1. Assignment of chemical shifts of compound (V). Chemical shifts are expressed in δ (p.p.m.)

isolated pure by silica-gel chromatography with ethyl acetate–ethanol (90:1, v/v) in 12% yield. It is a $C_{13}H_{19}NO_5$ compound (Found: *m/e* 269.12632. Calc. *m/e* 269.12915), corresponding to *N*-acetylanticapsin methyl ester + CH_2. The u.v. spectrum ($\lambda_{max.}$ 262 nm, ϵ 4170) is characteristic of an α-alkoxy enone [e.g. structure (VI) has $\lambda_{max.}$ 264 nm, ϵ 5000 (Lavie & Wilner, 1960)].

The i.r. spectrum of compound (V) in chloroform solution has the expected absorption: 3420 cm^{-1} (–NH), 1740 cm^{-1} (ester C=O), 1685 cm^{-1} (–C=C–C=O) and 1630 cm^{-1} (–C=C–).

The proton-magnetic-resonance spectrum shows a number of well-defined peaks, of which the chemical shifts are given in Scheme 1.

Decoupling experiments corroborated these assignments (Fig. 1). The two doublets centred at δ 6.85 p.p.m. (J_{AC}/2 Hz) are particularly noteworthy. These collapsed to a singlet on irradiation of the multiplet at δ 2.5 p.p.m.

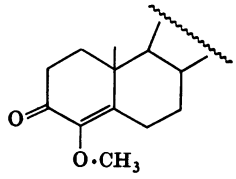
Additional evidence for the structure of compound (V) is furnished by its high-resolution mass spectrum shown in Scheme 2.

In addition to compound (V), a minor product (VII) was isolated in low yield only in admixture with compound (V). This mixture had u.v. and i.r. spectra identical with those of pure compound (V): the n.m.r. spectrum of the mixture displayed a new doublet at δ 5.69 p.p.m. (*J* 3 Hz) coupled with decreased intensity of the 5.85 p.p.m. double doublet. On the basis of this fact we have formulated compound (VII) as the isomeric α-methoxy ketone.

Thus compounds (V) and (VII) represent the two possible enol ethers of the 1,2-dione (VIII), the expected product of an acid-catalysed rearrangement of the epoxy ketone function of anticapsin.

The structure of these products confirms the carbon skeleton of anticapsin and, further, completely delineates the positional relationship of the epoxy ketone function and the side chain as shown in structure (II).

Roscoe & Abraham (1966) showed that shikimic



(VI)

