BIOSYNTHESIS OF CYCLOHEXIMIDE STEREOSPECIFIC INCORPORATION OF [1,2,3-$^{13}C_3$] MALONATE

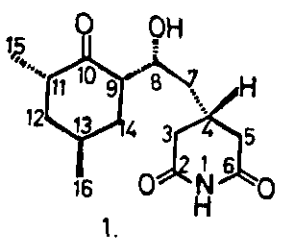
Hisao Shimada, Hiroshi Noguchi, Yoichi Iitaka and Ushio Sankawa*

Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan

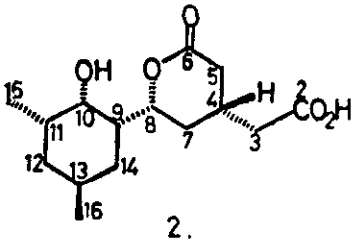
The stereochemical course of the incorporation of an intact malonate unit into the glutarimide ring of cycloheximide was investigated by using [1,2,3-$^{13}C_3$] malonate as a precursor. The labelled patterns of enriched cycloheximide and its derivatives revealed that the incorporation of an intact malonate unit was completely stereospecific. The pro-S acetate unit and methine carbons(C-4,5,6) of cycloheximide were shown to be labelled by intact [1,2,3-$^{13}C_3$] malonate.

The biosynthesis of cycloheximide(1) has been extensively investigated by Czeck workers.[1] The results of incorporation and degradation studies with ^{14}C-labelled precursors showed that the two methyl groups(c-15,16) were derived from methionine and the other carbon atoms from malonate. An interesting feature of cycloheximide biosynthesis is the intact incorporation of a malonate unit into the glutarimide ring. Cycloheximide(1) can be regarded as a unique polyketide possessing an intact malonate unit as a starter. The stereochmical course of the incorporation of an intact malonate unit was investigated by the same Czeck group. Cycloheximide(1) labelled by [^{14}C] carbon dioxide or [1-^{14}C] acetate was converted into dihydrocycloheximide acid lactone(2),[2] which was submitted to selective decarboxylation reactions to determine the radioactivities of the carboxyl and lactone carbonyl carbons. The results indicated that an intact malonate unit was incorporated into the glutarimide ring in partially stereospecific manner, and pro-S and pro-R acetate units were labelled by an intact malonate unit in the ratio of 2.2:1.[3]

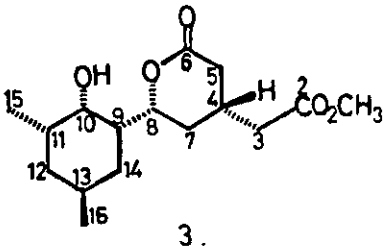
Our initial interest in cycloheximide biosynthesis was the application of [2-^{13}C,2-$^{2}H_3$] acetate to tracing the fate of acetate hydrogen in the biosynthetic methylation at C-11 and 13, and to clarify the further details of cycloheximide biosynthesis.[4] However, preliminary feeding experiment with [1-^{14}C] acetate in Streptomyces naraensis[5] revealed that acetate was a poor precursor even in pulse feeding experiments. The specific incorporation ratios of [1-^{14}C] acetate in 8 experiments under varied feeding conditions were 0.5-3.1%. In contrast, [2-^{14}C] malonate was found to be a better precursor and spcific incorporation ratios in 3 experiments with single feeding



1.



2.



3.

were 16-18%. It became clear that [^{13}C] acetate could not be used as a precursor and, therefore, we reinvestigated the stereochemical course of malonate incorporation into the glutarimide ring with [1,2,3-$^{13}C_3$] malonate. Since the labelling patterns of the two prochiral acetate units in the glutarimide ring (C-2,3 and C-5,6) were expected to be different, the stereospecificity in the incorporation of [1,2,3-$^{13}C_3$] malonate would be easily detected by ^{13}C-NMR.

