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Biosynthesis of Hibarimicins

I. ^{13}C-Labeling Experiments

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Biosynthesis of hibarimicin was studied based on the feeding experiments with ^{13}C labeled acetates. All carbons in the aglycon, except for the methoxy carbons, were derived from acetate. The carbon framework of the aglycon was proved to be constructed by dimerization of an intermediate which was biosynthesized *via* the decarboxylation and skeltal rearrangement starting from an undecaketide. The rearrangement was confirmed by detecting the long range (three-bond) coupling between two carbons in the difference spectra of selective ^{13}C decoupled INADEQUATE of hibarimicin B labeled with sodium [1,2-$^{13}C_2$] acetate.

Some kinds of protein kinases and phosphatases are important target enzymes for cancer chemotherapy. In the screening program of protein tyrosine kinase (PTK) inhibitors, we found hibarimicins in the culture broth of an actinomycete *Microbispora rosea* subsp. *hibaria* TP-A0121[1,2]. Hibarimicins A, B, C, D and G specifically inhibit PTK but show little effect on protein kinases A (PKA) and C (PKC). They also show moderate inihibitory activity against Gram-positive bacteria and potent cytotoxicity against B16-F10 (murine malanoma) and HCT-116 (human colon carcinoma)[1]. Hibarimicin is structurally consisting of five or six deoxyhexoses and a common aglycon which has eight condensed rings with two *n*-propyl side chains. The aglycon is pseudo-symmetrical and the chromophore is a highly oxidized naphthylnaphthoquinone (Fig. 1).

This study was attempted to elucidate the biosynthetic pathway of hibarimicins using ^{13}C labeled precursors in order to obtain the biologically active minimum fragment from the biosynthetic intermediates.

Materials and Methods

Fermentation and Feeding Experiment

A loopful of a mature slant culture of *Microbispora rosea* subsp. *hibaria* TP-A0121 was inoculated into a 500-ml K-1 flask containing 100 ml of the seed medium consisting of soluble starch (Wako Chemical Co.) 1.0%, glucose 0.5%, NZ-case (Humco Scheffield Chemical Co.) 0.3%, yeast extract (Difco Laboratories) 0.2%, trypton (Difco Laboratories) 0.5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). The flask was incubated for 4 days at 30°C on a rotary shaker (200 rpm). One-ml aliquot of the seed culture was transferred into thirty 500-ml K-1 flask each containing 100 ml of the production medium

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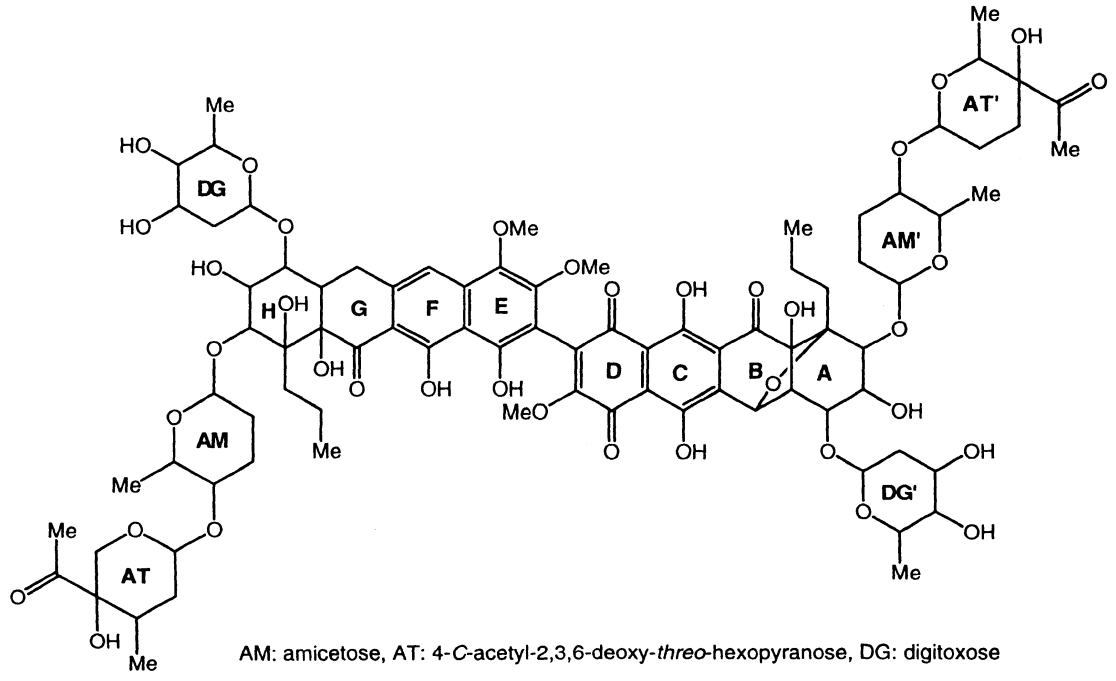
Fig. 1. Structure of hibarimicin B.



