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80. Biosynthesis of the Antibiotic Verrucarin E
Use of [1-^{13}C]-, [2-^{13}C]-, [1,2-^{13}C]- and [2-^{13}C,2-$^{2}H_3$]-Acetates

Verrucarins and Roridins, 37th Communication [1]

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Summary

The origin of the carbon skeleton of verrucarin E (**1**) from acetate as precursor is confirmed. Incorporation studies with [1,2-^{13}C]-acetate have demonstrated that two acetoacetate units couple together as shown in pattern **A** *(Scheme 2)* and not as in **B**. Analysis of the deuterium distribution in both verrucarin E (**1**) isolated after the incorporation of [2-^{13}C,2-$^{2}H_3$]-acetate and in sodium acetate obtained after *Kuhn-Roth* oxidation of the metabolite demonstrated that C(7) is derived from the starter unit of one of the acetoacetate moieties. The deuterium exchange in verrucarin E (**1**) occurring during fermentation was investigated.

Recent developments in ^{13}C-NMR. spectroscopy and the availability of ^{13}C-labelled precursors have made this technique more attractive and much favoured over the tedious and cumbersome ^{14}C-tracer procedure [2]. By this technique the intact acetate residues can be recognized by feeding with [1,2-^{13}C]-acetate: the broad band-decoupled ^{13}C-NMR. spectrum of the metabolite displays ^{13}C-coupled satellites in addition to the singlet resonances due to natural abundance for the adjacent C-atoms derived from the same two-carbon fragments. Acetate 'starter units' of acetate-malonate polyketide-derived compounds can be detected by feeding [2-^{13}C,2-$^{2}H_3$]-acetate. Observation of a seven line signal in the ^{13}C-NMR. spectrum of the methyl group in the derived natural product indicates retention of all three deuterium atoms [3].

Verrucarin E (**1**), an antibiotic isolated from *Myrothecium verrucaria* (strain S 833) [4] incorporates activity from isotopically labelled acetates and does not originate from proline, glutamic acid or its biogenetic equivalent, nor from 5-aminolevulinic acid [5]. The origin of C(4), C(6), C(7) and C(8) (numbering shown for **1** in *Scheme 1*) in **1** was defined through chemical degradation as outlined in *Scheme 1*.

The results were rationalized by proposing that verrucarin E (**1**) is derived from two independent acetoacetic units and that one carboxylic C-atom is lost. The

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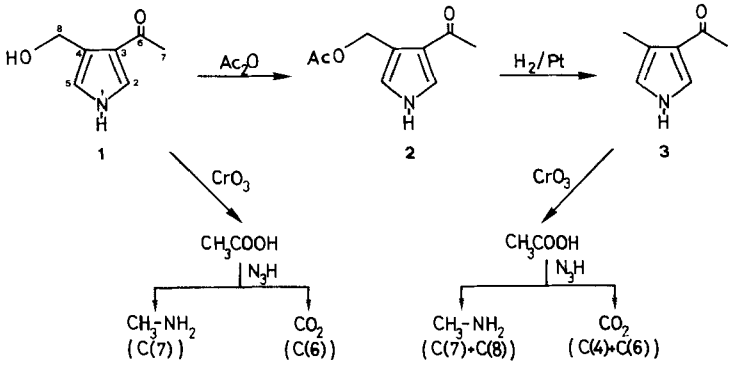
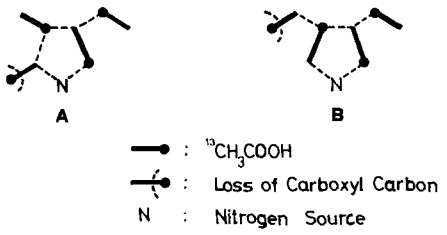
Scheme 1



Scheme 2



ambiguity as to whether two acetoacetate units couple together as shown in the arrangement **A** or pattern **B** *(Scheme 2)* could not be clarified completely from the available data at that time. Incorporation studies with [1-^{13}C]-, [2-^{13}C]- and [1,2-^{13}C]-acetates into verrucarin E (**1**), using *Myrothecium verrucaria* (strain S 833)[1]) have been carried out. An incorporation experiment with [1,2-^{13}C]-acetate was mandatory for differentiating between pattern **A** and pattern **B** (see *Scheme 2*). An additional experiment with [2-^{13}C,2-$^{2}H_3$]-acetate was carried out to determine if C(7) was indeed derived from the methyl group of an acetate starter unit.