Streptomyces naraensis was precultured for 2 days in 50 ml soybean powder-glucose medium in 500 ml Erlenmeyer flask on a rotary shaker (200 rpm) at 28°. [1,2,3-$^{13}C_3$] malonate (124 mg) was fed to five flasks on the second day of production culture using the same medium. The supernatant of culture broth obtained on centrifugation was extracted with chloroform, and the chloroform extract was chromatographed on silica-gel column. Cycloheximide that eluted from the column with chloroform-methanol (9:1) was recrystallized from isoamylacetate to give pure cycloheximide (1) (42.6 mg). In the ^{13}C-NMR spectrum of cycloheximide (1) enriched with [1,2,3-$^{13}C_3$] malonate all signals except for those of two methyl groups (C-15,16) showed ^{13}C-^{13}C couplings. The signals except for those of the prochiral acetate units (C-2,3 and C-5,6) were assigned with the aid of ^{13}C-^{13}C coupling constants, the natural abundance off-resonance spectrum and the spectrum of cycloheximide (1) enriched with [2-^{13}C] malonate. The signals arizing from the prochiral acetate units (C-2,3 and C-5,6) could not be assigned at this stage, because they showed the same coupling constant (J=48 Hz). However, only one of the methylenes (C-3 or C-5) of the prochiral acetate units showed couplings with both of the adjacent carbons, indicating that the incorporation of an intact malonate unit is completely stereospecific.

In order to clarify which of the prochiral acetate units is derived from an intact malonate unit, cycloheximide(1) enriched with [1,2,3-$^{13}C_3$] malonate was converted into dihydrocycloheximide acid lactone(2).[2] In order to obtain unambiguous evidence for the conformation of 2, we carried out X-ray analysis. The crystals of 2 grown from ethylacetate was found suitable for X-ray analysis. The crystal is orthorhombic, space group $P2_12_12_1$ with four molecules in a cell of dimensions, a=10.429(5), b=27.810(11), c=6.281(3)Å, D_x=1.26 g.cm^{-3}, V=1494.6Å^3. A total of 1701 reflections were recorded using a Philips PW 1100 diffractometer with CuKα radiation. The structure