AM: amicetose, AT: 4-*C*-acetyl-2,3,6-deoxy-*threo*-hexopyranose, DG: digitoxose

consisting of D-mannose 4.0%, Pharmamedia (Trader's Protein) 2.0% and $CaCO_3$ 0.3%. The pH of the medium was adjusted to 7.0 before autoclaving. Fermentation was carried out under the same condition described for the seed culture except for the addition of labeled acetates. After 24 hours of the transfer of the seed culture, 1 ml of 10.1% solution of sodium [1-^{13}C], [2-^{13}C] or [1,2-$^{13}C_2$] acetates (99% ^{13}C; ISOTEC Inc.) were added to each flask. Fermentation was continued for 9 days after the addition of labeled precursors.

Preparation of Labeled Hibarimicins

Labeled hibarimicins were extracted with ethyl acetate (1.5 liters×2) from the cultured broth. The organic layer was concentrated to give dark red gum. The gum materials were applied to an ODS (YMC ODS-AM 120-S50) column chromatography (700×40 mm, i.d.) with the eluent of CH_3CN-0.15% potassium phosphate buffer (pH 3.5). The fractions containing hibarimicin B were pooled and concentrated *in vacuo* at 35°C until CH_3CN was completely removed. Resulting aqueous suspension was extracted with ethyl acetate (50 ml×3). The organic layer was evaporated to a thick solution and the remaining solvent was removed by N_2 stream to give red powder. Final purification of the labeled hibarimicin B was achieved by ODS silica gel TLC developed with CH_3CN-H_2O (1 : 1). The red band corresponding to hibarimicin B was scratched off and extracted with ethyl acetate. The solvent was removed by flushing N_2 to give pure labeled hibarimicin B.

NMR

^{13}C-NMR spectra were measured in $^{12}CDCl_3$ at 100 MHz and 67.8 MHz on JEOL JNM-GX400 and GX270 spectrometers, respectively. Pulse sequence of the difference spectrum of selective ^{13}C decoupled 1D-INADEQUATE was as follows: for ^{13}C: delay-$(\pi/2)_x$-1/4J-$(\pi)_x$-1/4J{sel^{13}C}-$(\pi/2)_y$-$(\pi/2)_\varphi$-acquisition-delay-$(\pi/2)_x$-1/4J-$(\pi)_x$-1/4J-$(\pi/2)_y$-$(\pi/2)_{-\varphi}$-acquisition; for ^{1}H: broad band decoupling during all the period. 1/4J was set at 35 ms (J=7.1 Hz).

Results and Discussion

Biosynthetic origin of hibarimicin was determined by stable isotope incorporation experiments. Labeled hibarimicin B was obtained by the fermentation of *Microbispora rosea* subsp. *hibaria* TP-A0121 with ^{13}C labeled acetates, solvent extraction and chromatographic purification. ^{13}C-NMR spectra of the labeled and non-

Table 1. Relative ^{13}C enrichment in hibarimicin B obtained by feeding experiment with ^{13}C-labeled acetates.

