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A ^{13}C NMR Study on Trichodermin. Spectral Assignments, Spin-Lattice Relaxation, and Biosynthetic Incorporation of Sodium [1-^{13}C]-Acetate *

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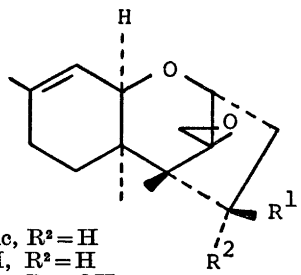
Unambiguous assignments of the ^{13}C FT NMR spectra of trichodermin and some related compounds have been obtained using various experimental techniques. Biosynthetic incorporation of sodium [1-^{13}C]-acetate into trichodermin has been studied by means of ^{13}C NMR spectroscopy. The results are in accordance with the earlier proposed biosynthesis for this compound. Spin-lattice relaxation times gave information on the molecular reorientational correlation times in chloroform solutions of trichodermin and on the internal motion of the methyl groups.

Trichodermin (*1*), an antifungal antibiotic first isolated in 1964 by Godtfredsen *et al.*[1] from the culture fluid of a strain of *Trichoderma viride*, is a member of the trichothecane group of antibiotics. Structurally *1* is a derivative of the tetracyclic 12,13-epoxy-trichothec-9-ene skeleton (*4*).[1,2]



1: R^1 = OAc, R^2 = H
2: R^1 = OH, R^2 = H
3: R^1 = H, R^2 = OH
4: R^1 = R^2 = H

The successful applications in recent years of ^{13}C pulse Fourier transform (FT) NMR spectroscopy as a tool for stereochemical and conformational analyses, biosynthetic, metabolic, and molecular interaction studies of biologically important molecules [3] prompted us to study some of these properties for trichodermin and some of its derivatives. In this paper we report the biosynthetic incorporation of sodium [1-^{13}C]-acetate into trichodermin (*1*) along with complete spectral assignments of the ^{13}C NMR spectra of *1*, trichodermol (Roridin C) (*2*) and 4-epi-trichodermol (*3*). In order to gain information on the steric effects of the methyl groups directly bonded to the rigid skeleton and on the behaviour of the molecular tumbling or *1* in solution we have measured the spin-lattice relaxation times (T_1) for *1* in a solution of chloroform. These data also confirmed and/or aided in the spectral assignments inferred from other methods. While this work was in progress, two papers dealing with the assignments of the ^{13}C chemical shifts in a series of trichothecane derivatives [4,5] and with the biosynthesis of trichothecolone from [2-^{13}C]-mevalonic acid [4] have appeared. It is found that a few of the spectral assignments in the earlier paper [4] are in error. However, these did not interfere with conclusions regarding the biosynthesis from [2-^{13}C]-mevalonic acid.

EXPERIMENTAL

Materials. *1* was isolated according to earlier reported procedures [1] and recrystallized twice

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from pentane (m.p. 46.0–46.5 °C uncorr.). *2* was prepared through saponification of *1*[1] and recrystallized twice from diethyl ether-hexane (m.p. 118–118.5 °C uncorr.). *3* was obtained from sodium borohydride reduction of trichodermone[1] (m.p. 199 °C uncorr.). ^{13}C-enriched *1* was obtained according to the following procedure: Spore material of *Trichoderma viride*, ND-8, from an eight days old agar-flask (Leo S-96) was transferred to shake-flasks with 250 ml of Saboraud fermentation medium. 4 h after inoculation, 0.6 ‰ sterile sodium [1-^{13}C]-acetate (Stohler Isotope Chemicals, 91.9 % enriched) was added to this medium. After two days growth at 28 °C on a reciprocal shaking-table, ^{13}C enriched *1* (8 % incorporation) was isolated. The sample solutions (*ca.* 0.2–1.2 M in $CDCl_3$) were contained in 12 mm o.d. tubes sealed under vacuum.

Spectra. Proton decoupled ^{13}C NMR spectra were obtained at 25.16 MHz and at a temperature of 33 °C on a Varian XL-100-15 spectrometer equipped with an S124-XL Fourier transform accessory. The ^2H resonance of the solvent ($CDCl_3$) served as internal field/frequency lock. Single frequency (off-resonance and selective) and noise modulated proton decoupling (100.1 MHz) were achieved by means of the system Gyrocode spin decoupler. Free induction decays were accumulated into 8 K data points and processed using a Varian 620 L 16 K computer. ^{13}C chemical shifts are relative to internal TMS and were obtained from noise-decoupled spectra with spectral widths of 5120 Hz and 2560 Hz in 4096 points. A pulse width corresponding to a flip angle of 68° and a delay time of 1 s between each data acquisition was used throughout. Selective

