

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

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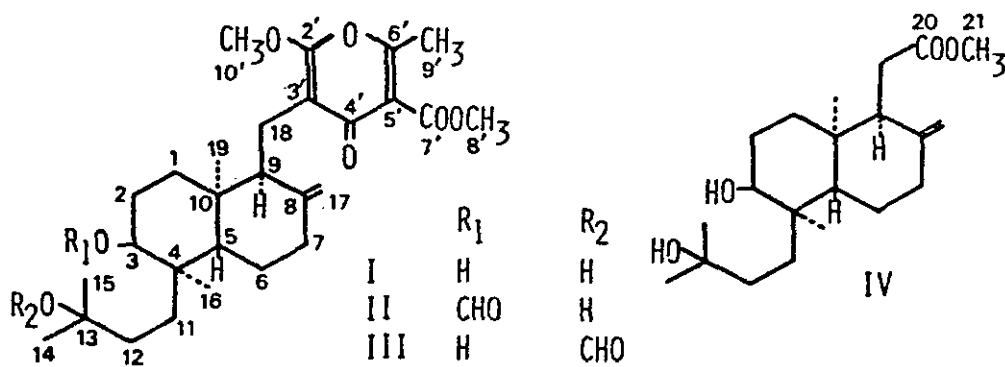
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DEDICATED TO PROFESSOR DOCTOR ADOLF BUTENANDT ON THE 75TH BIRTHDAY

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 capsii* 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 itself³ 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.



When applied to the young tobacco leaves, these compounds induced similar symptoms 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 ^1H noise decoupling spectra and multiplicities in ^1H 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.

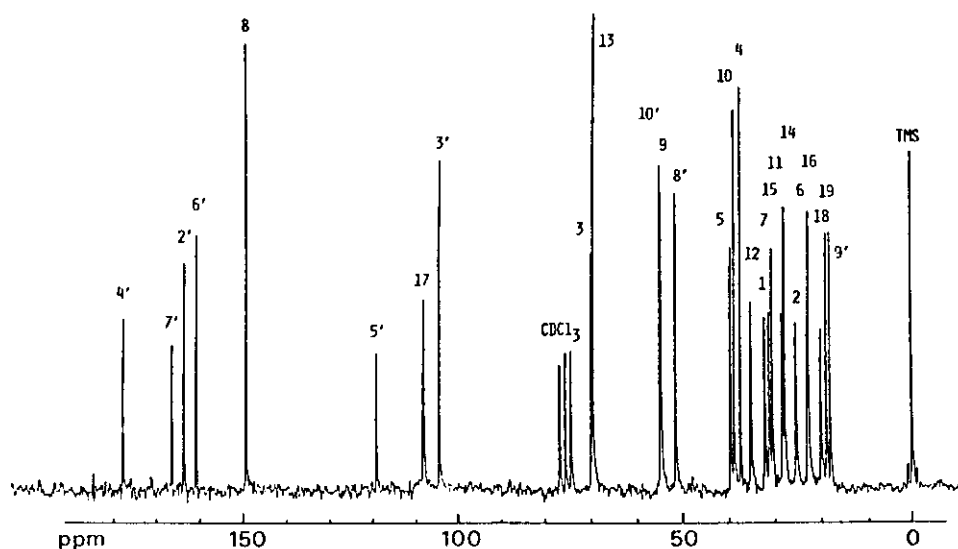


Fig. 1. ^{13}C -nmr spectrum of colletotrichin

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 quaternary 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