Position	δ (ppm)	^{13}C Intake [1-^{13}C]-acetate	^{13}C Intake [2-^{13}C]-acetate	J_{C-C} (Hz)	Position	δ (ppm)	^{13}C Intake [1-^{13}C]-acetate	^{13}C Intake [2-^{13}C]-acetate	J_{C-C} (Hz)
1	152.05	1.8	1.0	64	1'	187.82	1.7	1.0	55
2	107.93	0.9	1.8	70	2'	125.62	0.9	1.7	76
3	153.32	1.8	1.0	70	3'	158.40	1.8	1.1	76
4	138.56	0.9	2.0	68	4'	184.82	0.8	1.7	56
5	135.44	1.7	1.0	68	5'	116.28	1.8	1.1	56
6	111.97	1.0	2.3	66	6'	150.90	0.9	1.8	68
7	139.34	1.9	0.9	66	7'	147.98	1.8	1.0	68
8	27.79	0.9	1.5	33	8'	67.85	1.0	1.5	34
9	44.51	1.8	0.9	33	9'	55.79	1.7	1.1	34
10	76.18	1.0	1.7		10'	77.03	1.1	1.7	
11	70.75	2.1	1.0	38	11'	75.34	1.8	0.8	39
12	86.60	1.2	2.0	38	12'	85.44	0.9	1.8	39
13	79.44	2.2	1.1	40	13'	82.69	2.0	0.9	43
14	77.19	1.0	1.8		14'	85.73	1.0	1.9	
15	203.44	1.5	0.9		15'	195.54	1.6	1.0	
16	110.54	0.8	2.0	65	16'	124.84	0.9	1.7	68
17	164.34	2.0	1.0	65	17'	157.23	1.8	1.0	68
18	108.37	0.9	1.7	64	18'	112.98	0.9	1.8	55
19	37.11	1.0	1.5	40	19'	34.25	0.8	1.5	43
20	18.00	2.0	0.8	35	20'	16.58	1.5	0.7	35
21	15.12	0.9	1.7	35	21'	14.88	0.9	1.4	35
*3-OMe	60.87	1.0	1.0		*3'-OMe	60.79	1.0	1.0	
*4-OMe	61.12	1.0	1.0						
DG1	98.63	0.9	1.0		DG1'	98.50	1.1	1.0	
DG2	34.81	1.1	0.8		DG2'	35.29	1.0	0.8	
DG3	67.27	1.0	1.1		DG3'	67.12	1.0	0.9	
DG4	72.83	1.1	0.9		DG4'	72.59	1.1	1.1	
DG5	65.15	1.0	1.1		DG5'	65.00	1.0	0.9	
DG6	17.85	0.9	0.8		DG6'	17.66	0.7	0.8	
AM1	103.24	1.0	1.1		AM1'	103.24	1.0	1.1	
**AM2	30.53	1.1	0.9		**AM2'	30.38	1.0	0.8	
AM3	29.43	1.0	0.8		AM3'	29.49	1.0	0.8	
AM4	78.75	1.0	1.0		AM4'	78.88	1.0	0.9	
AM5	75.42	1.2	0.9		AM5'	75.21	1.1	1.0	
AM6	18.12	1.0	0.8		AM6'	18.09	1.0	0.8	
AT1	98.91	1.1	1.0		AT1'	98.91	1.1	1.0	
AT2	24.81	0.9	0.8		AT2'	24.81	0.9	0.8	
AT3	27.79	0.9	1.0		AT3'	27.79	0.9	1.0	
AT4	78.62	1.1	1.0		AT4'	78.62	1.1	1.0	
AT5	66.75	1.0	1.1		AT5'	66.68	1.0	1.1	
AT6	14.64	0.8	0.8		AT6'	14.64	0.8	0.8	
AT7	210.60	0.8	0.9		AT7'	210.60	0.8	0.9	
***AT8	25.01	1.0	1.0		***AT8'	25.04	1.0	1.0	

* defined as 1.0

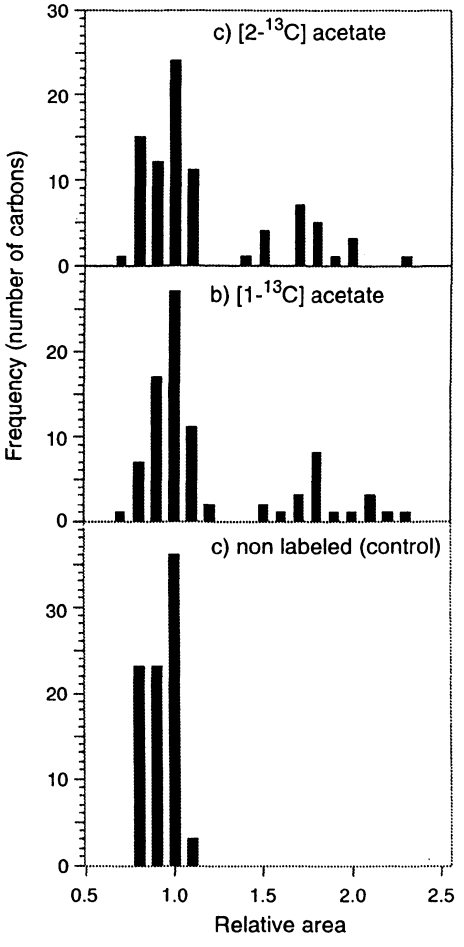
, * exchangeable

labeled hibarimicin B were recorded in $^{12}CDCl_3$ at 100 MHz at 23°C. Proton decoupling was used in gated mode to suppress NOE and the pulse interval time was set at 60 seconds. The relative peak intensities of all carbons were determined by normalizing the average area of the three methoxy carbons attached to C-3, C-3′ and C-4 as 1.0 (Table 1). Assignment of ^{13}C signals was accomplished by following our previous report[2].