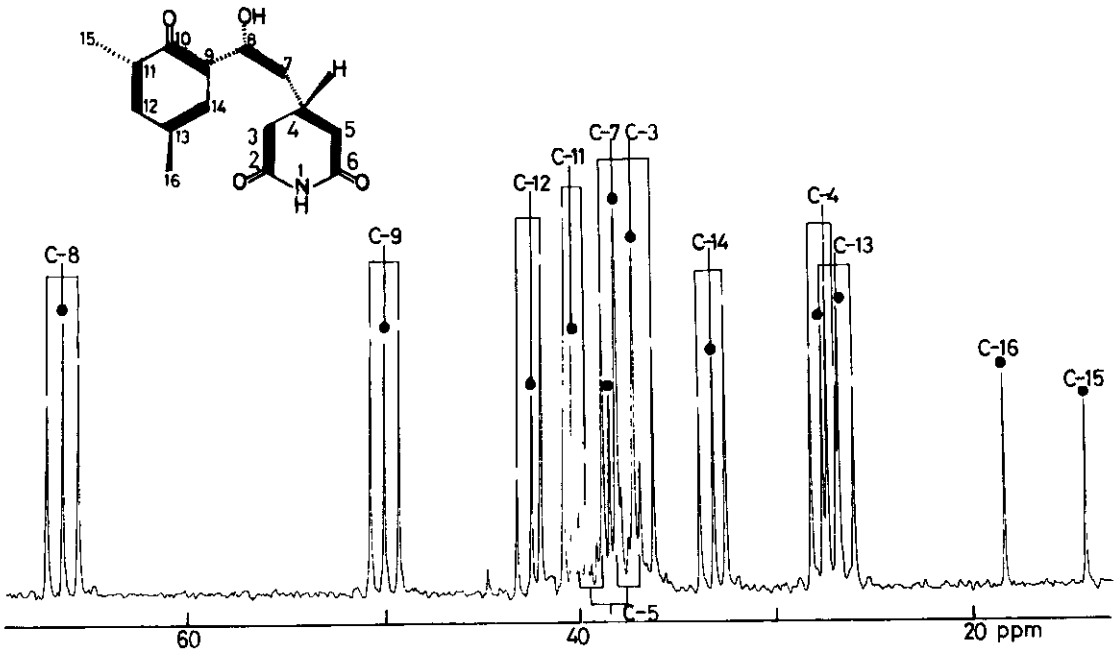


Fig. 1 ^{13}C-NMR spectrum of cycloheximide(1) enriched with [1,2,3-$^{13}C_3$] malonate[6a]

was solved by the direct method(MULTAN) and refined by the block-diagonal least-squares method (HBLS). The final R index was 0.087. Thus emerged structure(Fig. 2) shows that the dimethylcyclohexanol and acetic acid groups are both equatorial for the lactone ring, indicating that the acetic acid group(C-2,3) corresponds to the pro-R acetate unit(C-2,3) in cycloheximide(1) and the pro-S acetate unit(C-5,6) forms a part of the lactone ring.

In the ^{13}C-NMR spectrum of 2 enriched with [1,2,3-$^{13}C_3$] malonate, the ^{13}C-^{13}C coupling constants of the two pairs of carbon atoms in question(C-2,3 and C-5,6) are not identical. A methylene signal observed at 36.1 ppm(J=33,51 Hz) shows couplings with a methine signal at 28.9 ppm(J=33 Hz) and a carbonyl signal at 170.0 pppm(J=55 Hz), indicating that they are derived from an intact malonate unit. A methylene signal at 40.3 ppm(J=56 Hz) and a carbonyl signal at 172.9 ppm(J=55 Hz) are assigned to the other pair of the acetate units. This assignment is also supported by the fact that the satellite signals of carbonyl carbon at 170.0 ppm are smaller than those of the other carbonyl carbon (172.9 ppm)(Fig. 3). This is due to an extensive exchange of carboxyl carbon of malonyl CoA with non-

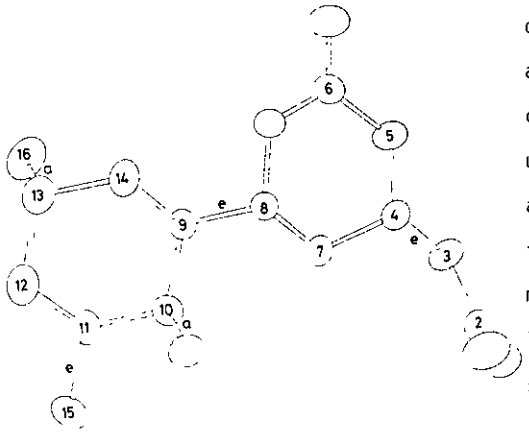


Fig. 2 ORTEP drawing of dihydrocycloheximide acid lactone(2)