Though 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

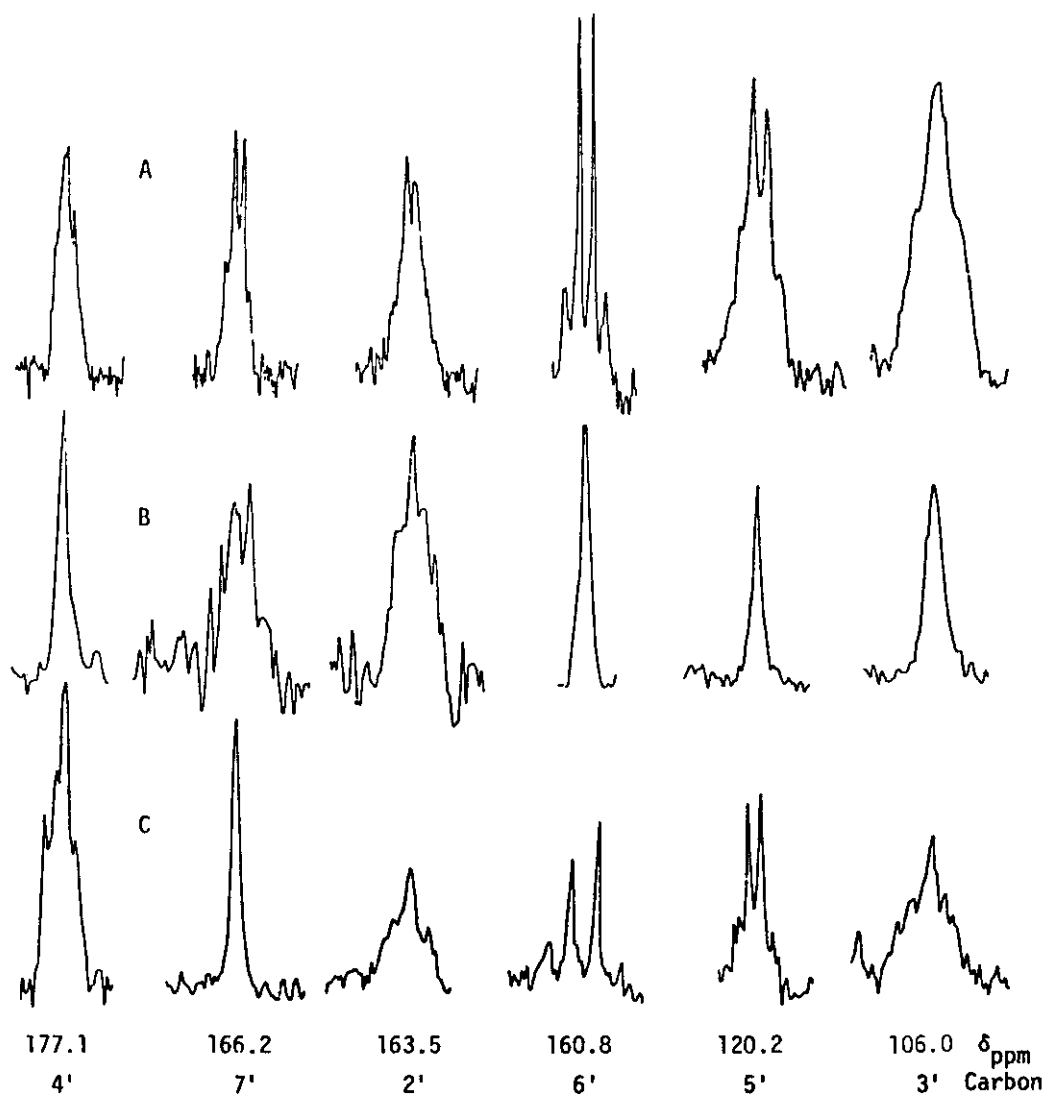


Fig. 2. ^{13}C -nmr spectra of colletotrichin

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_β elimination between these two carbons.

