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# Use of Singly and Doubly Labelled [$^{13}$C]Acetate in the Elucidation of the Structure of Carolic Acid

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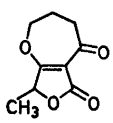
$^{13}$C NMR spectra of unenriched and $^{13}$C enriched carolic acid, isolated from *Penicillium charlesii* NRRL 1887, have been measured in deuteriochloroform and in alkaline solution. Carolic acid is shown to be a mixture of two isomers. The only acceptable assignments of the $^{13}$C NMR signals is achieved by considering carolic acid as a mixture of (*E*)- and (*Z*)-5-methyl-3-(2′-tetrahydrofurylidene)tetrahydrofuran-2,4-dione (*2*). The structure proposal containing a seven-membered ring can, therefore, be rejected.

Carolic acid,[1] a major metabolite of *Penicillium charlesii* and *P. fellutanum*,[2] possesses the molecular formula $C_9H_{10}O_4$ and has been formulated as *1* by Clutterbuck *et al.*[3] The seven-membered ring is an unusual feature in itself. It has been argued that *1* most satisfactory explains the IR absorptions at 1760 cm$^{-1}$ ($\gamma$-lactone) and 1710 cm$^{-1}$ (ketonic carbonyl) [4] as well as the $^1$H NMR spectrum.[5] Dean [6] has suggested that none of the published evidence suffices to eliminate structure *2* for carolic acid and states that if carolic acid is indeed an $\alpha,\beta$-unsaturated ketone, then carbonyl absorption at 1710 cm$^{-1}$ favours the idea that the carbonyl group is contained in a five-membered ring. Burrows and Turner [7] find that some of the signals in the $^1$H NMR spectrum of carolic acid are doubled indicating the existence of at least two isomers. The absolute configuration of carolic acid has been established to be *R*.[8]



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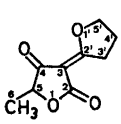

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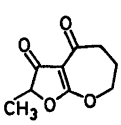


*3*

In order to clarify the problems related to the structure of carolic acid we have recorded the $^{13}$C NMR spectra in deuteriochloroform of carolic acid as well as of carolic acid enriched with [1-$^{13}$C]-, [2-$^{13}$C]- and [1,2-$^{13}$C]acetate. In aqueous solution the non-lactonic ring in carolic acid is opened and an $\alpha$-acyltetronic acid is formed. In non-aqueous media carolic acid appears as a compound having no active hydrogen. For the sake of completeness we also report the $^{13}$C NMR spectra of the unenriched and the enriched samples in alkaline solutions.

## RESULTS AND DISCUSSION

The $^{13}$C NMR spectrum of carolic acid in $CDCl_3$ clearly demonstrates the existence of two isomers in the ratio of approximately 4:5. Each isomer shows only one signal downfield to 195 ppm. Since normal $\alpha,\beta$-unsaturated ketonic carbon atoms always give values of chemical shift in this area,[9] it can be concluded that carolic acid is either a mixture of *1* and an isomer of *2* or a *cis/trans* mixture of *2*. The possibility of carolic acid existing as a mixture of either *1* or *2* together with a third structural possibility *3* can be excluded since such mixtures will give rise to the presence of three signals downfield to 195 ppm.

Feeding experiments with $^{14}$C-labelled acetate added to *P. charlesii* [10,11] have demonstrated that C-2 and C-3 together with the seven-membered ring in *1* or *3*, or the tetrahydrofurylidene ring in structure *2*, is formed from a subunit bearing the normal polyketide labelling pattern. This work has been repeated using [1-$^{13}$C]-, [2-$^{13}$C]-, and [1,2-$^{13}$C]acetate as precursors.[12]

*Table 1.* $^{13}C$ NMR chemical shifts, $\delta_i$(ppm), [a] and $^{13}C,^{13}C$ spin spin coupling constants $^1J_{ij}$ (Hz) [b] of carolic acid (*2*), enriched with [1,2-$^{13}C$]acetate, in $CDCl_3$.

| | C-2 | C-3 | C-4 | C-5 | C-6 | C-2′ | C-3′ | C-4′ | C-5′ |
|---|---|---|---|---|---|---|---|---|---|
| *Z*-form | | | | | | | | | |
| $\delta_i$ | 167.24 | 95.64 | 195.70 | 79.27 | 17.02 | 187.31 | 33.46 | 21.70 | 77.51 |
| $^1J_{ij}$ | 76.2 | | | | | 37.1 | | 32.2 | |
| *E*-form | | | | | | | | | |
| $\delta_i$ | 170.29 | 95.05 | 199.01 | 79.66 | 17.02 | 186.61 | 33.27 | 21.70 | 78.03 |
| $^1J_{ij}$ | 78.1 | | | | | 38.1 | | 32.2 | |

[a] Uncertainty: ± 0.07 ppm. [b] Uncertainty: ± 0.5 Hz.