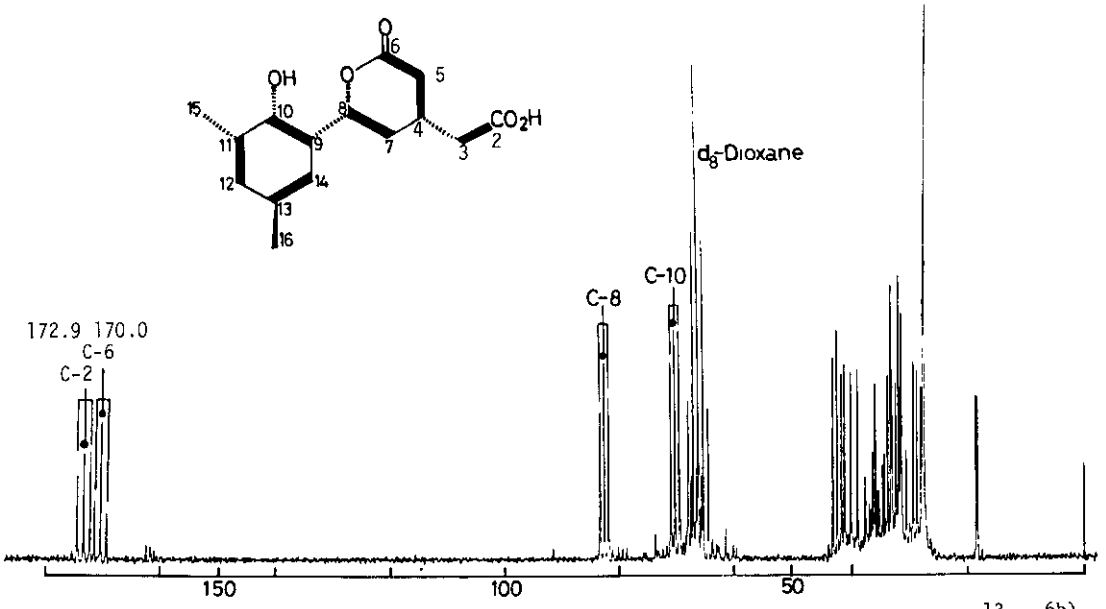


Fig. 3 ^{13}C-NMR spectrum of dihydrocycloheximide acid lactone(2) enriched with [1,2,3-$^{13}C_3$] malonate 6b)

