CARBON-13 NUCLEAR MAGNETIC RESONANCE STUDIES OF COLLETOTRICHINS AND THEIR BIOSYNTHESIS

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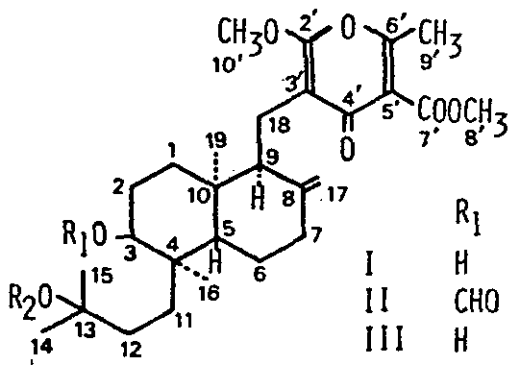
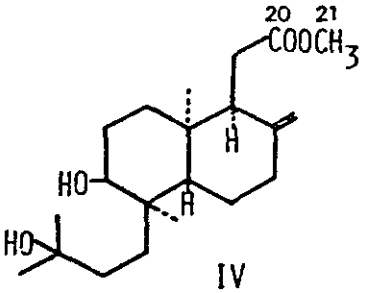
The ^{13}C-nmr spectra of three related secondary fungal metabolites, colletotrichin (I), colletotrichin B (II), and C (III), isolated from *Colletotrichum nicotianae* have been studied. Labelling patterns of these compounds derived from ^{13}C-formate, 1-^{13}C-, 2-^{13}C-, and 1,2-^{13}C-acetates, and 5-^{13}C-mevalonate have been determined. These compounds were proved to be biosynthesized in combination of acetate-mevalonate-geranyl-geranyl pyrophosphate route with acetate-polyketide route. Moreover, a new pathway, which involves cyclization of geranyl-geranyl pyrophosphate into I, II, and III was proposed.

Colletotrichin (I) is a phytotoxic substance isolated from *Colletotrichum capsi* by J. F. Grove et al.[1] and *C. nicotianae* by A. Suzuki et al.[2] and the structure has been independently determined by X-ray analysis of itself[3] and its acetate.[4] In addition to I, we isolated two closely related compounds,[5]

colletotrichins B (II) and C(III) from the culture filtrate of *Colletotrichum nicotianae* and established their structures in comparison of those of II and III with the physical and chemical data of I.



	R_1	R_2
I	H	H
II	CHO	H
III	H	CHO



IV

When applied to the young tobacco leaves, these compounds induced similar symptons on the leaves to the diseased lesion of tobacco plants infected with *C. nicotianae*, indicating the participation of these substances in the pathogenicity of the fungus.[5] Further it has been reported that colletotrichins inhibited the growth of lettuce and rice seedlings, and the oxidation of NADA and succinate by the mitochondria of rat liver.[6]

The structures of colletotrichins consisting of unique norditerpene and polysubstituted γ-pyrone moieties together with the interesting biological activities have stimulated us to study the biosynthesis of colletotrichins by means of ^{13}C-nmr.[7]

ASSIGNMENT OF ^{13}C-NMR SIGNALS OF COLLETOTRICHINS

The chemical shifts in ^{1}H noise decoupling spectra and multiplicities in ^{1}H off resonance spectra of I, II, and III together with C_{20}-acid methyl ester (IV) which was obtained on the treatment with alkaline hydrogen peroxide

of I, II, and III are shown in Table.

