

A NEW TREMORGENIC METABOLITE RELATED TO VERRUCULOGEN FROM PENICILLIUM VERRUCULOSUM

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Abstract - The isolation and structural elucidation of a new tremorgenic metabolite, acetoxyl verruculogen from Penicillium verruculosum is described.

Results on 1,2-¹³C-acetate biosynthetic feeding data are also presented.

We have isolated a new tremorgenic metabolite (I) from Penicillium verruculosum together with the known mycotoxin, verruculogen (II).² Both compounds cause severe tremorgenic reactions in mice on either oral or intraperitoneal administration.³ The structural elucidation of this new compound by X-ray crystallographic analysis as well as preliminary results on the biosynthetic studies of the mycotoxins are the subject of this paper.

Both toxins were produced in infected medium consisting of shredded wheat and Difco mycological broth supplemented with yeast extract in stationary cultivation at 28°C for 17-20 days. The metabolites were extracted with chloroform and purified by silica gel column chromatography (chloroform-acetone=93:7). The new metabolite was separated from verruculogen by preparative tlc (ethyl acetate-benzene=1:1) and recrystallized from ethyl acetate-hexane; mp 221-224°C (dec.), $[\alpha]_D^{25}$ -27.2 (CHCl₃) and M⁺, m/e 569.2371 (m/e calculated for C₂₉H₃₅N₃O₉, 569.2373). The UV spectrum suggests a 6-O-methylindole system; $\lambda_{\text{max}}^{\text{EtOH}}$ 226, 227, 295 nm (ϵ 14,500, 10,500, 9,300). The ir and proton nmr spectra show close similarities to those of verruculogen (II), whose structure has been determined by X-ray analysis. The elemental composition suggested the new compound (I) was an acetoxyl derivative of (II) (C₂₇H₃₃N₃O₇). The combined ir, ms and nmr data also supported this conclusion: $\nu_{\text{max}}^{\text{nujol}}$ 1735 and 1235 cm⁻¹ (ester), m/e 527 (M⁺-COCH₃), and δ (CDCl₃) 2.10 ppm (3H, s). The C-13 nmr spectrum of (I) exhibited 29 carbon signals. Comparison of the C-13 nmr spectra of (I) and (II), indicated that all of the carbon signals except those originating from the pyrrolidine ring were at the same chemical shift positions. These shifts are tabulated in Table 1.^{4,5} The additional signals in the spectrum of (I) at 20.9 and 170.0 ppm were assigned to the methyl and carbonyl carbons of an acetoxyl group respectively.

The spectral evidence thus indicates that the acetoxyl function is located on the pyrrolidine ring of verruculogen. This conclusion was verified by acid treatment of I that afforded 4-hydroxyproline as a hydrolytic product identical in tlc mobility with an authentic sample.⁶

The stereochemistry of I was conclusively established by X-ray crystallographic analysis.

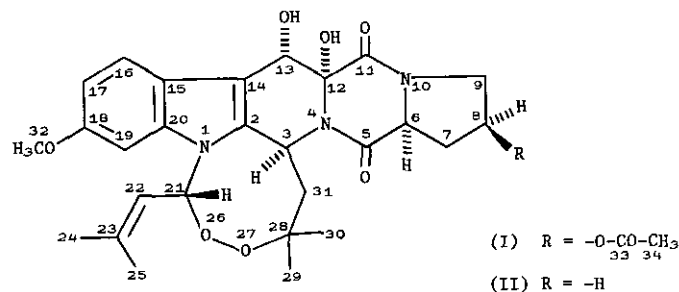
Crystals of (acetoxo-verruculogen) are orthorhombic with $a = 10.026(2)$, $b = 10.320(2)$, and $c = 27.823(5)$ Å. Systematic extinctions conformed to the common chiral space group $P2_12_12_1$ and a calculated and measured density of 1.31 g/cc indicated one molecule of composition $C_{29}H_{35}O_9N_3$ formed the asymmetric unit. All unique reflections with $2\theta \leq 114^\circ$ were surveyed with graphite monochromated $CuK\alpha$ (1.54178 Å) radiation. Of the 2214 reflections investigated, 1955 (88%) were judged observed ($F_o^2 \geq 3\sigma(F_o^2)$) after correction for Lorentz, polarization and background effects.

The angular dependence of the reflections was removed as they were converted to normalized structure factors. Phasing of the 300 largest normalized structure factors was accomplished using a multi-solution, weighted tangent formula approach with three origin defining and seven special reflections in the starting set.⁷ The positions of 33 nonhydrogen atoms were easily determined and confirmed by a least-squares refinement.⁸ The remaining eight nonhydrogen atoms were located on a subsequent Fourier synthesis. Full-matrix least-squares refinements with anisotropic temperature factors for all nonhydrogen atoms and isotropic temperature factors for 29 hydrogen atoms have converged to a standard crystallographic residual of 0.041 for the observed reflections. Six methyl hydrogens on C(36) and C(41) could not be located. See reference 9 for further crystallographic details.