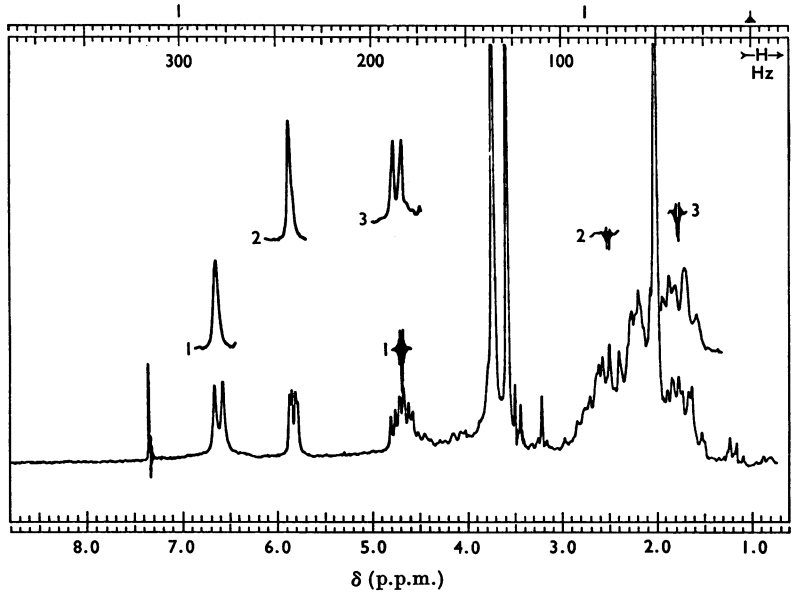
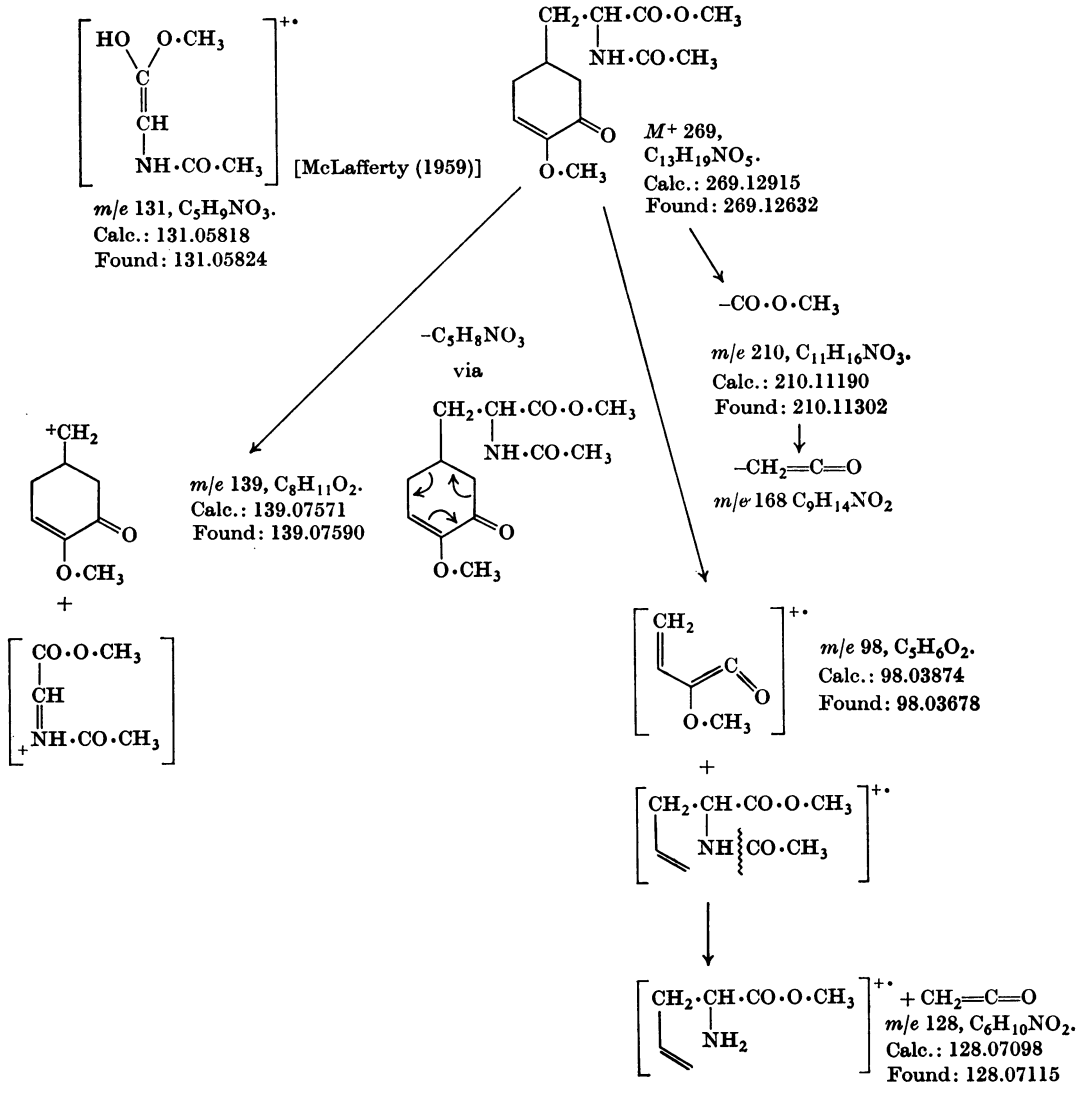


Fig. 1. 100 MHz proton-magnetic-resonance spectrum and decoupling experiments on compound (V) in $CDCl_3$ with tetramethylsilane as an internal standard. Irradiation of the multiplet at δ 4.72 p.p.m. resulted in the collapse of the doublet of the amide proton at δ 6.7 p.p.m. to a singlet, and partial collapse of the multiplet of the methylene of the side chain at δ 1.82 p.p.m. Subsequent irradiation in this region resulted in the collapse of the multiplet at δ 4.72 p.p.m. to a doublet. Irradiation of the methylene proton centred around δ 2.5 p.p.m. caused the two doublets (H_A) centred at δ 5.85 p.p.m. to collapse to a broad singlet.