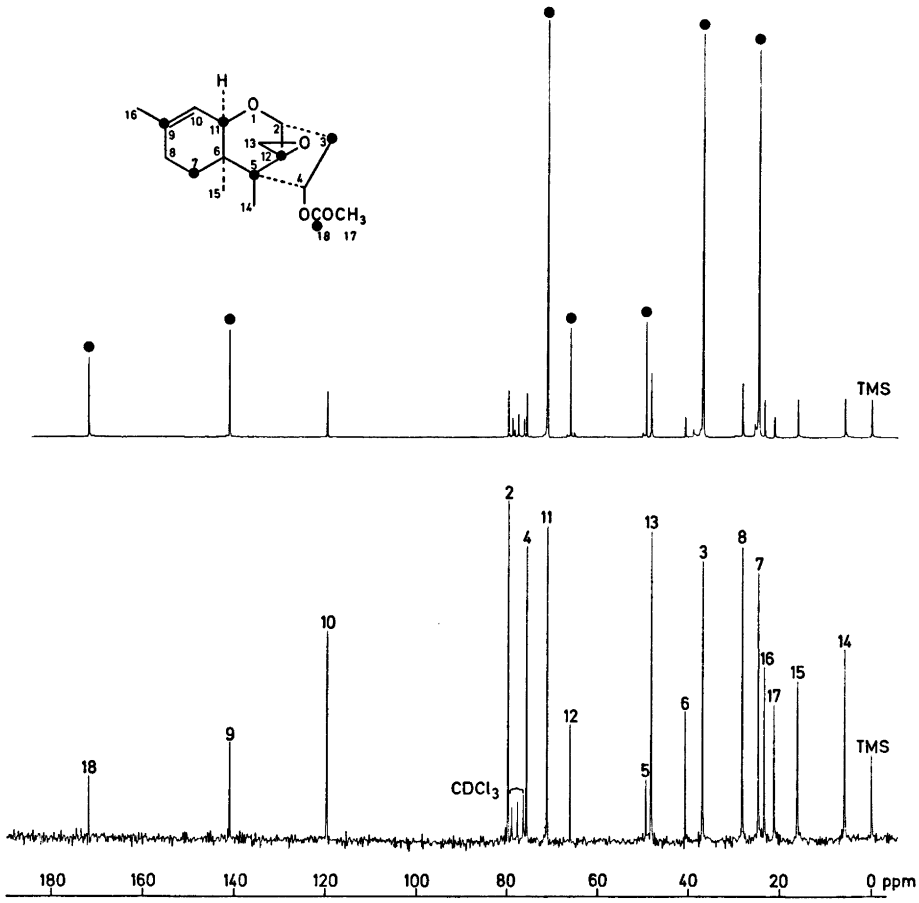

Fig. 1. 25.16 MHz proton noise decoupled ^{13}C FT NMR spectra of trichodermin *1* (lower part. 0.27 M in $CDCl_3$, 4500 transients) and biosynthetically ^{13}C-enriched ($CH_3{}^{13}COONa$) *1* (upper part, 0.13 M in $CDCl_3$, 2100 transients). Enriched carbons are indicated by ●.

^{13}C-{^{1}H} decoupling experiments were performed using the ^{1}H data obtained from the assigned 100 MHz ^{1}H spectrum.[1]

Spin-lattice relaxation times, T_1, were determined using the Freemann-Hill modification [6] of the inversion-recovery experiments,[7] *i.e.* applying a three-pulse sequency according to $(...T, 90°, T, 180°, \tau, 90°_{\tau}...)_N$. The 90° pulse samples the completely relaxed spectrum (S_∞), whereas the $90°_{\tau}$ pulse samples the partially relaxed spectrum (S_τ). The difference between the two corresponding free induction decays (FID's) is automatically generated in the computer so the final spectrum after Fourier transformation is equivalent to S_∞-S_τ. N usually ranged from 100 – 300 and normally 8 – 10 different τ values were used for the determination of T_1. T was always chosen to be greater than five times the longest T_1 for the carbons considered. The data collection was entirely automated with overnight runs using a Sykes Compu/Corder 120 magnetic tape cassette for storing the FID's. The resulting data, $S_\infty - S_\tau$, were fitted to the exponential function $S_\infty - S_\tau = 2S_\infty \times \exp(-\tau/T_1)$, to give T_1 using least-squares analyses, on a CDC 6400 computer system. The resulting T_1 values are the means of at least two independent measurements and were obtained with an accuracy of at least within 5 – 10 %.

