Studies on Cochleamycins, Novel Antitumor Antibiotics

III. Biosyntheses of Cochleamycins: Incorporation of ^{13}C- and ^{2}H-Labeled Compounds into Cochleamycins

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Biosynthetic studies using ^{13}C- and ^{2}H-labeled compounds revealed that the carbon skeletons of cochleamycins A and B were derived from eight acetic acid units and one propionic acid unit with the introduction of an acetoxy group at C-10, which was replaced by an isobutyryl residue derived from valine in cochleamycins A2 and B2.

In previous papers, we reported the taxonomy of the producing organism of cochleamycins, and the production, isolation, biological activities, physico-chemical properties and structures of cochleamycins[1,2]. This paper reports the results of biosynthetic studies on these antibiotics using ^{13}C- and ^{2}H-labeled precursors.

The unique polycyclic skeletons of cochleamycins (Fig. 1) described in the previous paper[2] stimulated us to reveal their biosynthetic pathways. Since the framework of cochleamycin A (**1**) is identical with that of cochleamycin B (**2**) except for the linkage between C-4 and C-16, **2** is assumed to be formed by a reductive transannular cyclization of **1**.

Since the production of cochleamycins began between 48 and 72 hours after starting cultivation[1], labeled substrates were separately added to the producing cultures at 48 hours (100 mg/100 ml medium in a 500 ml flask) and the fermentation was continued for a further 3 days.

Incorporations of [1-^{13}C] or [2-^{13}C]acetate into **1**

Fig. 1. Total structures of cochleamycins.

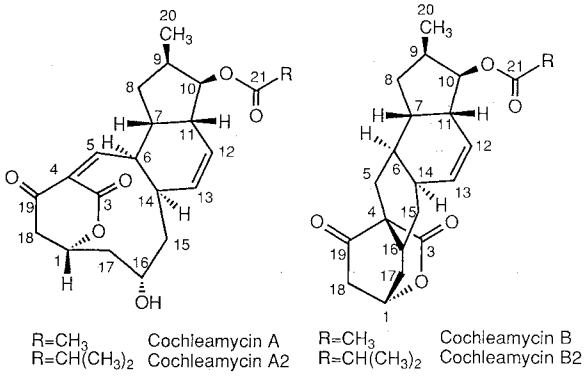
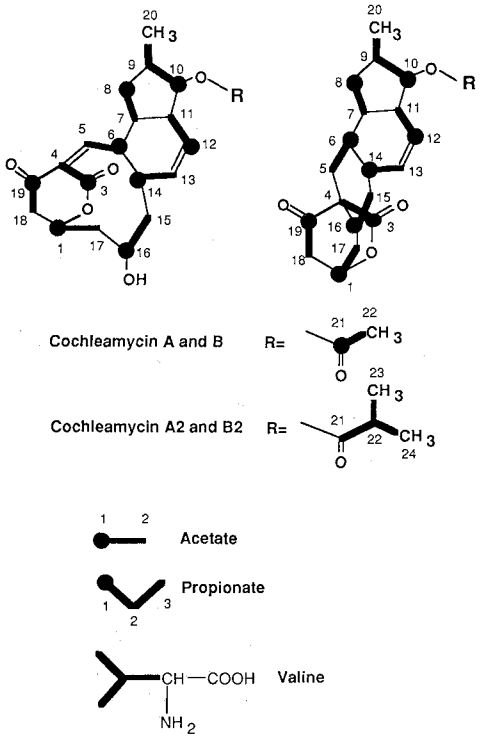


Fig. 2. Incorporation of labeled precursors into cochleamycins.



and **2** were determined from the ^{13}C NMR spectra of these compounds and the results are summarized in Table 1. The ^{13}C NMR spectra of **1** and **2** produced in the presence of [1-^{13}C] or [2-^{13}C]acetate were compared with those of unlabeled **1** and **2** by taking C-10 as a standard which was labeled by [3-^{13}C]propionic acid (*vide infra*). The peaks due to C-1, -3, -6, -8, -12, -14, -16, -19 and -21 were enhanced 3~7 folds by [1-^{13}C]-acetate, while those of C-4, -5, -7, -9, -11, -13, -15, -17, -18 and -22 were enriched 3~7 folds by [2-^{13}C]acetate in **1** and **2**. The feeding experiment using [1,2-^{13}C]acetate followed by analysis of ^{13}C-^{13}C couplings established

Table 1. ^{13}C enrichment in cochleamycins A and B obtained from feeding experiments with ^{13}C-labeled precursors.