The histograms for the relative signal areas of hibarimicin B labeled with [1-^{13}C] or [2-^{13}C] acetate showed two groups of peaks (Fig. 2): one of them centered around 1.0 and the other around 1.7~1.8 whereas the histogram for non-labeled hibarimicin showed one peak centered around 1.0. There was no carbon whose signal area was 1.3 in any cases. We judged that the carbon atoms whose relative intensity was larger than 1.4 were derived from the labeled precursor. For some carbons, the signal intensities could not be measured directly due to peak overlapping. Hibarimicin B possesses two 4-*C*-acetyl-2,3,6-trideoxy-*threo*-hexopyranosyl residues (abbreviated as AT and AT′) in symmetrical positions, and the chemical shifts of C-AT1~4, 6, 7 and C-AT1′~4′, 6′, 7′ are completely identical, respectively. The intensities of each overlapped pairs were 1.6~2.2, except for C-AT3 and C-AT3′, for the compounds labeled with [1-^{13}C] and [2-^{13}C] acetates. Intensity of each carbon was calculated by dividing equally since it was obvious that the both carbons were arising from the common precursor. In addition, the signal of C-AT3 and C-AT3′ was overlapped with that of C-8. The combined area intensity of these carbons was not enhanced by feeding [1-^{13}C] acetate (the sum of intensity was 2.7). Individual intensities were thus obtained by dividing the signal area equally. In case of the feeding experiment of [2-^{13}C] acetate, the corresponding combined intensity was 3.5, and we concluded that C-8 was enriched but C-AT3 and C-AT3′ were not. Thus, the intensities of C-AT3, C-AT3′ and C-8 were determined as *ca.* 1.0, 1.0 and 1.5, respectively. It was reasonable that C-8 was enriched by feeding [2-^{13}C] acetate in consideration of the following findings: (1) one-bond ^{13}C-^{13}C coupling (33 Hz) was observed between C-8 and C-9 in INADEQUATE experiment when [1,2-$^{13}C_2$] acetate was fed and the signal of C-9 was enhanced when [1-^{13}C] acetate was fed; (2) the combined intensity (C-AT3+C-AT3′+C-8) 3.5 seems to be too small for the enhancement of C-AT3 and C-AT3′ because the both carbons are biosynthetically equivalent and should be enriched at the same time.

Distribution of the enriched carbon is summarized in Fig. 3. Acetate was not incorporated into three methoxy carbons at C-3, 3′ and 4′ and the carbohydrate moieties, while all

Fig. 2. Histograms of relative abundance of ^{13}C in hibarimicin B obtained by feeding experiments.



the carbons of the aglycon framework were labeled by the acetate. Alternate alignment of enriched carbons in the aglycon moiety obtained by the feeding experiments with [1-^{13}C] and [2-^{13}C] acetates indicated that hibarimicin was biosynthesized through the polyketide pathway.

The result of the 2D-INADEQUATE experiment of hibarimicin B fed with [1,2-$^{13}C_2$] acetate demonstrates the symmetric distribution of a set of two polyketide chains and the tail-to-tail coupling between C-2 and C-2′ (Fig. 3). This symmetric carbon framework indicates that the aglycon is constructed through the oxidative dimerization of the half fragments at C-2 and C-2′. Similar dimeric compounds can be seen in fungal and plant metabolites such as xanthomegnin[3] and stipandrol[4].

Irregular distribution of the labeled carbons was observed in A, B, G and H rings. Although C-14 and C-15 were derived from the carbonyl and methyl carbons of acetate, respectively, and directly bonded each other (Fig.

Fig. 3. Incorporation pattern of ^{13}C-labeled acetate.