The chemical shifts in the ^{13}C-NMR. spectrum of **1** have been assigned previously by *Gossauer & Suhl* [6]. The signals for olefinic pairs C(2) and C(5), and C(3) and C(4) were differentiated through an unambiguous synthesis of verrucarin E (**1**) labelled at C(2) and C(3). Our results from enriched samples obtained from incorporation experiments are in complete agreement with their assignments. Intensity of the signals for C(2), C(4) and C(6) in the ^{13}C-NMR. spectrum was enhanced as compared to the natural abundance in [1-^{13}C]-acetate-enriched verrucarin E (**1**) and for C(3), C(5), C(7) and C(8) in [2-^{13}C]-acetate enriched verrucarin E (**1**), respectively. This alternate labelling pattern with the acetate precursors *(Scheme 3)* confirms the conclusions of the previous study with ^{14}C-labelled precursors [4]. In the ^{13}C-NMR. spectrum of [2-^{13}C]-acetate enriched **1**, a

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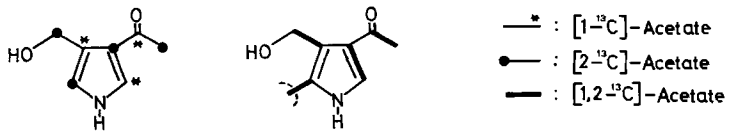
Scheme 3



1,3-coupling ($^2J_{3,7}$= 14 Hz) was also evident *(cf. Fig. 1)*. It establishes a relationship between C(3) and C(7). From this experiment we also assigned unambiguously the chemical shift of C(3). This 1,3-coupling across the carbonyl C-atom is well known [7] [8]. The ^{13}C-NMR. spectrum of verrucarin E (**1**) enriched from [1,2-^{13}C]-acetate was rather complicated in the olefinic region (C(2), C(3) and C(4)) *(cf. Fig. 2)*. The anticipated characteristic triplets are seen for C(6), C(7) and C(8). The presence of the characteristic signal for C(8) – the signal for C(4) is too complex and not clearly defined owing to overlapping – can only be explained if it is derived from an intact acetate unit. This observation clearly rules out the hypothetical mode of coupling of two acetoacetate units according to the pattern **B**. The satellites for C(5) are of low intensity and most likely arise by coupling between two enriched C-atoms (C(4) and C(5)) from different acetate units. This interacetate unit coupling can only occur when there is a high probability of two acetate units being incorporated into adjacent positions in any one molecule. This has been recognized as a problem with the organisms where the incorporation of acetate is very efficient [2]. The dilution of the labelled acetate with unlabelled acetate before feeding was proposed and used to circumvent this problem of two acetate units coming together [9]. However, the actual validity of this dilution technique has been questioned by a recent statistical treatment [10]. Ion intensities in the molecular ion region of the mass spectra of natural abundance, [1-^{13}C]- and [2-^{13}C]-acetate enriched verrucarin E (**1**) are given in *Table 1*. A substantial proportion of the biosynthetically enriched samples are multiply labelled. There are even a significant number of molecules in which all the four acetate units are derived from the added precursor, so accounting for the extensive ^{13}C, ^{13}C-coupling. Addition of a large amount of exogenous acetate likely represses the production of endogenous acetate by feedback inhibition mechanism, so that until this pool falls below a certain threshold level and endogenous acetate production recommences most of the metabolite is produced from exogenous precursor.