Carbon atom	relative ^{13}C intensities[a]					Coupling constant (Hz)		Acetate unit
	[1-^{13}C]Acetate		[2-^{13}C]Acetate		[3-^{13}C]Propionate	[1,2-$^{13}C_2$]Acetate		CH_3CO_2H
	A	B	A	B	B[b]	A	B	
1	7.0	3.9	0.6	0.7	1.0	35.2	34.6	17-1
3	3.6	6.0	0.6	1.4	1.4	66.0	47.5	3-4
4	0.6	0.8	3.5	4.9	0.6	66.0	47.5	
5	0.6	0.8	3.3	4.4	1.5	39.5	34.0	5-6
6	4.1	7.6	0.8	1.2	2.0	39.5	34.0	
7	0.9	1.1	3.2	6.5	1.1	33.0	32.6	7-8
8	5.8	3.9	1.1	1.4	0.8	33.0	32.6	
9	0.9	0.6	1.8	1.2	0.8			
10	1.0	1.0	1.0	1.0	1.0			
11	0.7	0.8	3.5	7.0	1.0	39.0	39.0	11-12
12	5.1	4.9	0.8	1.3	1.0	39.0	39.0	
13	0.5	0.6	3.5	5.1	1.4	43.5	40.0	13-14
14	4.3	4.3	0.9	1.3	1.3	43.5	40.0	
15	0.8	0.6	3.4	4.6	1.4	39.0	33.8	15-16
16	3.5	10.4	0.6	1.6	1.3	39.0	33.8	
17	0.5	0.9	4.8	5.9	1.8	35.2	34.6	
18	0.7	1.2	3.4	7.2	1.8	39.6	38.0	18-19
19	3.7	4.7	0.9	1.1	1.2	39.6	38.0	
20	1.2	0.6	2.0	1.6	18.5			
21	4.3	6.8	0.9	1.3	0.7	58.5	59.5	21-22
22	0.5	0.7	3.7	3.4	2.0	58.5	59.5	

[a] Peak height ratio of ^{13}C enriched to natural abundance.
[b] In this experiment, cochleamycin A was obtained only in a trace amount preventing to take ^{13}C NMR spectra.

Table 2. ^{13}C enrichment in cochleamycins A2 and B2 obtained from feeding experiments with ^{13}C-labeled precursors.

Carbon atom	relative ^{13}C intensities[a]					Coupling constant (Hz)	Acetate unit
	[1-^{13}C]Acetate		[2-^{13}C]Acetate		[3-^{13}C]Propionate	[1,2-$^{13}C_2$]Acetate	CH_3CO_2H
	A2	B2	A2	B2	B2[b]	A2[c]	
1	7.9	6.4	1.7	1.1	1.6	35.9	17-1
3	4.1	4.4	0.7	1.4	1.2	66.2	3-4
4	0.5	0.5	2.4	2.8	0.6	66.2	
5	1.6	0.6	3.7	2.9	0.8	39.5	5-6
6	4.9	2.7	1.4	1.0	0.5	39.5	
7	1.0	0.6	2.5	2.5	0.5	33.1	
8	6.1	2.9	0.7	1.3	0.6	33.1	
9	1.1	0.9	1.7	1.2	0.8		
10	1.0	1.0	1.0	1.0	1.0		
11	0.5	0.5	2.5	3.3	0.8	39.5	11-12
12	6.8	3.8	1.0	1.2	1.3	39.5	
13	0.8	1.0	2.5	2.1	1.0	40.4	13-14
14	3.8	3.6	0.7	1.2	1.0	40.4	
15	1.0	0.8	2.7	2.9	1.1	39.5	15-16
16	11.5	3.1	1.4	0.9	1.1	39.5	
17	0.6	0.5	2.8	3.0	0.8	35.9	
18	1.0	0.5	2.7	2.3	0.9	39.5	18-19
19	5.1	5.4	0.9	1.2	0.9	39.5	
20	1.5	1.0	1.5	1.3	11.2		
21	0.9	1.1	0.7	1.4	0.6		
22	0.8	0.4	0.5	1.2	0.8		
23	0.9	1.0	1.0	1.2	0.6		
24	1.2	0.8	0.9	0.8	1.0		