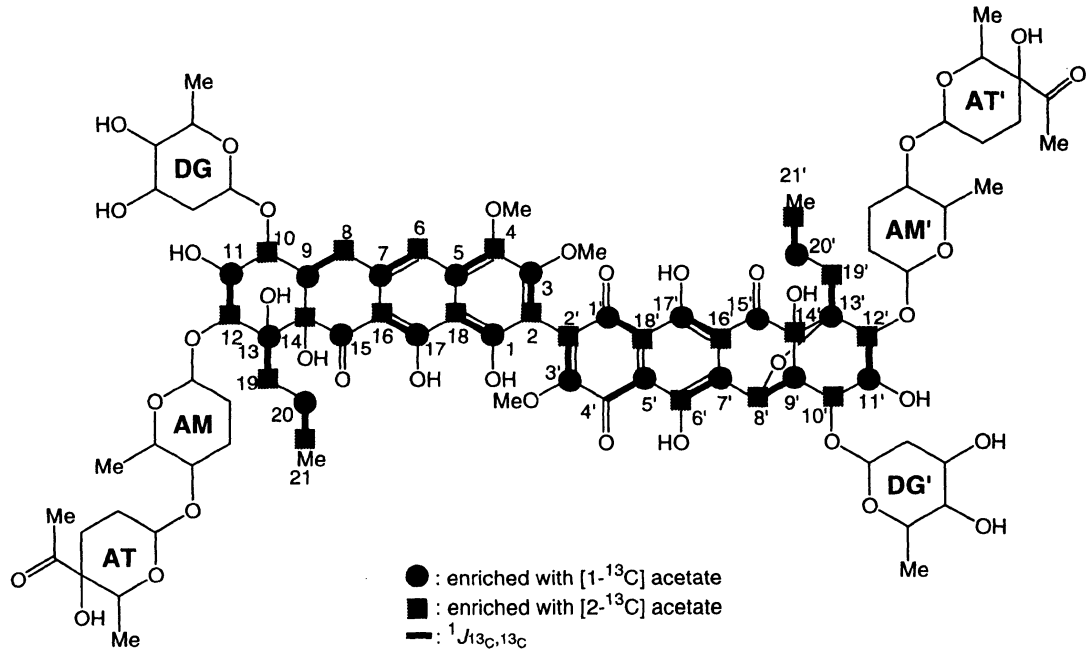


Fig. 4. Proposed biosynthetic pathway of hibarimicins.

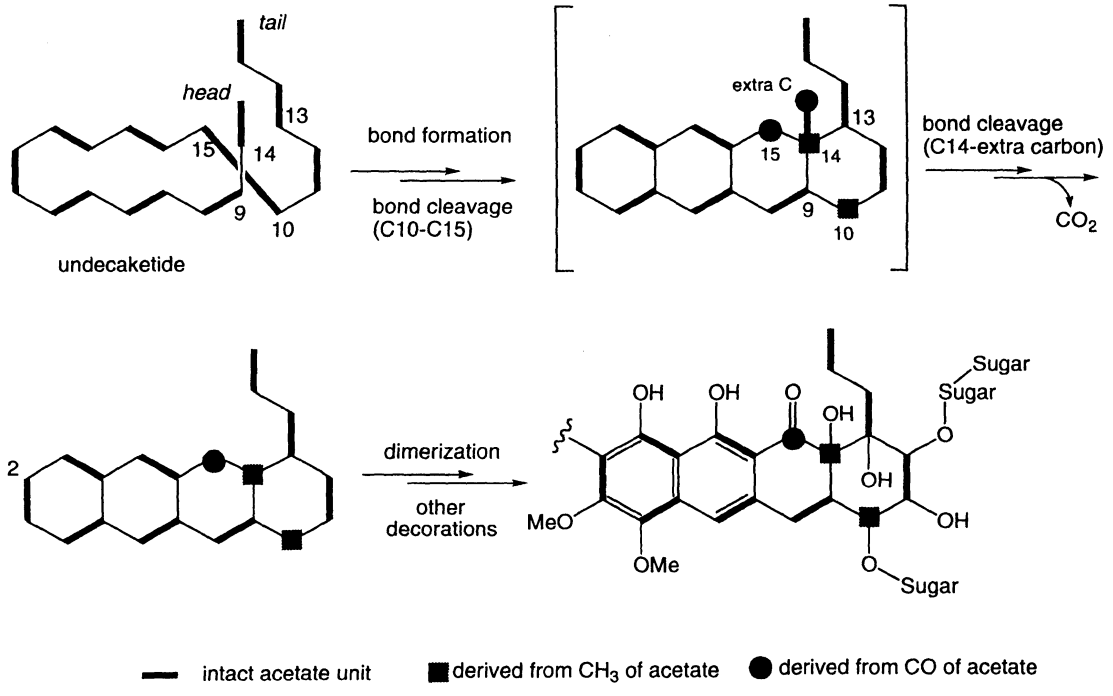
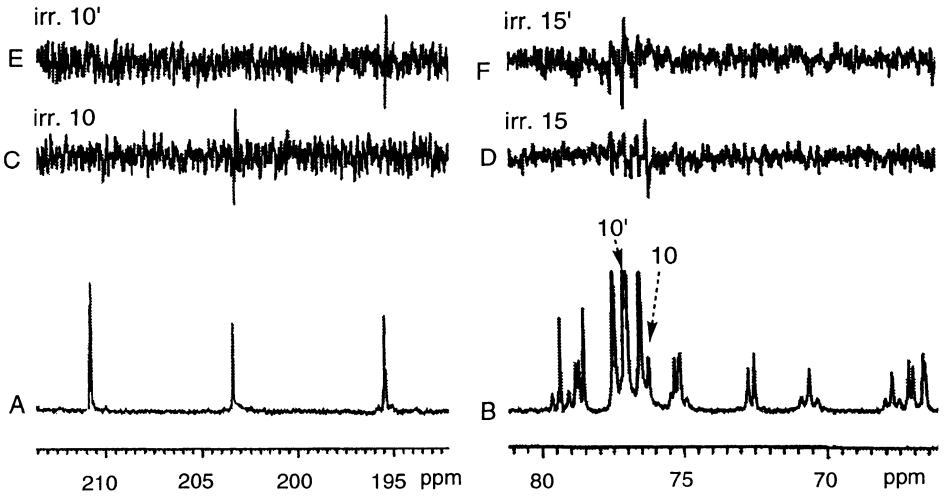