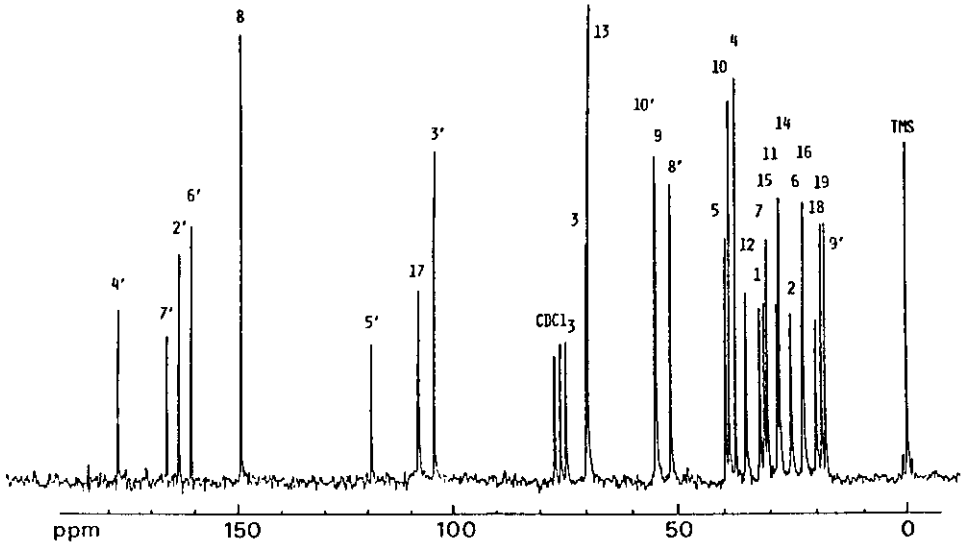


Fig. 1. ^{13}C-nmr spectrum of colletotrichin

Since the signals at δ_C 25.6 and 35.7 of I shifted 2.7 and 1.7 ppm, respectively, in II and III which were replaced hydrogen with formyl on hydroxyl at C-3 and C-13, the former was assigned to C-2 and the latter to C-12. The signal at δ_C 20.1 shifted downfield in IV was unambiguously assigned to C-18. The signal at δ_C 23.1 appeared at rather high field as methylene carbon was assigned to C-6 from the literature value.[8] Since the signal intensity at δ_C 109.6 was enhanced by irradiating at exomethylene protons (δ_H 4.4), C-17 was assigned to this signal. Three signals at δ_C 32.6, 31.7, and 28.7 assigned to C-1, -7, and -11 were not distinguished from each other.

Among the quarternary methyl carbons, two signals at δ_C 28.2 and 31.0

were assigned to C-14 and C-15 from the high field shift in the ^{13}C-nmr spectrum of III, whereas the signals at δ_C 22.8 and 18.9 originating from C-16 and C-19 were not differentiated from each other.

The signal at δ_C 71.6 appeared as a doublet was assigned to the oxygen-bearing C-3 from the chemical shift and in comparison with that of II. The remaining two doublet signals at δ_C 40.2 and 56.0 were assigned to C-5 and C-9, respectively, from the literature values.[8]

Thugh two singlets at δ_C 38.0 and 39.4 were not distinguished from each other, the remaining two singlets at δ_C 149.6 and 71.2 were assigned to C-8 and C-13, respectively, from characteristic chemical shifts.

The signals due to a polysubstituted γ-pyrone moiety were assigned by the measurement of proton coupled spectra with NOE and by an application of long-range selective proton decoupling experiments.[9]

Two signals at δ_C 160.8 and 177.1 were appeared as a singlet by irradiation at the frequency of C-9' methyl protons (δ_H 2.34). From this result together with the splitting patterns in the proton coupled spectrum, the former was assigned to C-6' and the latter to C-4'.

Irradiation at methoxy protons (δ_H 3.88) eliminated the splitting of the signal at δ_C 166.2 assigned to C-7'. On the other hand, the signal at δ_C 163.5 due to C-2' was rather broad due to the coupling with C-18 methylene protons.

Discrimination between the signals at δ_C 106.0 and 120.2 was also accomplished by long-range selective proton decoupling experiments irradiating at methyl protons (δ_H 2.34). In this case, the signal of C-5' at δ_C 120.2 was observed as a singlet which indicated that any other coupling proton except for C-9' methyl protons was absent. On the other hand, the signal at δ_C 106.0 assigned to C-3' was rather broad due to couplings with protons