[a] Peak height ratio of ^{13}C enriched to natural abundance.
[b] In this experiment, cochleamycin A2 were obtained only in a trace amount preventing to take ^{13}C NMR spectra.
[c] In this experiment, cochleamycin B2 were obtained only in a trace amount preventing to take ^{13}C NMR spectra.

the incorporation pattern of intact acetate units. Three carbons C-9, C-10 and C-20, which were not enriched by either [1-^{13}C] or [2-^{13}C]acetate, were assumed to be derived from propionic acid. The feeding experiment using [3-^{13}C]propionate followed by ^{13}C NMR studies revealed the enhancement of the signal for C-20 of **2**. The amount of **1** obtained in this experiment, however, was too poor to give useful ^{13}C NMR information. The labeling patterns of **1** and **2** are shown in Fig. 2. Thus the frameworks of **1** and **2** were unambiguously clarified to be derived from eight acetate units and one propionate unit with the introduction of an acetoxyl group at C-10.

The labeling patterns of cochleamycins A2 (**3**) and B2 (**4**) were identical with those of **1** and **2** except for the incorporation of the isobutyryl side chain (C-21 ~ C-24) at C-10 as shown in Table 2. The four carbons of this unit were not enhanced by either [^{13}C]acetate or [^{13}C]propionate suggesting that valine would be the origin of the isobutylate unit as in some metabolites[3,4]. The feeding experiment using [2,3,4,5-$^{2}H_8$]valine as seen in Fig. 3, revealed that 65% of ^{1}H was replaced by ^{2}H at the C-22, -23 and -24 positions, indicating the biosynthetic origin of these carbons was apparently valine (Fig. 2).

Fig. 3. (a) 500 MHz ^{1}H NMR spectrum of natural cochleamycin A2. (b) 76.8 MHz ^{2}H NMR spectrum of cochleamycin A2 derived from [2,3,4,5-$^{2}H_8$]D,L-valine.

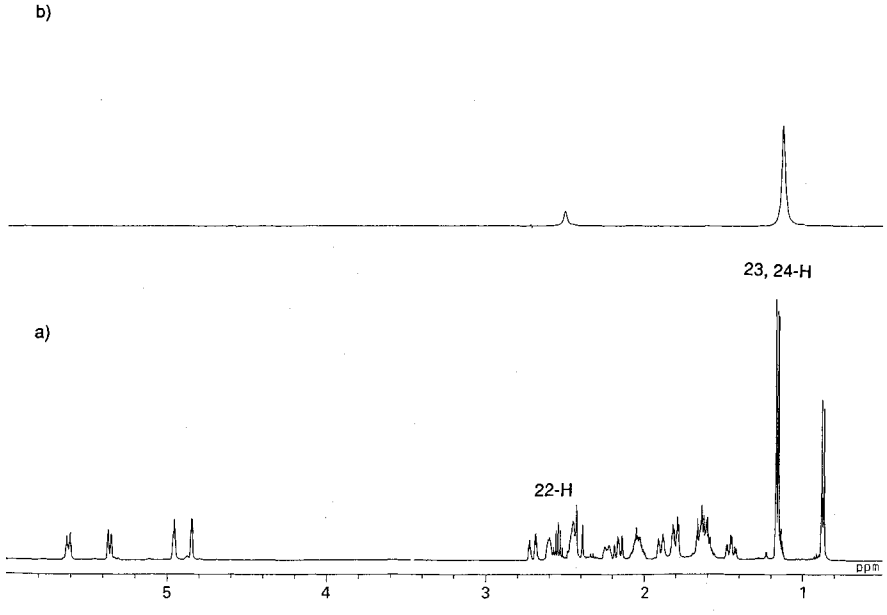


Fig. 4. A possible biosynthetic pathway for cochleamycin A and B.