The ^{13}C-NMR. spectrum of verrucarin E (**1**) derived from [2-^{13}C,2-^{2}H$_3$]-acetate also displayed signals indicating extensive incorporation of more than one acetate unit per molecule. The signals for C(7) consisted of several triplets. The centre peak corresponds to the expected singlet for C(7) derived from [2-^{13}C]-acetate in

Table 1. *Ion intensities in the molecular ion region of verrucarin E* (**1**)

Verrucarin E (**1**)	M^+	M^++1	M^++2	M^++3	M^++4
Natural abundance	100	10.25	1.19	–	–
[1-^{13}C]-Acetate enriched	100	11.1	2.92	0.86	–
[2-^{13}C]-Acetate enriched	100	12.35	4.78	2.65	0.7

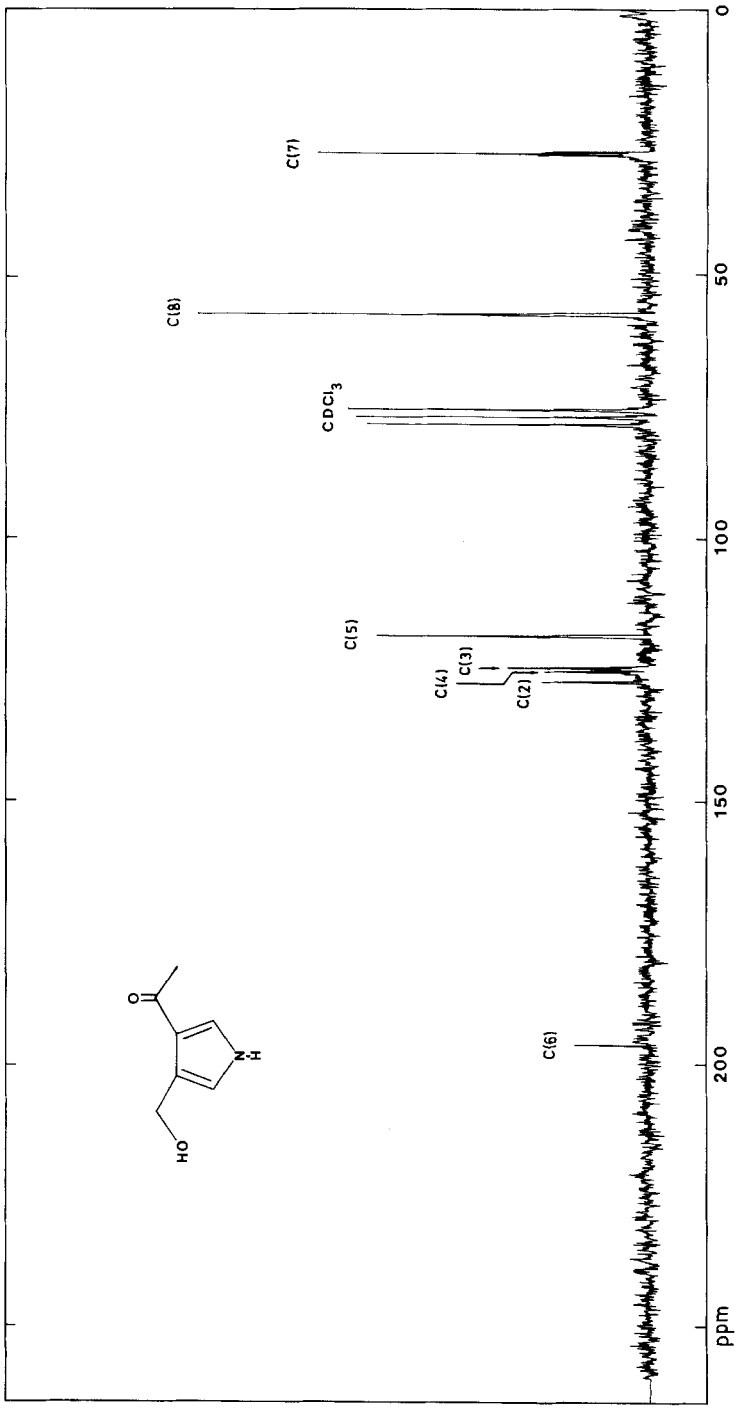


Fig. 1. *^{13}C-NMR. spectrum of [2-^{13}C]-acetate enriched verrucarin E* (**1**)