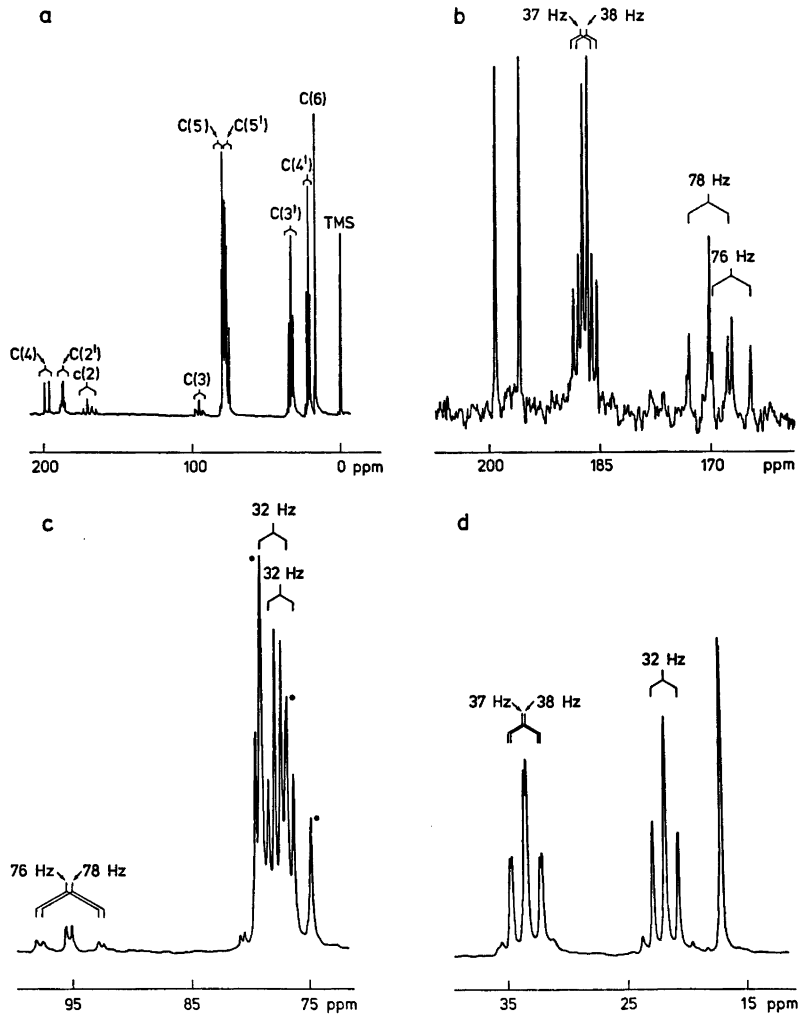
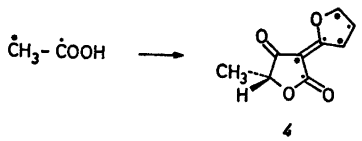



*Fig. 1.* $^{13}C$ NMR spectrum in $CDCl_3$ of carolic acid enriched with [1,2-$^{13}C$]acetate. Trace b–d are details of the spectrum shown in trace a. The lines marked with an asterisk contain signals from $CDCl_3$.

The $^{13}$C spectrum of the enriched carolic acid shows that none of the carbon atoms responsible for the lines downfield to 195 ppm are enriched, giving rise to the conclusion that the two isomers of carolic acid both possess an $\alpha,\beta$-unsaturated carbonyl group at C-4. The only possibility open is, therefore, to consider carolic acid as a *cis/trans* mixture of *2*. This structure proposal makes it possible to explain the various aspects of the $^{13}$C NMR spectra and gives, as further evidence, the only reasonable assignments of the observed signals.

The $^{13}$C chemical shifts obtained for carolic acid (*2*) are listed in Table 1. The assignments of the lines are based upon the gated decoupled spectrum and the spectra of the $^{13}$C enriched molecules. The spectrum of *2* enriched with [1-$^{13}$C]acetate exhibits an increase of the intensity of the lines at 21.70, 167.24, 170.29, 186.61 and 187.31 ppm compared to the intensity of the lines in the spectrum of the unenriched compound. The spectrum of *2* enriched with [2-$^{13}$C]acetate shows increased intensity of the lines at 33.27, 33.46, 77.51, 78.03, 95.05 and 95.64 ppm. The degree of enrichment amounts in both cases to approximately 1.0 %.

The $^{13}$C NMR spectrum of *2* enriched with [1,2-$^{13}$C]acetate is shown in Fig. 1. The signals from the enriched carbon atoms are composed of doublets arising from the $^{13}$C,$^{13}$C spin spin coupling constants (given in Table 1). The two lines in the doublets are distributed symmetrically around the lines from the carbon in natural abundance carolic acid. The only carbon atoms, unaffected by introduction of $^{13}$C-labeled acetate, are C-4, C-5, and C-6. In accordance with the proposed biosynthesis [12] the reaction pathway may, therefore, be rationalized as shown in Scheme 1.




*4*

*Scheme 1.*

Independently, Holker [13] has reached the same conclusion concerning the biosynthesis of a structurally related tetronic acid, terrestric acid, using the same technique.