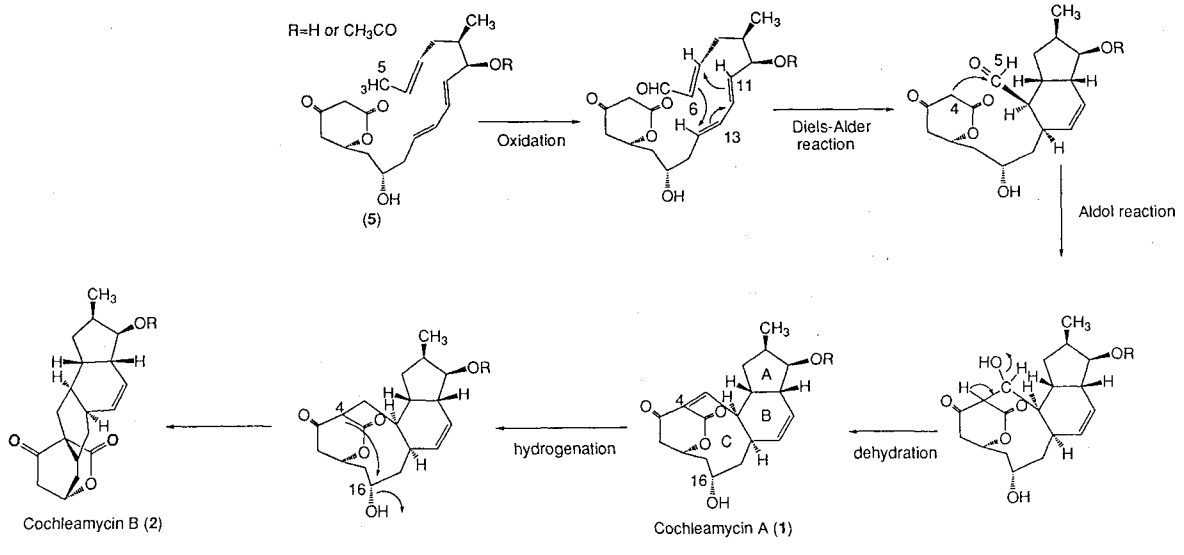
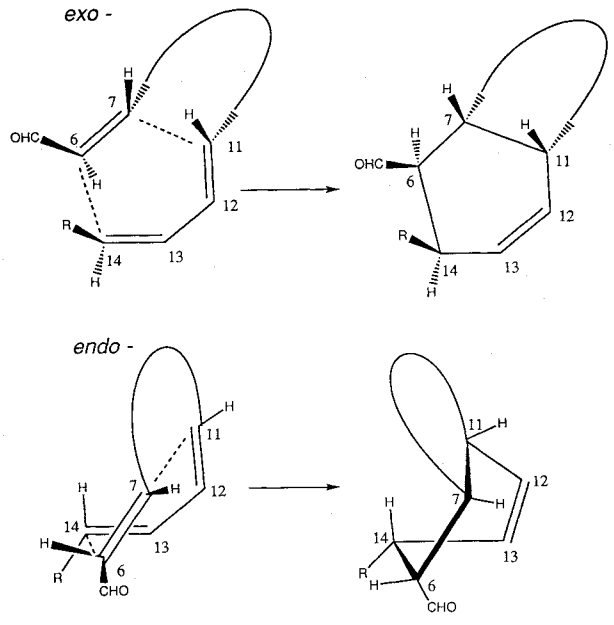


Fig. 5. Stereochemical aspects in Diels-Alder process.