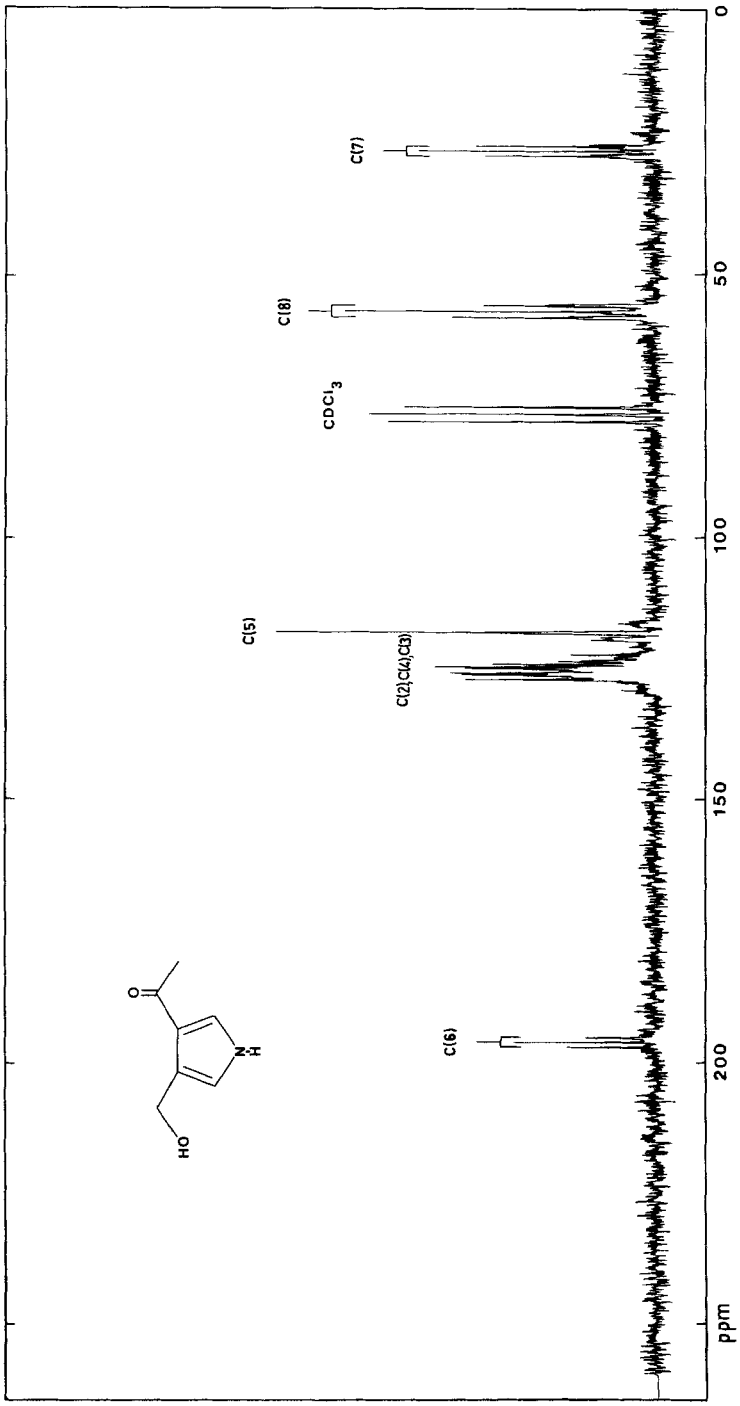


Fig. 2. *^{13}C-NMR. spectrum of [1,2-^{13}C]-acetate enriched verrucarin E* (1)

