Biosynthesis of Verruculogen, a Tremorgenic Metabolite of *Penicillium verruculosum*: Stereochemical Course of Peroxide Ring Formation

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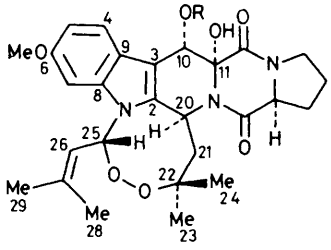
The stereochemical course of the formation of the eight-membered peroxide ring in verruculogen, a tremorgenic metabolite of *Penicillium verruculosum*, was established by incorporation of ^{13}C-, ^{2}H-, and ^{18}O-labelled precursors.

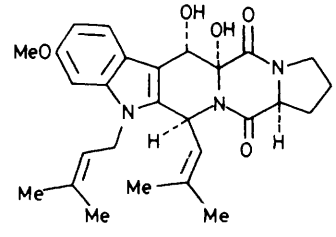
Verruculogen (**1**), a metabolite of *Penicillium verruculosum*,[1] is one of a number of mycotoxins capable of eliciting a sustained tremoring response in vertebrate animals.[2] Its possible role, together with that of other tremorgens, in animal neurological disorders has been investigated.[3] The structure and relative stereochemistry of (**1**) were determined by *X*-ray crystallography[4] and the absolute configuration followed when (2*S*)-proline was obtained from the acidic hydrolysis of a related compound, fumitremorgin A (**2**).[5] Incorporation studies with ^{14}C-labelled precursors established that verruculogen is formed from (2*S*)-tryptophan, (2*S*)-proline, mevalonate, and (2*S*)-methionine.[6] The co-existence of verruculogen (**1**) and fumitremorgin B (**3**)[7] in cultures of *Aspergillus caespitosus*[8] points to a possible biogenetic relationship between the two metabolites, and prompted us to investigate the biosynthesis of verruculogen and especially the stereochemical course of the peroxide ring formation.

The relevant ^{1}H and ^{13}C n.m.r. data for verruculogen (**1**) are collated in Table 1. The complete assignments will be described in a subsequent publication. The signals of the proton-bearing carbon atoms were correlated with specific proton resonances in a two-dimensional (2D) (^{13}C,^{1}H) chemical shift correlation experiment.[9] The method, however, does not allow the correlation of the resonances at δ_C 24.16 (C-23) and 25.88 (C-29), as a result of the small difference in the corresponding proton chemical shift values [δ_H 1.703 (s) and 1.714 (d), respectively]. This ambiguity was resolved when irradiation at δ_H 0.991 in a ^{13}C {^{1}H} selective population inversion (S.P.I.)[10] experiment affected the resonance at δ_C 24.16 (C-23). The assignment of the signals at δ_H 1.703 and 0.991 (and thus δ_C 24.16 and 27.07) to the prochiral



(**1**) R = H

(**2**) R = $CH_2CH{=}CHMe_2$

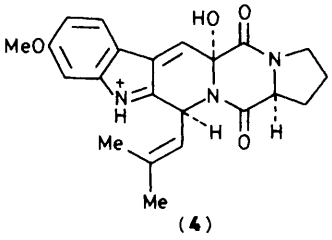


(**3**)

Table 1. N.m.r. data (^1H and ^{13}C) for verruculogen (**1**).[a]

Carbon atom	δ_C	$^1J_{CC}$/Hz[b]	$\Delta\delta$[c]	δ_H	J_{HH}/Hz
6	156.36 (s)		0.018		
10	68.63 (d)			5.629 (dd)	2.6, 1.1
11	82.55 (s)		0.016		
20	48.85 (d)	35.5		6.033 (dd)	10.1, 1.1, 1.1
21	51.18 (t)	35.3		1.990 (dd)	13.4, 1.1
				1.656 (dd)	13.4, 10.1
22	82.06 (s)	38.8	0.042	—	
23	24.16 (q)			1.703 (s)	
24	27.07 (q)	38.5		0.991 (s)	
25	85.81 (d)	51.8	0.026	6.623 (d)	8.0
26	118.52 (d)	51.4		5.027 (dqq)	8.1, 1.3, 1.4
28	18.75 (q)	42.2		1.979 (d)	1.3
29	25.58 (q)			1.714 (d)	1.4
30	55.73 (q)		0.026	3.818 (s)	

[a] Recorded with a Bruker WM-500 spectrometer; solvent $CDCl_3$. [b] Values obtained from the proton-decoupled ^{13}C spectrum of verruculogen derived from [1,2-$^{13}C_2$]acetate. [c] ^{18}O-Induced upfield isotope shift in p.p.m.