The $^{13}$C chemical shift of C-2 differs about 3 ppm in the two isomers. Brouwer and Stothers [14] have reported $^{13}$C chemical shifts of the methyl esters of (*Z*)- and (*E*)-3-methoxyacrylic acid and concluded that the chemical shift of the ester carbon atom in the *E*-isomer is shifted 3 ppm downfield compared to the corresponding value in the *Z*-isomer. If this result is extended to *2*, the assignment of the lines to the two isomers are as given in Table 1. The differences of the $^{13}$C,$^{13}$C spin spin coupling constants and the small differences in intensity of the lines given by the two isomers have been used for this assignment. The possibility of errors in this assignment caused by change of the nuclear Overhauser effect between the two isomers may therefore exist, except in case of C-2 and C-3.

The variation in $^{13}$C chemical shift between the two isomers of *2* can be explained in terms of change in the electronic environment of the carbon atoms. The distances from C-2 and C-4 to O-1′ are distinctly different for the two isomers, thus giving rise to quite different contributions to the chemical shifts for the respective carbon atoms in the two isomers. The electronic environment around the other carbon atoms is expected to be only slightly different in the two isomers. It must, therefore, be possible to conclude that the difference in $^{13}$C chemical shifts between the two isomers should be much larger for C-2 and C-4 than for the other carbon atoms. As can be seen from Table 1 this is in agreement with the experimental data.

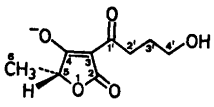
The chemical shifts observed for the carbon atoms in *2* are in agreement with results obtained from related compounds.[9,15] In case of C-2′ the shift value is exceptionally high for an ethylenic carbon atom, but reasonable when taking into consideration that the $\pi$-electrons are highly delocalized and that C-2′ is attached to the strongly electron-withdrawing alkoxy group.

The $^1J_{CC}$ coupling constants of *2* given in Table 1 show the usual values for $sp^3$ hybridized carbon atoms [15] in case of $^1J_{C-2',C-3'}$ and $^1J_{C-4',C-5'}$. The values found for $^1J_{C-2,C-3}$ are in the range normally found for coupling between $sp^2$ hybridized carbon atoms [15] supporting the idea of having a very high degree of delocalization of the $\pi$-electrons in *2*.

*Table 2.* $^{13}C$ NMR chemical shifts, $\delta_i$(ppm), [a] and $^{13}C,^{13}C$ spin spin coupling constants $^1J_{ij}$ (Hz), [c] of the open form (*5*) of carolic acid in alkaline solution.

| | C-2 | C-3 | C-4 | C-5 | C-6 | C-1′ | C-2′ | C-3′ | C-4′ |
|---|---|---|---|---|---|---|---|---|---|
| $\delta_i$ | 177.46 | 96.09 | 200.00 | 77.64 | 16.70 | 199.35 | 33.35 | 27.55 | 61.53 |
| $^1J_{ij}$ | 76.2 | | | | | 41.0 | | 37.0 | |

[a] Uncertainty: ±0.07 ppm. [b] Uncertainty: ±0.5 Hz.



*5*

In alkaline solution carolic acid is hydrolyzed to give the open structure *5*. The chemical shifts of the carbon atoms have been determined for *5* as well as for the three $^{13}C$ enriched analogues. The results are given in Table 2. The assignment of the lines have been performed by use of the lines in the $^{13}C$ spectra of the $^{13}C$ enriched molecules and the gated decoupled spectrum. The lines due to C-2′ and C-5 show the existence of an exchange of the directly attached protons with deuterium. The rate of exchange differed in the two cases ranging from a few hours for H-2′ to a few days for H-5.

The chemical shifts of the carbon atoms in *5* are characterized by showing two signals due to α,β-unsaturated carbonylic carbon atoms and assigned to C-4 and C-1′. This implies that the excess charge is delocalized to a high degree in the bonds between C-1′, O-2, and C-4. From the $^1J_{CC}$ coupling constants in *5*, given in Table 2, it can be seen that the degree of delocalization is recognized in the value of $^1J_{C-2,C-3}$.

The $^{13}C$ NMR spectrum of the open structure of carolic acid in acid solution is complicated due to the existence of two forms, related to two different ways of forming hydrogen bonds in α-acyltetronic acids. This problem is currently under investigation.

## EXPERIMENTAL

The $^{13}C$ NMR spectra were recorded on a Jeol FX60 spectrometer. 8 K data points were used with a pulse length of 6 μs corresponding to a 60° flip angle. The spectral width was 4000 Hz. The magnetic field was stabilized by internal deuterium lock on the signal from the solvents. The probe temperature was 30 °C. All chemical shifts were measured as δ (ppm downfield to TMS). The sample concentrations used were 100 mg carolic acid in either 1.3 ml $CDCl_3$ or 1.3 $D_2O$ + 0.05 ml 40 % NaOD.

Carolic acid was isolated in adequate amounts from single flask cultures of *Penicillium charlesii* NRRL 1887. Details concerning addition of $^{13}C$-labeled acetate and isolation will be discussed in another paper.[13]

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