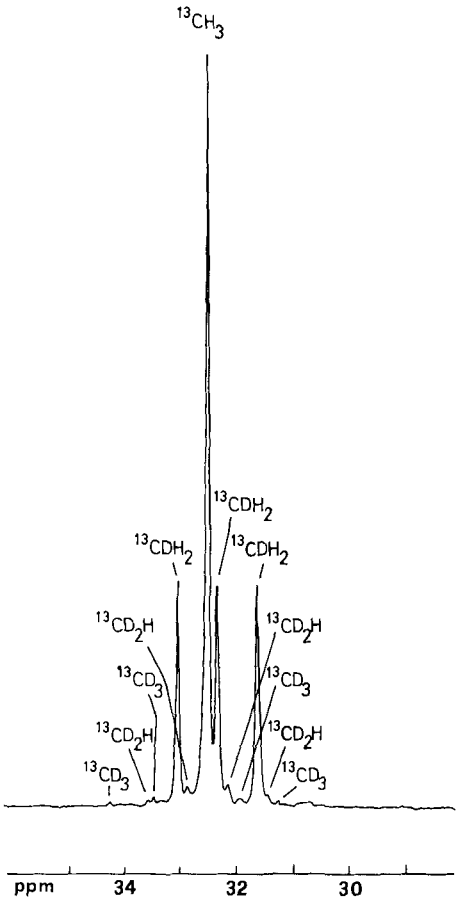


Fig. 3. *^{13}C-NMR. spectrum of sodium acetate obtained from verrucarin E* (**1**) *derived from [2-^{13}C, 2-$^{2}H_3$]-acetate by* Kuhn-Roth *oxidation*

molecules in which C(3) originates from unlabelled acetate. The outer two signals of the triplet result from 1,3-coupling between C(3) and C(7) in molecules in which both atoms are enriched with ^{13}C. Further splitting is due to the presence of $^{13}C^2H^1H_2$ and $^{13}C^2H_2{}^1H$ species for C(7). This indicates that extensive exchange of deuterium has occurred, but does not rule out the possibility of C(7) being derived from a starter unit, since such an exchange can take place in the metabolic equilibrium between acetate and malonate. The deuterium coupling results in a triplet and a quintuplet, respectively, for these species. Additionally, the substitution of deuterium for hydrogen results in an upfield shift of about 0.25 ppm for each deuterium. The resulting complex signal for C(7) made it impossible to detect any signals for any $^{13}C^2H_3$ species which may have been present.

In order to simplify the signal for C(7), a *Kuhn-Roth* oxidation of the $^{13}C^2H_3$-labelled preparation of verrucarin E (**1**) was performed and C(7) isolated as the methyl group of sodium acetate. In the ^{13}C-NMR. spectrum of this specimen *(Fig. 3)*, the effects of deuterium substitution could be observed without the interference of ^{13}C, ^{13}C-coupling. The spectrum shows a central singlet corresponding to

Table 2. *Exchange of hydrogen for deuterium at C(7) of verrucarin E* (**1**) *after incubation in a deuteriated medium as determined by* ^{1}*H-NMR. spectroscopy*

C-Atoms and groups	C(2)	C(5)	C(7)	OH	NH
% Exchange	33	33	23	100	60

$^{13}C^1H_3$. A deuterium triplet corresponding to $^{13}C^2H^1H_2$ is centered 0.23 ppm upfield from the singlet owing to the deuterium isotope effect on the ^{13}C chemical shift. In a further 0.22 ppm upfield position is the centre signal of the quintuplet corresponding to $^{13}C^2H_2{}^1H$, shifted by the isotope effect of two deuterium atoms. Another peak, albeit very small, appears 0.22 ppm upfield from the centre of the quintuplet. This signal can be assigned as the centre of a heptuplet due to the presence of $^{13}C^2H_3$ at C(7). Additional lines of these multiplets are labelled as shown in *Figure 3*.