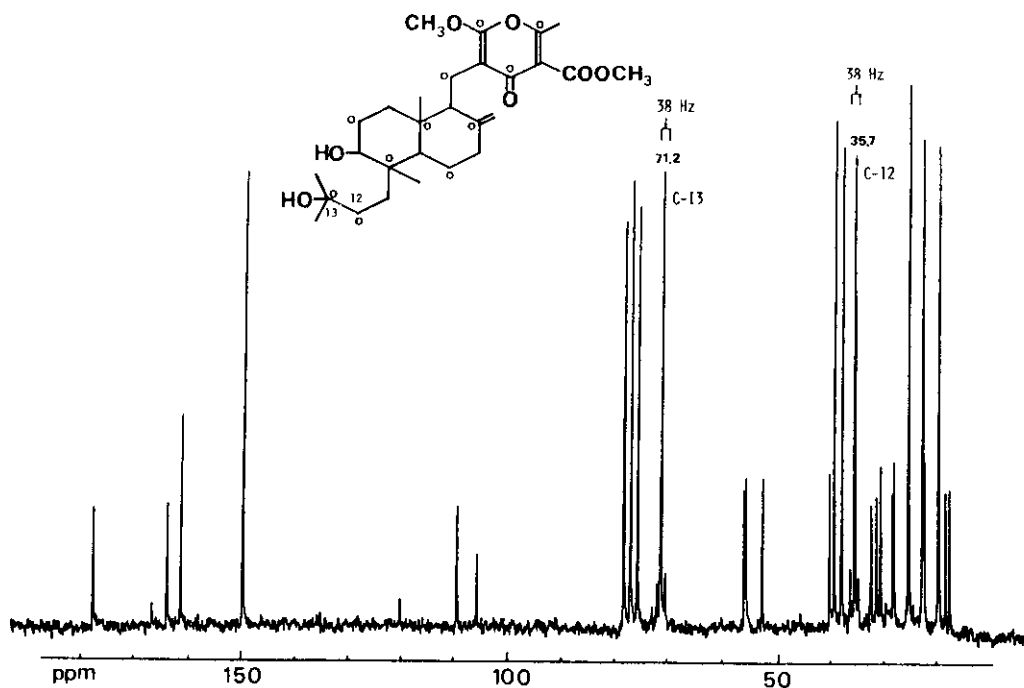


Fig. 3. ^{13}C -nmr spectrum of 1- ^{13}C -acetate enriched colletotrichin

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.

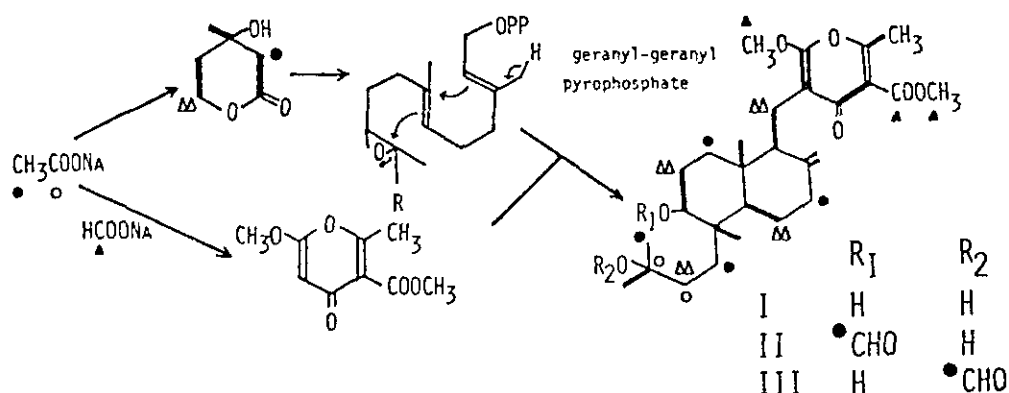
In the ^{13}C -nmr spectrum of I biosynthesized from 90 % enriched 1,2- ^{13}C -acetate that was diluted two fold with unlabelled acetate, 20 signals with ^{13}C - ^{13}C coupling were observed, indicating that 10 acetate units were incorporated. In addition to these carbons, the signals for C-1, -7, -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 cyclization)¹¹ with acetate-polyketide route.



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.