METHODS, RESULTS AND DISCUSSION

Spectral assignments. As shown in Fig. 1, the 17 different carbon atoms in *1* give rise to separate signals in the proton noise-decoupled spectrum. The complete assignment of the signals to the individual carbon atoms in *1* was performed by combined use of various techniques, *e.g.* off-resonance ^{1}H decoupling, selective ^{1}H decoupling, and spin-lattice relaxation. Comparison with the ^{13}C spectra of *2* and *3* gave useful information concerning the correct assignment of C2 and C4, while comparison with model compounds aided the assignment of the olefinic carbons and the allylic methyl carbon. The complete assignments for *1*, *2*, and *3* are listed in Table 1.

Application of T_1 relaxation times as a tool in the assignment of ^{13}C spectra has been used occasionally.[9] In the present study the observation of an unusual high field shift for one of the methyl carbon resonances (5.82 ppm) along with its small T_1/T_1^{CH} ratio (*vide infra*) indicated this methyl group to be in a sterically

Table 1. ^{13}C and ^{1}H chemical shifts for trichodermin and some of its derivatives [a].

Carbon	δ_C *1*	*2*	*3*	δ_H *1*	*2*
2	79.20	78.80	78.10	3.82	3.81
3	36.72	40.18	36.47	1.93 2.54	1.82 2.59
4	75.17	74.03	80.24	5.58	4.34
5	49.01	49.18	47.10	–	–
6	40.49	39.85	40.44	–	–
7	24.54	24.48	25.55	1.42	1.47
8	28.07	28.05	27.81	1.98	1.99
9	140.11	140.06	139.70	–	–
10	118.76	118.84	119.53	5.42	5.40
11	70.60	70.35	70.30	3.62	3.50
12	65.53	65.77	66.23	–	–
13	47.85	47.55	49.09	2.83 3.13	2.79 3.09
14	5.82	6.21	9.89	0.72	0.77
15	16.01	15.81	15.75	0.93	0.82
16	23.21	23.21	23.26	1.72	1.72
17	21.12	–	–	2.07	–
18	170.91	–	–	–	–

[a] Chemical shifts are in ppm relative to internal TMS for both ^{13}C and ^{1}H with an accuracy within ± 0.05 ppm; ^{1}H chemical shifts are taken from Ref. 1. Trichodermin (*1*), 0.27 M in $CDCl_3$; trichodermol (*2*), 0.27 M in $CDCl_3$; 4-epi-trichodermol (*3*), 0.21 M in $CDCl_3$.

crowded position.[10] Thus this methyl carbon could be identified as C14 which has a more hindered internal rotation than C15. Furthermore, substitution of the α-acetoxy group in the 4 position with an α-hydroxy group resulted in a downfield shift of 0.39 ppm for C14 while substitution with a β-hydroxyl group gave a downfield shift of 4.07 ppm for this carbon indicating release in the steric effects for C14. Selective ^{13}C-{^1H} decoupling of the C14 methyl protons showed that the assignment of the C14 and C15 methyl protons in the ^1H spectrum reported by Godtfredsen *et al.*[1] has to be reversed.

Off-resonance decoupling experiments gave information regarding the number of protons bonded to the individual carbons (quaternary, tertiary, secondary, and primary) through the splittings of the individual lines (singlet, doublet, triplet, and quartet, respectively). However, various interesting effects were observed in the off-resonance decoupled spectra. For the methylene carbon C8 a residual doublet splitting due to long-range three-bond $^{13}C-^1H$ coupling between C8 and H10 was observed during selective decoupling of the methylene protons. However, no such coupling was observed between C7 and H10. This allowed a distinction between C7 and C8.[11] Off-resonance decoupling of the AB spins of an ABX system may produce second-order effects in the X spectrum which give rise to an increase in the number of lines expected or to line broadening.[12,13] The system most often showing this effect consists of strongly coupled vicinal protons which have two different couplings ($^1J_{C-H}$ and $^2J_{C-H}$) to one of the bonded carbons.[12] In the off-resonance decoupled spectra of *1* second order effects were observed for C13. Computer simulations demonstrated that the chemical shift nonequivalent geminal protons bonded to C13 are responsible for the second order splittings. In Fig. 2 an example of one of the off-resonance decoupled C13 spectra is shown along with the computer simulated spectrum. The parameters used in the simulation are given in the figure legend.