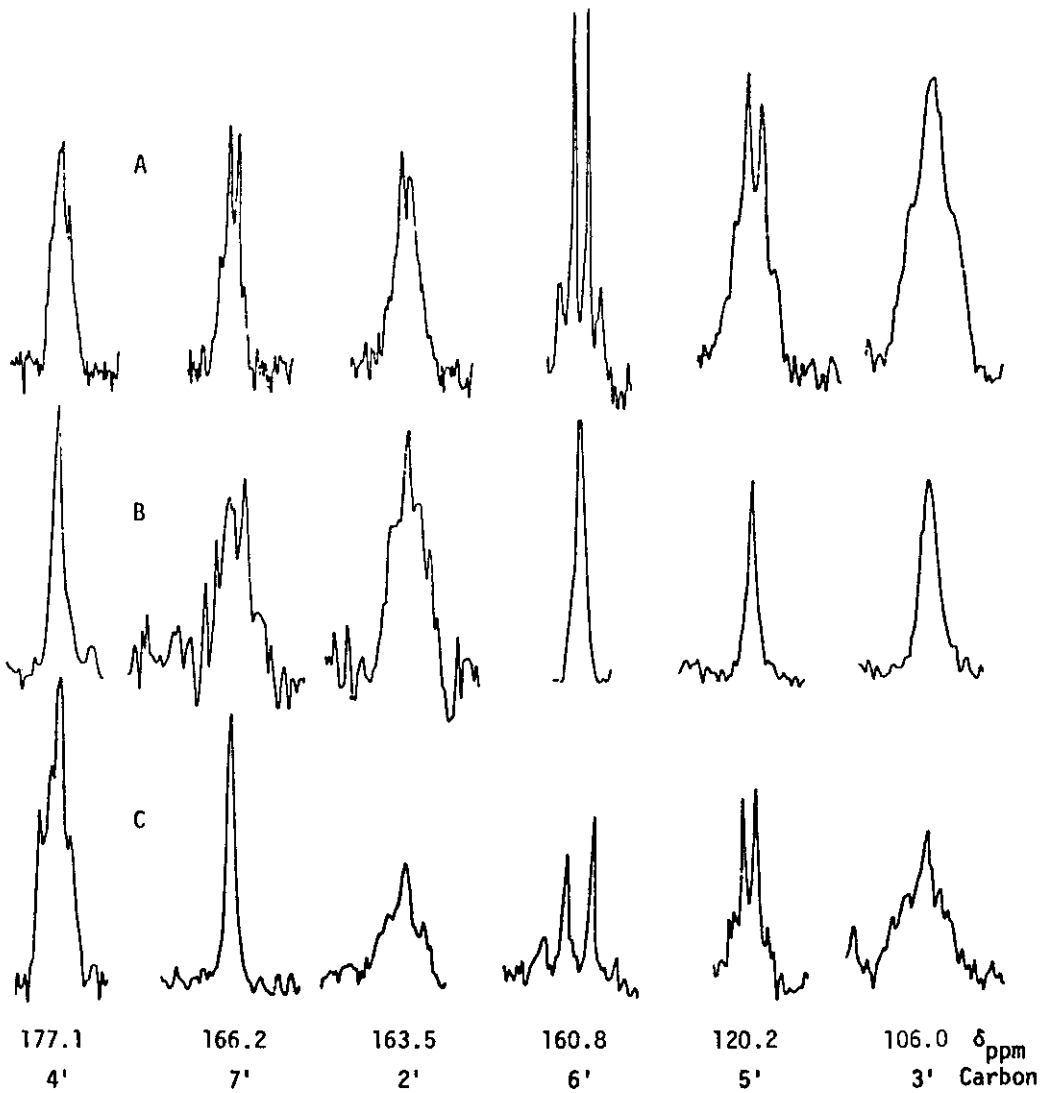


Fig. 2. ^{13}C-nmr spectra of colletotrichin

A: Proton coupled spectrum with NOE

B: Long-range selective proton decoupling spectrum, C-9' H irrd.

C: Long-range selective proton decoupling spectrum, C-8' and C-10' H irrd.

attached to C-9 and C-18.

Among the remaining three signals, one at δ_C 18.0 was assigned to C-9' and the others at δ_C 52.7 and 56.2 to C-10' and C-8', respectively, from the chemical shifts and multiplicities.

The signals at δ_C 160.6 of II and 161.0 of III appeared as a doublet were assigned to formyl carbon of each compound, respectively.

BIOSYNTHESIS OF COLLETOTRICHINS

In order to get ^{13}C-labelled colletotrichins, *C. nicotianae* was inoculated into 500 ml Erlenmeyer flasks containing 120 ml of the medium (sucrose 2.0 %, KCl 0.1 %, $MgSO_4$ 0.05 %, KNO_3 0.05 %, and yeast extract 0.1 %) and incubated aerobically at 26.5 °C. After 2 days, ^{13}C-labelled precursors were separately added. I was isolated after a further 8 days from the culture filtrate according to the same method as reported before,[5] whereas II and III were obtained from 3-day culture.