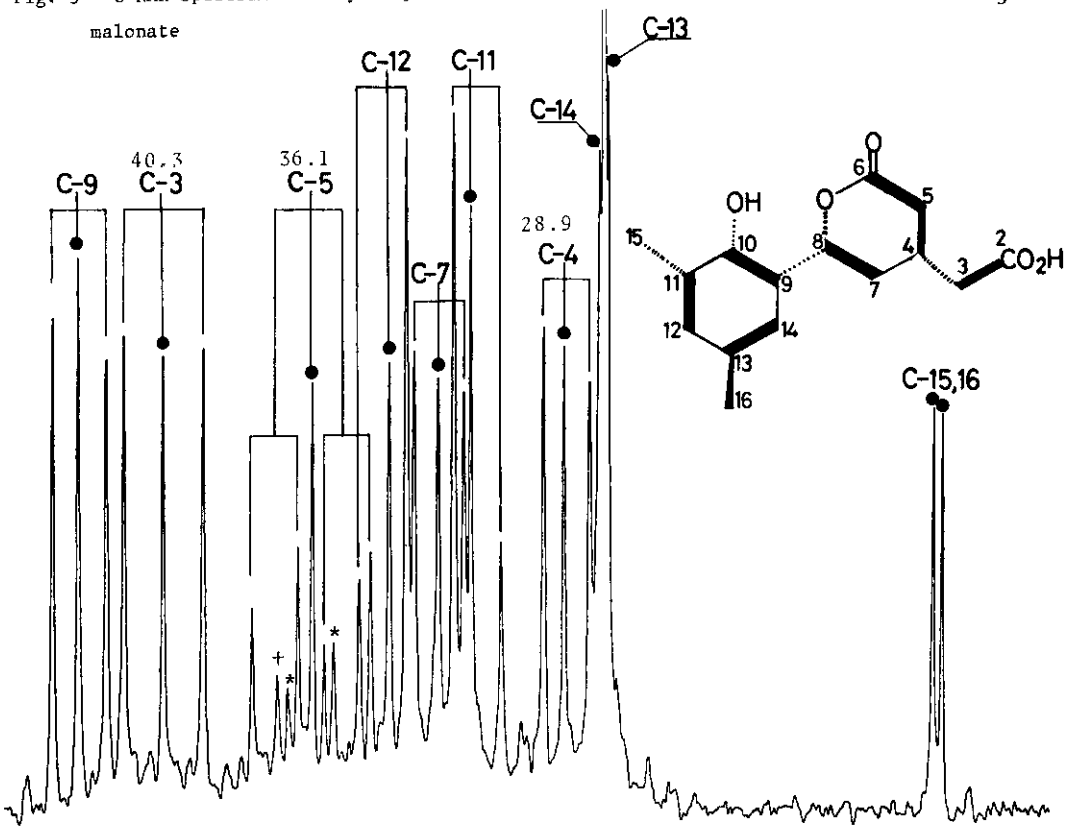


Fig. 4 Enlarged ^{13}C-NMR spectrum of dihydrocycloheximide acid lactone(2) enriched with [1,2,3-$^{13}C_3$] malonate. * Signals of C-5 only coupled with C-4. + Unidentified signal.6b)

labelled carbon dioxide through the interconversion reaction between malonyl CoA and acetyl CoA. However, the complete assignment of ^{13}C-NMR signals for the two pairs of carbon atomes(C-2,3 and C-5,6) were again not possible. 2 was methylated with diazomethane in methanol-ether to give a methyl ester(3). Two carbonyl carbon signals were observed at 170.6 and 171.5 ppm, respectively. The undecoupled natural abundance ^{13}C-NMR spectrum of 3 showed a multiplet centered at 171.5 ppm (Fig. 5a), which was collapsed into a triplet like signal by the selective irradiation of methoxyl protons(δ 3.68)(Fig. 5b). The signal at 171.5 ppm was thus assigned to the carbomethoxyl carbon (C-2) and the signal at 170.6 ppm to the lactone carbonyl carbon(C-6). The ^{13}C-NMR spectrum of enriched 3 shows two triplets centered at 170.6 ppm(J=51 Hz) and 171.5ppm(J=57 Hz). The values of the coupling constants are similar to those observed in the ^{13}C-NMR spectrum of 2 enriched with [1,2,3-$^{13}C_3$] malonate(Table 2; 52,55 Hz). Moreover, the intensities of the satellite signals of lactone carbonyl(170.6 ppm) are smaller than those of carbomethoxyl(171.5 ppm), indicating that the carbon atoms labelled by intact [1,2,3-$^{13}C_3$] malonate are present in the lactone ring. The results unambiguously demonstrates that only the pro-S acetate unit of the glutarimide ring is derived from an intact malonate unit. The complete assignments of ^{13}C-NMR spectra of 1 and 2 are shown in Table 1 and 2. The results obtained by the Czeck group which indicated partially stereospecific incorporation of an intact malonate unit were presumably caused by the lack of selectivity in the decarboxylation reactions.

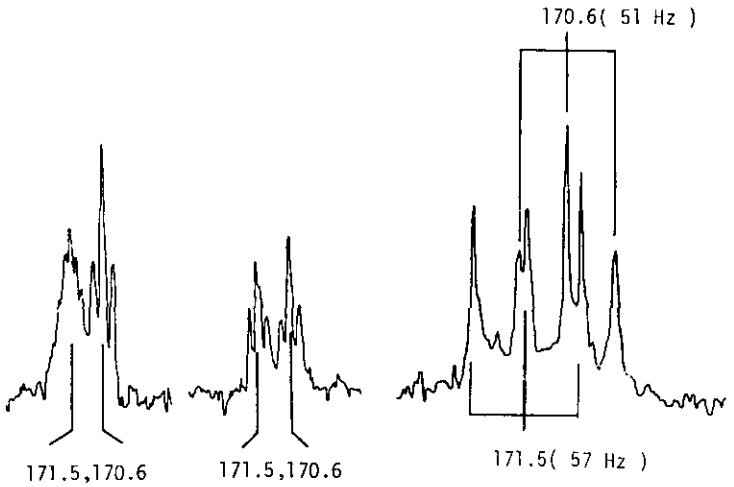


Fig.5 Signals of carbonyl carbons(C-2,5) of the methyl ester(3)
(a) Nondecoupled natural abundance signals[6c]
(b) Signals selectively irradiated at methoxyl protons[6c]
(c) Signals of enriched sample[6d]