It was not possible using the above-mentioned techniques to determine the correct assignment of the signals at 49.01 ppm and 40.49 ppm for the quaternary carbons C5 and C6. However, the spectrum of biosynthetically ^{13}C-enriched

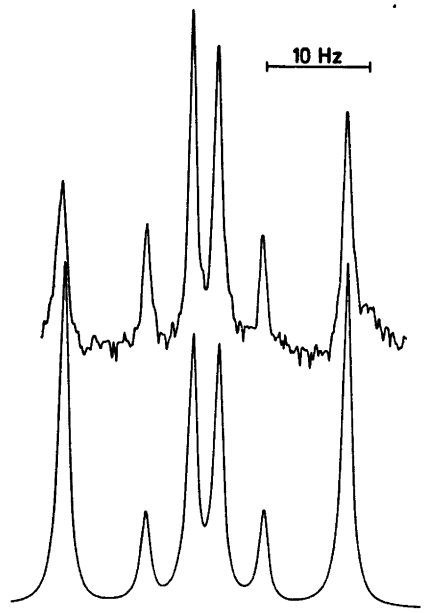

Fig. 2. Experimental (upper) and simulated (lower) $^{13}C-\{^1H\}$ off-resonance decoupled spectrum for the carbon atom C13. The experimental parameters are: $\nu_A = 283$ Hz, $\nu_B = 313$ Hz, $^2J_{AB} = 4.05$ Hz, $^1J_{^{13}C-H_A} = {}^1J_{^{13}C-H_B} = 174.5$ Hz, $\nu_2 = 368$ Hz and $\gamma H_2/2\pi = 890$ Hz.

trichodermin (*1*) (*vide infra*) showed $^{13}C-^{13}C$ satellites centered around the C14 signal with a coupling constant $^1J_{^{13}C-^{13}C} = 40.44$ Hz due to enrichment at C5. Hence, the observation that the intensity of the 49.01 ppm resonance augmented for the enriched material, while the 40.49 ppm resonance remained unchanged established that the former arise from C5.

The assignments of the ^{13}C spectra reported here for *1*, *2*, and *3* are in agreement with those given for the ^{13}C spectra of some trichothecanes by Breitenstein and Tamm [5] while they derivate for some carbons from the spectra of *2* and *3* assigned by Hanson *et al.*[4] For the spectrum of *2* the opposite assignment of C2 and C11 is proposed while in the spectrum of *3* not only the assignments for C2 and C11 are in error, but also that for C4. Our assignment was verified from selective decoupling experiments along with the ^1H chemical shift data for *1* and *2* (Table 1). Furthermore, the change of the hydroxyl group from the 4α- to the 4β-position is expected to affect the C4 and C2 chemical shifts to a greater extent than the C11 shift.

Biosynthesis. The availability of ^{13}C enriched materials and the recent progress in experimental ^{13}C NMR spectroscopy have greatly simplified biosynthetic studies of microbial metabolites using specifically ^{13}C labelled compounds as starting materials and ^{13}C NMR as a detection scheme.[14,15] In this work the fungus *Trichoderma viride* has been grown in a suitable medium containing $CH_3{}^{13}COONa$, thus allowing ^{13}C-enriched *1* to be isolated (see Experimental). The ^{13}C spectrum of the enriched compound (Fig. 1) immediately permitted identification of the enriched carbon positions. The amount of ^{13}C incorporated into each of these positions was determined to be *ca.* 8 %. Due to the relatively high level of ^{13}C incorporation, it was possible to observe $^{13}C-{}^{13}C$ couplings between directly bonded carbons where at least one of the positions has been enriched. Thus the observation of $^{13}C-{}^{13}C$ satellites centered around C14 gave the correct assignment of C5 and C6 (*vide supra*). Furthermore, the appearance of an AB $^{13}C-{}^{13}C$ satellite spectrum around C5 and C12 (Fig. 1) with a coupling constant $^1J_{^{13}C-^{13}C} = 41.85$ Hz confirmed this assignment. The enriched positions are indicated on the structural formula of Fig. 1 and the distribution is in full accordance with the proposed biogenesis (ring closure of a farnesyl pyrophosphate unit).[4,16]