In the ^{13}C-nmr spectrum of 5-^{13}C-mevalonate labelled I, the signal intensities of carbons, C-2, -6, -12, and -18, were increased by approximately two fold. This result revealed that the terpene moiety of colletotrichin was biosynthesized from 4 moles of mevalonate, indicating that I was biosynthesized *via* geranyl-geranyl pyrophosphate.

The label of 1-^{13}C-acetate was efficiently incorporated into C-2, -4, -6, -8, -10, -12, -13, and -17 in the terpene moiety as well as C-2', -4', and -6' in the γ-pyrone moiety. This result was well consistent with that of incorporation of 4 moles of mevalonate as shown in Scheme 1. Further the coupling constant,[10] J=38 Hz, observed between adjacent C-12 and C-13 verified the assignment and supposed C_1 elimination between these two carbons.

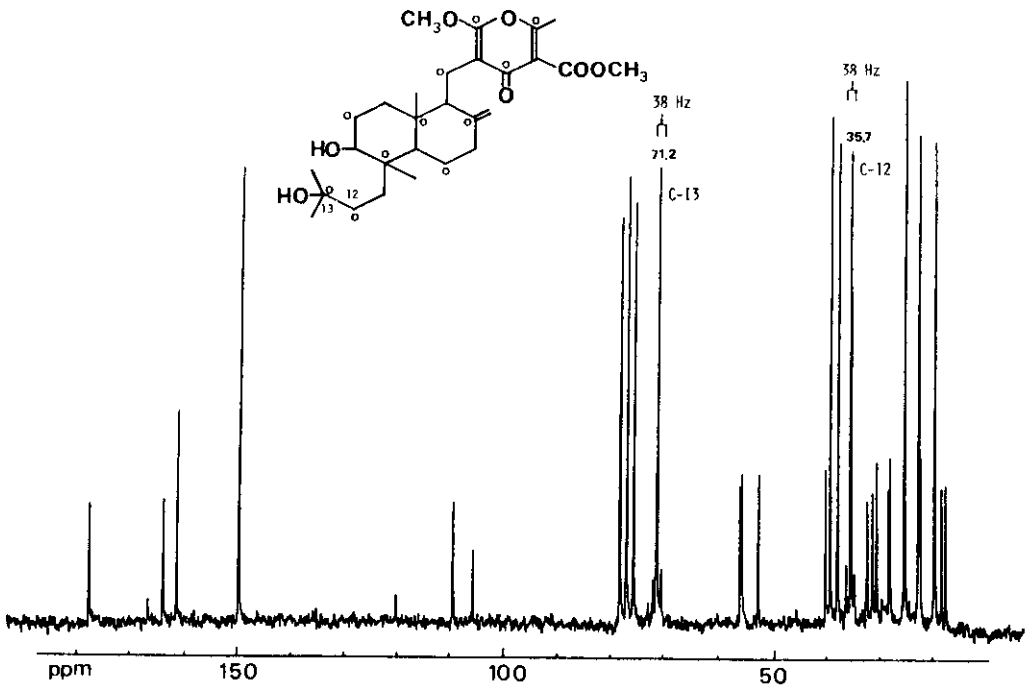


Fig. 3. ^{13}C-nmr spectrum of 1-^{13}C-acetate enriched colletotrichin

In the ^{13}C-nmr spectrum of 2-^{13}C-acetate enriched I, the signal intensities of carbons, C-3', -5', -9', -1, -3, -5, -7, -9, -11, -14, -15, -17, and -19, were increased.