The presence of a small but detectable amount of $^{13}C^2H_3$ species at C(7) proves that it is incorporated directly from acetate as the starter unit of the acetoacetate chain giving rise to C(2), C(3), C(6) and C(7). The extensive exchange of deuterium observed is probably due to the conversion of acetate to malonate followed by the reverse reaction in this equilibrium process. In each cycle of this process one deuterium can be exchanged. An alternative explanation is the exchange of deuterium from C(7) after incorporation of $^{13}C^2H_3$ from the fully labelled acetate. This exchange *via* enolization could occur quite easily since the methyl group is adjacent to a ketone conjugated to the aromatic pyrrole.

To test how much exchange at C(7) of verrucarin E (**1**) could occur in the fermentation broth, unlabelled verrucarin E (**1**) was treated for 48 h with a sterile medium prepared with D_2O and the other D_2O-exchanged components. The exchange of deuterium for hydrogen was measured in the re-isolated verrucarin E (**1**) by ^{1}H-NMR. spectroscopy. The signal of $H_2C(8)$ was used as internal standard for measuring the integration. The amount of exchange for the other H-atoms is shown in *Table 2*. The H-atoms at C(7) have exchanged to the extent of 23%. This result demonstrates that the extensive exchange of deuterium at C(7) of verrucarin E (**1**) in the [2-^{13}C,2-$^2H_3$]-acetate experiment could be partly due to exchange after formation of the metabolite.

Support of these investigations by the *'Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung'* (Projects No. 2.435.0.75, 2.629.0.76) and by *Sandoz AG.*, Basel, is gratefully acknowledged.

Experimental Part

General. The melting points (m.p.) were measured on a *Kofler* block and are corrected. The 90-MHz-^{1}H-NMR.- and 22.63-MHz-^{13}C-NMR. spectra were recorded on a *Bruker*-WH-90 spectrometer with *Fourier* transform in our spectral laboratory by Mr. *K. Aegerter.* The mass spectra were measured by Mr. *A. Raas* at the Institute of Physical chemistry, University of Basel, on a *Hitachi-Perkin-Elmer* model RMU7 instrument.

Incorporation studies. Spores of *Myrothecium verrucaria* (S 833) were fermented following [5]. The solutions of [1-^{13}C], [2-^{13}C], [1,2-^{13}C] and [2-^{13}C,2-$^2H_3$] enriched acetates (1 g in 7 ml of sterilized

water) were added to the growing cultures (1 l of medium in each case) after 5 days. The fermentation was terminated after 8 days and both the mycelium and the medium were extracted with ethyl acetate (3 × 1 l). The organic layer was washed with 10% Na_2CO_3-solution (500 ml) and with water (2 × 1 l) and evaporated i.V. The residue was dissolved in CH_2Cl_2 (5 ml) and passed through an aluminium oxide column (5 g, developed with CH_2Cl_2/methanol 99:1). The isolated verrucarin E (**1**) was crystallized from benzene, m.p. 91–92°. Yield of crystallized verrucarin E (**1**) was 25–30 mg/l. The ^{13}C-NMR. spectra were recorded on these samples.

Sodium [2-^{13}C,2-$^{2}H_3$]-acetate [11]. A solution of 1 g of sodium [2-^{13}C]-acetate in 20 ml of 98% D_2O was heated in a sealed tube at 150° for 48 h. The solution was lyophilized to dryness. The process was repeated 3 times to yield sodium [2-^{13}C,2-^{2}H]-acetate with a methyl group composed of 81.1% C^2H_3, 17.9% $C^2H_2{}^1H$, and 1.0% $C^2H^1H_2$ as determined by mass spectral analysis of the *p*-nitrobenzyl ester.

Incubation of verrucarin E (**1**) *in a deuteriated medium.* The ingredients for 50 ml of medium were dissolved in 10 ml of 98% D_2O and stirred overnight at RT. The solution was lyophilized and the residue dissolved in 50 ml of D_2O. This solution was sterilized by filtration through a Millipore filter into a sterile 100 ml *Erlenmeyer* flask. A solution of 40 mg of unlabelled verrucarin E (**1**) in 0.5 ml of acetone was added and the mixture shaken at 210 rpm at 27° for 48 h. Re-isolation of verrucarin E (**1**) in the usual way yielded 1.1 mg of material.

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