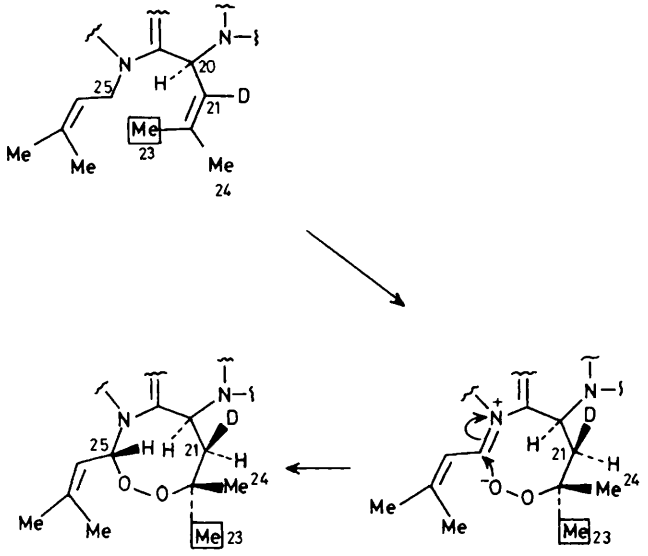


(**4**)

diastereotopic methyl groups, C-23 and C-24, respectively, is based on the nuclear Overhauser effect (n.O.e.) observed for H-20 on irradiation at δ_H 1.703 in a proton–proton n.O.e. experiment. The n.O.e. observed for H-26 in the same experiment assigns the signal at δ_H 1.714 to the pro-*E* methyl group (C-29).

Fermentation of cultures of *P. verruculosum*, IFO 30858 on Czapek-yeast extract medium (500 ml) in an atmosphere containing $^{18}O_2$ (50 atom % ^{18}O) over days 4—14, produced labelled verruculogen (**1**) (20 mg). The ^{18}O-induced isotope shifts observed in the proton-decoupled ^{13}C n.m.r. spectrum[11] (see Table 1) demonstrated that the oxygen atoms at C-6, C-11, C-22, and C-25 are derived from oxidative processes. The origin of the oxygen atom at C-10 (from water) and of that at C-11 (from oxygen gas) can be rationalised in terms of involvement of the indole nitrogen atom in the opening of a (10*S*,11*R*)-oxirane precursor. Subsequent *Re*-face attack of water at C-10 in the intermediate (**4**) generates the 10*S*,11*R*-chirality present in verruculogen.

The arrangement of intact acetate units in verruculogen derived from [1,2-$^{13}C_2$]acetate was deduced from the measured one-bond (^{13}C,^{13}C) coupling constants (Table 1) and is similar to that previously reported.[12] The results show that both the 27-pro-*Z* methyl group (C-28) and the 22-*Re* methyl group (C-24) form part of an intact acetate unit. The 27-pro-*E* and 22-*Si*) methyl groups are therefore derived from C-2 of mevalonate. However, although both the C-23 and C-29 signals are enhanced (enrichment factors of 9.6 and 8.8, respectively) in the proton-decoupled ^{13}C n.m.r. spectrum of verruculogen derived from [2-^{13}C]mevalonolactone, an enhancement is also observed for the C-24 signal (enrichment factor 3.3). A similar phenomenon was observed for fumitremorgin B (**3**) obtained from the same feeding experiment. As expected (3*RS*)-[2-^{13}C]mevalonolactone labels the 27-pro-*E* methyl group, C-29 (δ_C 25.36, enrichment factor 6.8) of fumitremorgin B (**3**), but it is the 22-pro-*Z* methyl group, C-23



Scheme 1. Stereochemical course of the eight-membered peroxide ring formation in verruculogen; the boxed Me is that derived from C-2 of mevalonolactone.

(δ_C 18.21) of the 2,2-dimethylvinyl moiety which is enriched (enrichment factor 6.5). In addition an enhancement is also observed for the signal of the 22-pro-*E* methyl group, C-24 (δ_C 25.55, enrichment factor 2.0) of (**3**). It is evident that the stereochemical integrity of the C-22 diastereotopic methyl groups is lost in the formation of fumitremorgin B (**3**). The mechanism of this process is under investigation.

The fate of the hydrogen atoms in the biosynthesis of verruculogen was studied by incorporation of [1-^{13}C,-2-$^2H_3$]acetate into the metabolite. The retention of ^2H two bonds removed from a ^{13}C atom can be detected by the characteristic β-isotope shifts in the resonance position of the ^{13}C nucleus.[13,14] The ^2H atoms retained at both C-21 and C-26, as indicated by the β-isotope shifts of −0.052 and −0.048 p.p.m. for the C-20 and C-25 resonances, respectively, are derived in each case from the 4-*Re* position of mevalonate. We now have to determine which of the two diastereotopic C-21 methylene hydrogen atoms is isotopically substituted by deuterium. The known absolute configuration of verruculogen, in conjunction with the observed (^1H,^1H) coupling