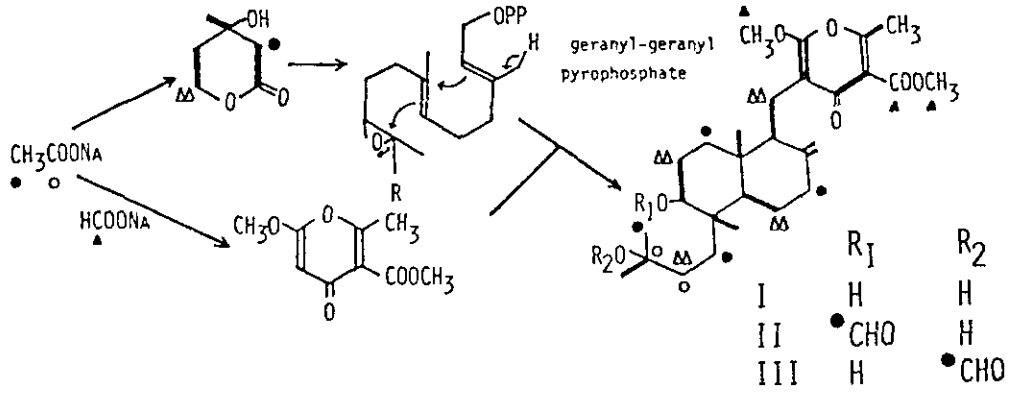
In the ^{13}C-nmr spectrum of I biosynthesized from 90 % enriched 1,2-^{13}C-acetate that was diluted two fold with unlabelled acetate, 20 signals with ^{13}C-^{13}C coupling were observed, indicating that 10 acetate units were incorporated. In addition to these carbons, the signals for C-1, -7, -11, -12, and -15 carbons have also enhanced intensities relative to the natural abundance of C-8' methoxy signal without ^{13}C-^{13}C coupling.

It is noteworthy that signal intensities of enriched carbons in C-11~15

of labelled I were almost the same extent as those in the remaining terpene moiety. Moreover, the patterns of acetates and mevalonate incorporation into C-11~15 revealed that one carbon corresponding to C-4 methylene of mevalonate derived from C-2 of acetate was eliminated in the course of the biosynthesis of I.

The ^{13}C-spectrum of I labelled with ^{13}C-formate showed that three carbon atoms, C-7', -8', and -10', were enriched.

Thus, it was indicated that I was biosynthesized in combination of acetate-*mevalonate-geranyl-geranyl pyrophosphate-terpene pathway* (*via* sacculatal type cylization)[11] with acetate-polyketide route.



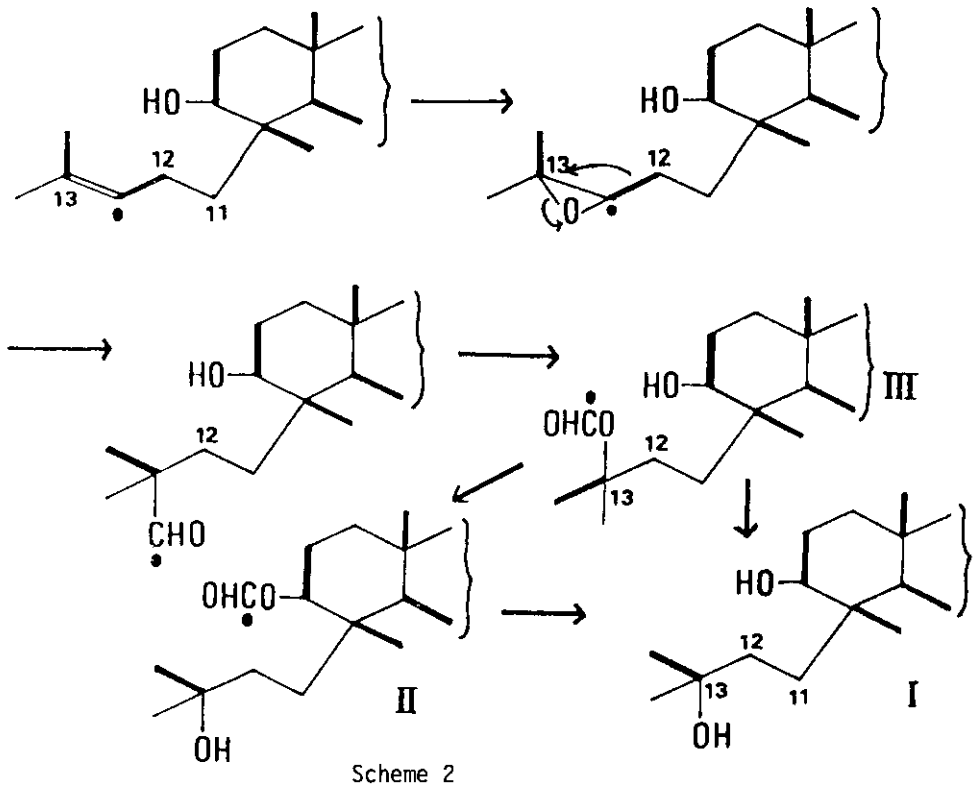
Scheme 1

The labelling patterns of II and III biosynthesized from ^{13}C-formate, 1-^{13}C-, 2-^{13}C-, and 1,2-^{13}C-acetates, and 5-^{13}C-mevalonate were consistent with those of I except for formyl groups.