Fig. 5. Detection of three-bond ^{13}C-^{13}C couplings between C-10 (C-10′) and C-15 (C-15′).



A and B: Proton decoupled conentional ^{13}C spectra of hibarimicin B.
C~F: Difference spectra of selective ^{13}C-decoupled INADEQUATE of hibarimicin B labeled with [1,2-^{13}C] acetate.

3), the one-bond ^{13}C-^{13}C coupling was not observed between them. This was also the case of C-14′ and C-15′. In addition, C-10 and C-10′ were derived from the methyl carbon of acetate but there was no carbon directly coupled to them. To explain these observations, we hypothesized that C-10 and C-15 were derived from a single acetate molecule and an extra carboxyl carbon was originally attached to C-14. As shown in Fig. 4, it was speculated that the half fragment was biosynthesized from an undecaketide through the decarboxylation of the extra carbon and the rearrangement around C-10 and C-15: formation of C-14~C-15, C-13~C14, C-9~C-10 and C-9~C14 bonds and cleavage of original C-10~C-15 bond.

To testify this assumption, we examined the detection of three-bond couplings, $^3J_{C10,C15}$ and $^3J_{C10',C15'}$, in the compound labeled with [1,2-$^{13}C_2$] acetate. In the long-range 2D-INADEQUATE experiments, no such couplings were detected due to the peak overlapping and low sensitivity. Consequently, detection of the long-range coupling was achieved by obtaining the difference spectra of selective ^{13}C decoupled 1D-INADEQUATE (Fig. 5). This experiment was designed to detect only the carbons coupled with the irradiated carbon. C-10, C-10′, C-15 and C-15′ were selectively irradiated during the second evolution time ($1/4J$=35 ms) of the first INADEQUATE pulse sequence followed by the second INADEQUATE sequence in which the phase of the reading pulse was inverted to the first one (see Materials and Methods). In this experiment, only C-15 or C-15′ gave clear doublet signals (4 Hz) when C-10 or C-10′ were irradiated, respectively. Although the irradiation of C-15 and C-15′ gave the signals due to C-10 and C-10′, they could not be distinguished from the solvent signals. These results confirmed that C-10 and C-15 and also C-10′ and C-15′ were derived from a single acetate unit, suggesting that the half fragment was originated from an undecaketide chain.

In conclusion, the aglycon of hibarimicin was shown to be biosynthesized through the polyketide pathway. It was suggested that the carbon framework of aglycon was constructed by dimerization of the half fragments which was generated *via* the decarboxylation and skeltal rearrangement starting from an undecaketide. Further study on the biosynthetic pathway is described in the following paper[5].

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