Spin-lattice relaxation times, T_1. In studies of molecular mobility, ^{13}C spin-lattice relaxation times offer useful information, especially in the cases where the relaxation mechanism is purely $^{13}C-{}^1H$ dipolar. In the absence of internal and anisotropic motions and for non-viscous solutions $[(\omega_H+\omega_C)^2\ \tau_R{}^2 \ll 1$, *i. e.* the extreme narrowing condition], the relaxation rate for carbons relaxed by the $^{13}C-{}^1H$ dipole-dipole interaction mechanism is given by [17]

$$1/T_1 = h^2\gamma_H{}^2\gamma_C{}^2\tau_R N r_{CH}{}^{-6} \qquad (1)$$

where long-range $^{13}C-{}^1H$ dipolar interactions have been neglected. τ_R is the correlation time for molecular reorientation, N the number of directly bonded protons, r_{CH} the C–H bond length (1.09 Å) and γ_H and γ_C the magnetogyric ratios for ^{1}H and ^{13}C, respectively. Thus, for molecules having a rigid skeleton (τ_R being the same for all C–H vectors), T_1 values may be a valuable tool for differentiation between backbone CH_2, CH, and quaternary carbon resonances and provide information complementary to that obtained from off-resonance ^{1}H decoupled spectra.

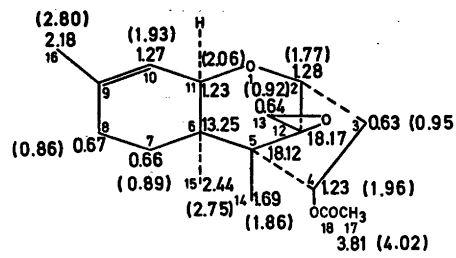

Fig. 3. ^{13}C Spin-lattice relaxation times T_1 (sec) for 1.17 M and 0.26 M solutions of *1* in $CDCl_3$. Values in parenthesis are for the solution of lower concentration.

The ^{13}C relaxation times measured for 0.26 M and 1.17 M solutions of *1* are shown in Fig. 3. The very short relaxation times for methylene and methine carbons, the CH carbons having relaxation times twice those of the CH_2 carbons, indicate essentially isotropic motion and almost complete dipole-dipole relaxation. Determination of the nuclear Overhauser enhancement (NOE) for all carbons in *1* confirmed a 100 % contribution of the $^{13}C-{}^1H$ dipole-dipole mechanism to the relaxation time for each resonance. Hence eqn. (1) may be used to calculate the reorientational correlation times giving 2.6×10^{-11} s and 3.6×10^{-11} s for 0.26 M and 1.17 M solutions, respectively.

The relaxation times for the quaternary carbons are considerably longer than T_1 for the protonated carbons due to the r^{-6} dependence of the dipole-dipole relaxation mechanism. Thus the protons most effectively contributing to the relaxation of the quaternary carbons are situated two bonds away from these carbons. From the number of such protons it is possible to rationalize the observed relaxation times.[9] The three quaternary carbons of *1*, C5, C6, and C12, have different numbers of "nearest" protons situated two bonds away (4, 6, and 3, respectively). Thus, it is expected that their relaxation times differ. It is observed that C6 has the shortest relaxation time as expected while T_1 for C5 and C12 is almost equal. However, three of the protons contributing to the relaxation of C5 are part of a methyl group which undergoes internal rotation (*vide infra*).

Table 2. $T_1/\overline{T_1^{CH}}$ ratios (see text) for the three methyl carbons C14, C15, and C16 in trichodermin, *1* (1.17 M and 0.26 M in $CDCl_3$).

Carbon	$T_1/\overline{T_1^{CH}}$ 1.17 M	0.26 M
C14	1.35	0.96
C15	1.95	1.42
C16	1.74	1.45

Therefore, the effective contribution to the relaxation from these protons is diminished due to the internal motion.[10] These qualitative estimates are in accordance with the actual observations and the results further confirm the correct assignment of C5 and C6.

Methyl groups attached to the rigid body of *1* may undergo internal motion and eqn. (1) is thus not applicable to these carbons. It has been shown, however, that the relaxation times of methyl carbons directly bonded to a rigid, isotropically tumbling body are bound to be within certain limits as expressed by [10]

$$1/3 \leq T_1/\overline{T_1^{CH}} \leq 3 \qquad (2)$$

locked methyl — freely rotating methyl

where $\overline{T_1^{CH}}$ denotes the mean relaxation time of the methine carbons. From this expression it appears that when the relaxation time of a methyl group approaches 1/3 $\overline{T_1^{CH}}$, the internal methyl group rotation is slow compared with the overall motion of the molecule, whereas the rotation is fast when T_1 approaches $3\overline{T_1^{CH}}$. In Table 2 the $T_1/\overline{T_1^{CH}}$ ratios are listed for the three methyl carbons in *1*, C14, C15, and C16. It is readily seen that the three methyl groups are restricted in their rotation. C14 is more restricted in its motion relative to C15 and C16 in accordance with the observation of an unusual high field shift for this methyl carbon.

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