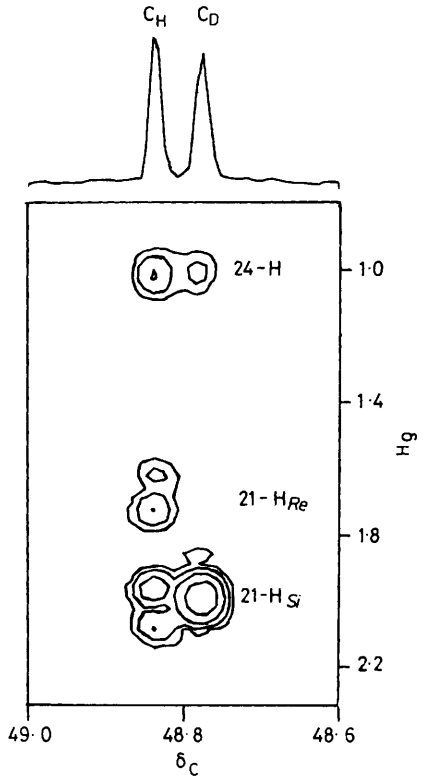


Figure 1. Part of the 2D long-range (^1H,^{13}C) chemical shift correlation spectrum of verruculogen derived from [1-^{13}C,2-$^2H_3$]acetate showing the two- and three-bond connectivity pattern for the C-20 resonance.

constants, allows us to assign the resonance at δ_H 1.990 (*J* 13.4 and 1.1 Hz) to the 21-*Si* proton, as the vicinal coupling constant of 1.1 Hz corresponds to the observed torsion angle of 80—90 °C. The resonance at δ_H 1.656 (*J* 13.4 and 10.1 Hz) is therefore assigned to the 21-*Re* proton. The stereospecificity of ^2H labelling at C-21 could not be determined by ^2H n.m.r. spectroscopy as the relevant resonances are obscured by the broad C-23 and C-28 resonances. However, the deuterium-induced β-isotope shift of the ^{13}C nucleus can be utilized in a long-range (^1H,^{13}C) chemical shift experiment to determine the stereochemistry of deuterium labelling at a methylene group.[15] The two- and three-bond connectivity pattern for *inter alia* the C-20 resonance (Figure 1) was established by 2D long-range (^1H,^{13}C) chemical shift correlation spectroscopy.[9]† The spectrum shows signals due to the two-bond correlation of C-20 of non-deuteriated verruculogen with both the 21-*Re* (δ_H 1.656) and 21-*Si* (δ_H 1.990) protons. The presence of a deuterium atom at C-21 causes an upfield shift of the C-20 resonance and this signal correlates only with that of the 21-*Si* proton proving that the deuterium atom is located in the 21-*Re* position.

Analysis of the foregoing results affords considerable insight into the stereochemistry of the biosynthetic processes by which verruculogen is elaborated. The eight-membered peroxide ring in verruculogen (**1**) presumably arises from dioxygenation of the co-metabolite, fumitremorgin B (**3**) by a dioxygenase enzyme as shown in Scheme 1.[16] This conversion could proceed by enzyme-induced substrate activation to form a radical cation at the 21,22 double bond.[16] Addition of a hydride ion (derived from NADPH) at C-21 generates the C-22 radical, which reacts with a dioxygen molecule to give the hydroperoxide. Our results show that the overall addition of the hydride ion and the dioxygen molecule must occur at opposite faces of the double bond *i.e.* the 21*Si*,22*Re*- and the 21*Re*,22*Si*-face, respectively, in order to generate the correct prochirality at C-21 and C-22 in verruculogen. The ring closure step could proceed by either an ionic or a radical mechanism.

The possibility of an intramolecular transfer of a hydrogen atom from C-25 to C-21 at the 21*Si*,22-*Re*-face of the double bond in the formation of the peroxide ring was ruled out by incorporation of (3*RS*)-[5-$^2H_2$]mevalonolactone into verruculogen. The β-isotope shifts observed in the proton-decoupled ^{13}C n.m.r. spectrum as well as the signals in the ^2H n.m.r. spectrum showed the presence of ^2H only at C-20 and C-25. It is not yet known which of the two diastereotopic C-5 protons of mevalonate is retained in either case.

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† Recorded with a Bruker WM-500 spectrometer (40 mg sample in $CDCl_3$) using the pulse sequence: 90_H–$t_1/2$–180_C–$t_1/2$–Δ_1–90_H,90_C–Δ_2–decouple protons, acquire ^{13}C, wih Δ_1 = 100 ms and Δ_2 = 40 ms. The matrix used for time-domain data was 128 × 8 K and that for 2D Fourier transformation was 256 × 16 K, to give a digital resolution in F_2 of 1.7 Hz per data point. 1152 Transients were accumulated for each of 128 t_1 increments, using spectral widths of 13 888 Hz in F_2 and 4237 Hz in F_1; total experiment time 56 h.

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