The possible biosynthesic pathway of **2** *via* **1** is suggested in Fig. 4. Oxidation of the allylic methyl group[5] of a plausible intermediate **5** possessing a *cis*, *trans*-diene moiety, followed by Diels-Alder reaction[6,7] and aldol condensation may lead to the formation of **1**. Reductive transannular cyclization at the C-4 and -16 positions of **1**, accompanied by elimination of the hydroxyl group from the C-16 position, may afford **2**. Although a "stepwise" mechanism for reductive transannular cyclization of **1** is illustrated in Fig. 4, a "concerted" mechanism usually known in the biosynthesis of terpenoids[8] would be possible as well. The stereochemical aspects at AB- and BC-ring junctions may be rationalized by *endo*-addition of the *trans*-olefin at the C-6 position to the 11-*trans*, 13-*cis*-diene, or otherwise by *exo*-addition of the *trans*-olefin to the 11-*cis*, 13-*trans*-diene in the intramolecular Diels-Alder process (Fig. 5). Attempts to obtain some intermediates are in progress.

Experimental

Labeled Compounds

Sodium [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$]acetates, sodium [3-^{13}C]propionate and [2,3,4,5-$^{2}H_8$]D,L-valine were purchased from MSD Isotopes.

Incorporation Studies

The incorporation studies were conducted as follows: The strain DT136 was inoculated into 100 ml of a medium consisting of glucose 2.5%, soy-bean meal 1.5%, dry yeast 0.2% and $CaCO_3$ 0.4% in a 500-ml Erlenmeyer flask (pH 7.2), and after culturing at 27°C for 48 hours on a rotary shaker, 100 mg of a labeled compound was added to each flask, and the incubation was continued for an additional 3 days. The isolation procedure for [^{13}C] and [^{2}H]cochleamycins was essentially the same as previously reported. From one liter culture broth, cochleamycins were obtained in yields of 3~5 mg. The yields of **1** in the [3-^{13}C]propionate feeding experiment, **3** in [3-^{13}C]propionate and [2,3-^{2}H]valine feeding experiments and **4** in the [1,2-$^{13}C_2$]acetate feeding experiment were too poor to be analyzed by ^{13}C NMR studies.

References

1) SHINDO, K.; M. MATSUOKA & H. KAWAI: Studies on cochleamycins, novel antitumor antibiotics. I. Taxonomy, production, isolation and biological activities. J. Antibiotics 49: 241~243, 1996
2) SHINDO, K.; H. IIJIMA & H. KAWAI: Studies on cochleamycins, novel antitumor antibiotics. II. Physicochemical properties and structure determination. J. Antibiotics 49: 244~248, 1996
3) OMURA, S.; K. TSUZUKI, Y. TANAKA, H. SAKAKIBARA, M. AIZAWA & G. LUKACS: Valine as a precursor of *n*-butyrate unit in the biosynthesis of macrolide aglycone. J. Antibiotics 36: 614~616, 1983
4) LEHNINGER, A. L.: Biochemistry. 2nd ed., pp. 576~578, Worth Publishers, New York, 1975
5) MANNERS, G. D. & L. JURD: The hydroquinone terpenoids of *Cordia alliodora*. J. Chem. Soc., Perkin I. 405~410, 1977
6) CANE, D. E. & C. YANG: Biosynthetic origin of the carbon skeleton and oxygen atoms of nargenicin A1. J. Am. Chem. Soc. 106: 784~787, 1984
7) OIKAWA, H.; Y. MURAKAMI & A. ICHIHARA: New plausible precursors of chaetoglobosin A accumulated by treatment of *Chaetomium subaffine* with cytochrome P-450 inhibitors. Tetrahedron Lett. 32: 4533~4536, 1991
8) HEFTMAN, E.: Steroid Biochemistry, pp. 1~11, Academic Press, New york, 1970