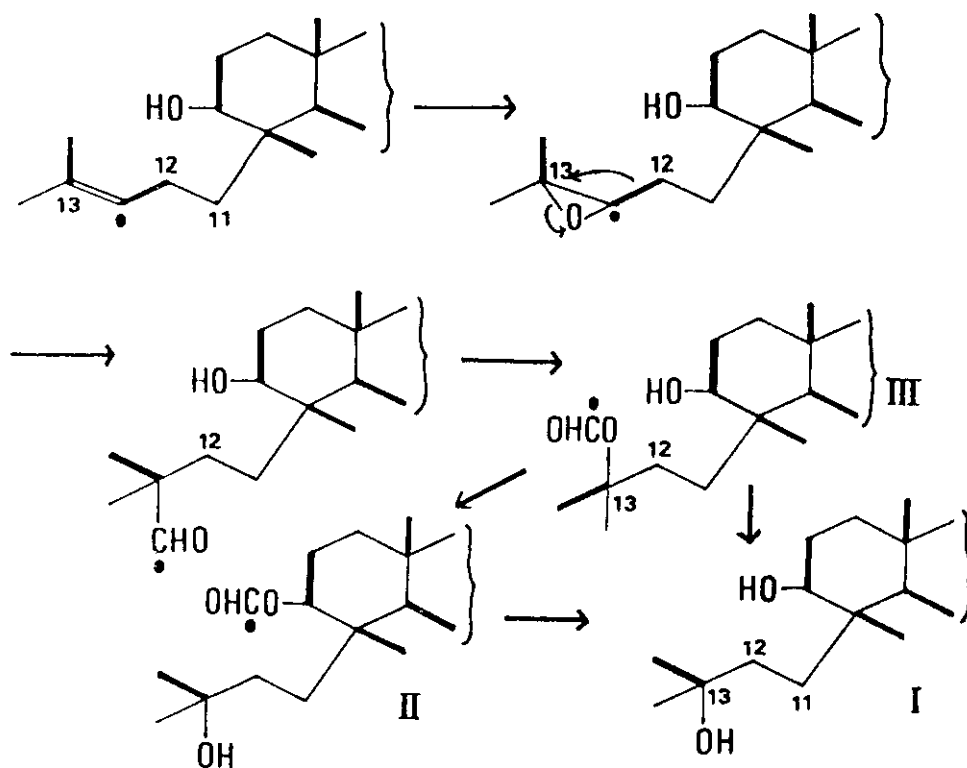


Table. Chemical Shifts and Coupling Constants of Colletotrichins and C₂₀ Acid Methyl Ester

Carbon		I	II	III	IV			$J_{13C-13C}^k$
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	○	Δ Δ	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	○	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	○	Δ Δ	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	○	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	○	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	○ ^f	Δ Δ	^h
C-13	s	71.2	70.9	84.3	71.3	○ ^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	○	Δ Δ	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	-	○	Δ	89
C-3'	s	106.0	105.4	105.5	-	● ^g	Δ	89
C-4'	s	177.1	176.6	176.4	-	○	Δ	54
C-5'	s	120.2	119.9	119.8	-	● ^g	Δ	54
C-6'	s	160.8	160.4	160.5	-	○	Δ	52
C-7'	s	166.2	165.6	165.7	-		Δ	
C-8' ^c	q	52.7	52.6	52.7	-		Δ	
C-9'	q	18.0	18.0	18.1	-	●	Δ	52
C-10' ^c	q	56.2	55.9	56.0	-		Δ	
R ₁ or R ₂	d	-	160.6	161.0	-	●	Δ	

Measured in CDCl₃ 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. Δ ¹³C-Formate. ○ 1-¹³C-Acetate. ● 2-¹³C-Acetate. Δ 1,2-¹³C-Acetate. Δ 5-¹³C-Mevalonolactone. ^k Coupling constants of 1,2-¹³C-acetate labelled I.

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