Contrary to our expectation that formate as C_1 unit might be incorporated into each formyl group of II and III, only three signals assignable to C-7', -8', and -10' carbons were enriched by approximately eight fold, whereas

in the ^{13}C-nmr spectra of both II and III labelled with 2-^{13}C- and 1,2-^{13}C-acetates, signal intensities of formyl carbons were enhanced.

From the data of labelled II and III together with those of I, the mechanism involving cyclization of geranyl-geranyl pyrophosphate, followed by epoxidation of terminal double bond, cleavage, and Baeyer-Villiger type reaction would account for the biosynthetic pathway of colletotrichins as shown in Scheme 2.



Scheme 2

Table. Chemical Shifts and Coupling Constants of Colletotrichins and C_{-20} Acid Methyl Ester

Carbon		I	II	III	IV				$J^{k}_{^{13}C-^{13}C}$
C-1[b]	t[i]	32.6	33.0	32.6	32.1		●	Δ	h
C-2	t	25.6	22.9	25.8	25.3	o		Δ ΔΔ	37
C-3	d	71.6	75.5	71.8	71.1		●	Δ	37
C-4[e]	s	38.0	37.7	38.0	37.6	o		Δ	37
C-5	d	40.2	41.0	40.2	40.2		●	Δ	35
C-6	t	23.1	22.9	22.9	22.9	o		Δ ΔΔ	35
C-7[b]	t	31.7	31.2	31.4	31.4		●	Δ	h
C-8	s	149.6	148.4	148.8	148.0	o		Δ	72
C-9	d	56.0	55.9	55.8	55.4		●	Δ	34
C-10[e]	s	39.4	38.4	39.2	39.3	o		Δ	36
C-11[b]	t	28.7	29.0	26.5	28.7		●	Δ	h
C-12	t	35.7	36.2	34.0	35.2	o[f]		Δ ΔΔ	h
C-13	s	71.2	70.9	84.3	71.3	o[f]		Δ	39
C-14	q	28.2	29.2	26.7	28.1		●	Δ	39
C-15	q	31.0	29.4	28.4	30.9		●	Δ	h
C-16[a]	q	22.8	22.9	22.8	22.2		●	Δ	37
C-17	t	109.6	109.6	109.4	110.1		●	Δ	72
C-18	t	20.1	19.9	20.0	34.4	o		Δ ΔΔ	34
C-19[a]	q	18.9	18.5	18.9	18.5		●	Δ	36
C-20	s	-	-	-	173.9				
C-21	q	-	-	-	51.4				
C-2'	s	163.5	163.0	163.0	-	o		Δ	89
C-3'	s	106.0	105.4	105.5	-		●[g]	Δ	89
C-4'	s	177.1	176.6	176.4	-	o		Δ	54
C-5'	s	120.2	119.9	119.8	-		●[g]	Δ	54
C-6'	s	160.8	160.4	160.5	-	o		Δ	52
C-7'	s	166.2	165.6	165.7	-			▲	
C-8'[c]	q	52.7	52.6	52.7	-			▲	
C-9'	q	18.0	18.0	18.1	-		●	Δ	52
C-10'[c]	q	56.2	55.9	56.0	-			▲	
R_1 or R_2	d	-	160.6	161.0	-		●	Δ	

Measured in $CDCl_3$ solution, in ppm downfield from internal TMS. [a,c,e] Assignment may be reversed. [b] Assignment may be changed. [f,g] Coupling constants ; J_f=38, J_g=12 Hz. [h]Uncoupled signal. [i] Multiplicity. ▲^{13}C-Formate. o 1-^{13}C-Acetate. ● 2-^{13}-C-Acetate. Δ 1,2-^{13}C-Acetate. ΔΔ 5-^{13}C-Mevalonolactone. [k]Coupling constants of 1,2-^{13}C-acetate labelled I.

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