Scheme 2. Elemental composition and tentative structures of some abundant ions in the high-resolution mass spectrum of compound (V).

acid, but not tyrosine, is a precursor of bacilysin; presumably anticapsin is produced similarly.

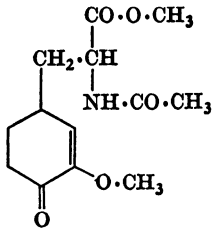
EXPERIMENTAL

Proton-magnetic-resonance spectra. Samples whose spectra were to be determined in D_2O were dissolved in D_2O, and solutions were freeze-dried. The samples were then redissolved in D_2O (10%, w/v), and their proton-magnetic-resonance spectra were determined. Chemical shifts were measured at 100MHz relative to tetramethylsilane as external reference in D_2O solution. The spectra in $CDCl_3$ were measured with tetramethylsilane as an internal standard. The spectra were recorded on a Varian HA 100 MHz instrument.

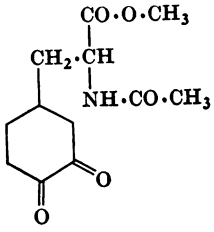
High-resolution mass spectra. The spectra were recorded with a CEC high-resolution model 21-110 instrument.

Optical measurements. Optical-rotatory-dispersion and circular-dichroism spectra were recorded on a Cary model 60 recording spectropolarimeter.

Preparation of crystalline anticapsin. A solution of



(VII)



(VIII)

850 mg of impure anticapsin was chromatographed on a column of 80 g of cellulose (Schleicher and Schuell no. 286 pulp). The column (120 cm × 5 cm) was eluted with propan-1-ol–water (7:3, v/v). Fractions (15 ml) were collected, evaporated *in vacuo* at 30°C and examined by t.l.c. on cellulose plates with the same solvent system. The first 21 fractions were discarded. Fraction 22 gave 152.0 mg of crystalline, chromatographically pure anticapsin. Fraction 23 gave 200 mg of approx. 85%-pure anticapsin and fraction 24 gave 131.0 mg of approx. 50%-pure anticapsin. Later fractions were complex mixtures. The material from fraction 22 was used for recording physical and analytical data.

Analysis. Anticapsin was dried at 80°C *in vacuo* and analysed (Found: C, 53.9; H, 6.7; N, 6.9; O, 32.0. Calc. for $C_9H_{13}NO_4$: C, 54.3; H, 6.6; N, 7.0; O, 32.1).

Hydrolysis of anticapsin: isolation of L-*tyrosine.* A solution of 50 mg of anticapsin in 20 ml of 6M-HCl was refluxed for 18 h and evaporated *in vacuo* to yield 53 mg of residue. This was chromatographed on a column (90 cm × 1.9 cm) of 50 g of Bio-Rad resin (AG 1-X4, 200–400 mesh, OH^- form) with water as solvent. The first twelve 20 ml fractions were discarded, and the solvent was changed to 0.1M-formic acid. Fraction 14 contained 18.6 mg of L-tyrosine as identified by amino acid analysis, paper chromatography, t.l.c. [cellulose; butan-1-ol–acetic acid–water (3:1:1, by vol.)] and optical rotatory dispersion.

Acid rearrangement of anticapsin and acetylation: isolation of compound (V). Into a stirred suspension of 750 mg of anticapsin in 100 ml of methanol was bubbled anhydrous HCl for about 5 min until refluxing occurred (approx. 64°C). After the solution had been allowed to cool, it was evaporated *in vacuo*. The residue was dissolved in 15 ml of anhydrous acetic anhydride, 15 ml of anhydrous pyridine was added and the mixture was allowed to stand at room temperature overnight. The residue (1.9 g) obtained after evaporation *in vacuo* was chromatographed on a column (95 cm × 3.1 cm) of 200 g of silica (Merck) (less than 0.08 mm in size) with the following solvents of increasing polarity: ethyl acetate (1262 ml), ethyl acetate–ethanol, (9:1, v/v) (900 ml) and ethyl acetate–ethanol (3:1, v/v) (1500 ml). Fractions (18 ml) were collected. Fraction 58 [ethyl acetate–ethanol (9:1, v/v)] gave 31 mg of a mixture of compounds (V) and (VII), and fraction 62 gave 90 mg of pure compound (V) [as determined by t.l.c. on silica with ethyl acetate–ethanol (3:1, v/v) as solvent and development with iodine].

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