Aknowledgement

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan. The authors thank Dr.T.Okuda of Tanabe Pharmaceutical Co. for kindly providing a strain of Streptomyces naraensis. The authors are indebted to Dr.K.Yamasaki of Hiroshima University for measuring ^{13}C-NMR. Thanks are also due to Dr.H.Seto of Institute of Applied

Microbiology, University of Tokyo for his kind advice.

Table 1. ^{13}C-chemical shifts and ^{13}C-^{13}C coupling constants of cycloheximide(1)[6a)]

Carbon	ppm *		J_{C-C}(Hz)
2	172.5(s)		48
3	37.2(t)	+	48
4	27.5(d)		32
5	38.5(t)	+	32,48
6	172.7(s)		48
7	38.1(t)	+	41
8	66.4(d)		40
9	50.1(d)	+	37
10	216.2(s)		37
11	40.5(d)	+	30
12	42.6(t)		30
13	26.7(d)	+	33
14	33.1(t)		33
15	14.2(q)		-
16	18.3(q)		-

* Multiplicity in the off-resonace decoupled spectrum

\+ Enriched with [2-^{13}C] malonate

Table 2. ^{13}C-chemical shifts and ^{13}C-^{13}C coupling constants of dihydrocycloheximide acid lactone(2)[6b)]

Carbon	ppm *	J_{C-C}(Hz)
2	172.9(s)	55
3	40.3(t)	56
4	28.9(d)	33
5	36.1(t)	33,51
6	170.0(s)	52
7	32.5(t)	35
8	82.6(d)	35
9	42.6(d)	37
10	70.5(d)	37
11	31.6(d)	33
12	33.9(t)	34
13	27.6(d)	+
14	27.9(t)	+
15	18.7(q)	-
16	18.4(q)	-

* Multiplicity in the off-resonance decoupled spectrum

\+ AB type coupling;exact values were not determined

References and Note

1) Z.Vaněk, M.Půža, J.Cudlín, M.Vodráček and R.W.Rickards, Folia Microbiol. 14 388 (1968); F.Johnson, "Fortschritte der Chemie Organischer Naturstoffe", Vol.29 , ed. by W.Herz, H.Griesebach and G.W.Kirby, Springer-Verlag, Wien, 1971, pp 140-208

2) E.C.Kornfeld, R.G.Hones and T.V.Parke, J.Amer.Chem.Soc. 71 150 (1949)

3) J.Cudlín, M.Půža, M.Vondráček, Z.Vaněk and R.W.Rickards, Folia Microbiol. 12 376 (1967)

4) U.Sankawa, H.Shimada, T.Sato and T.Kinoshita, Tetrahedron Lett. 483 (1977): U.Sankawa and H.Shimada, Tetrahedron Lett. 3375 (1978); M.J.Garson and J.Staunton, Chemical Society Review 8 539 (1979)

5) T.Okuda, K.Ashino, Y.Egawa and M.Suzuki, Chem.Pharm.Bull.(Tokyo) 6 711 (1958)

6) ^{13}C-NMR spectra were obtained using a JEOL FX-100 spectrometer at 25.2 MHz. Pulse widths of 5-7 μs with pulse intervals of 2-4.5 s and a spectral width of 6000 Hz were employed with 1800-10000 scans; a) 42 mg in 0.7 ml $CDCl_3$; b) 25 mg in 0.4 ml d_8-dioxane; c) 50 mg in 0.5 ml $CDCl_3$; d) 15 mg in 0.5 ml $CDCl_3$

Received, 17th October, 1980