A computer generated perspective drawing of the final X-ray model is presented in the Figure. All bond distances and angles⁹ agree well with generally accepted bond types. The molecular conformation is very similar to that of verruculogen which has been previously discussed.¹⁰ An intramolecular hydrogen bond is formed between O(37) and O(39) with a distance of 2.804 Å. We interpret the intermolecular separation between O(37) and O(38) of 2.698 Å to also be a hydrogen bond. All other intermolecular approaches correspond to van der Waals' interactions.

For biosynthetic studies ^{13}C -labeled toxins, (I) and (II), were prepared by feeding of [1,2- ^{13}C]-sodium acetate (90% enriched) as a precursor to the cultured medium of the mold.¹¹ Labeled metabolites were isolated and purified by conventional methods. In the ^{13}C -NMR spectrum of labeled (I), prominent ^{13}C - ^{13}C couplings between C-21-C-22, C-23-C-24, C-28-C-29 and C-31-C-3 are observed (Table) and enhancement of the signals at C-25 and C-30 was evident. These results establish that the two 5-C units attached to N-1 and N-4 are mevalonate derived as expected.

Other ^{13}C - ^{13}C couplings were observed between C-5-C-6 ($J_{C-C} = 53$ Hz) and C-8-C-9 ($J_{C-C} = 34$ Hz). Comparison of the relative intensities indicated however these satellite bands were less enriched. From these results we conclude that ^{13}C -labeled acetate after initial conversion into acetyl-S-CoA is introduced into proline through the well known pathway through glutamate and the TCA-cycle that results in more extensive dilution of the labeled acetate. Furthermore the absence of C-C coupling at C-7 clearly establishes the de novo synthesis of proline by this micro-organism. The exact

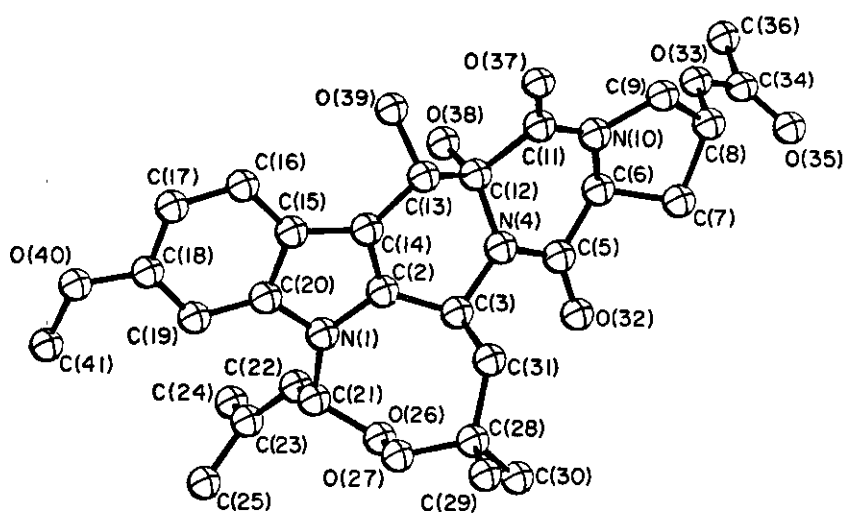


TABLE

Carbon No.	(I)			(II)		Δ (ppm)
	δ_c (ppm)	mult	J (Hz) c-c	δ_c (ppm)	J (Hz) c-c	
2	131.5	s		131.4		
3	48.4	d	36.0	48.9	35.8	
5	170.7	s	53.6	170.3	55.0	-0.4
6	58.6	d	53.7	57.2	55.1	-1.4
7	28.9	t		35.3		+6.4
8	22.5	t	33.9	70.7	38.0	+48.2
9	45.2	t	34.0	51.8	---a	+6.6
11	166.2	s		166.2		
12	82.6	s		82.7		
13	68.5	d		68.5		
14	105.8	s		105.7		
15	121.1	s		121.1		
16	121.7	d		121.7		
17	109.2	d		109.3		
18	156.2	s		156.4		
19	93.9	d		93.4		
20	136.2	s		136.2		
21	85.8	d	52.2	85.8	51.8	
22	118.6	d	52.2	118.6	52.0	
23	142.9	s	41.2	143.0	41.2	
24	18.7	q	41.1	18.7	41.5	
25	24.1	q		24.2		
28	82.0	s	38.9	82.0	38.1	
29	27.1	q	38.2	27.1	38.0	
30	25.5	q		25.5		
31	51.2	t	35.3	51.2	---a	
32	55.6	q		55.6		
33	---			170.0	59.6	
34	---			20.9	59.3	

^aBoth spectra were taken in $CDCl_3$ with an internal TMS standard. The chemical shift assignments of (I) were accomplished by comparison with the chemical shift values of known compounds off resonance decoupling and specific single frequency proton decoupling.^{4,5}

^aThe J-values could not be measured due to the overlapping of signals.



timing of the biochemical events is uncertain as to whether 4-hydroxyproline is biosynthesized prior to the formation of II or whether direct hydroxylation of II to I occurs.

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requesting Supplement to Publication for this article and submitting \$0.50 in the form of check, cash, or money order. Give your name and complete address (including zip code for mailing).

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11. Labeled precursor (90 atom % of ^{13}C) was diluted with one and a half fold of unlabeled sodium acetate and added to the cultured medium on 4th and 5th day after inoculation. Fermentation was continued for further 2 weeks.

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