BIOSYNTHESIS OF NAPYRADIOMYCINS

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Biosynthetic studies on napyradiomycins were carried out based on the incorporation of [2-^{13}C]acetate and [1,2-^{13}C]acetate. The alignment of acetate units suggested that the B and C rings of napyradiomycins are derived from a pentaketide, while ring A and the side chain may be synthesized from mevalonate.

The napyradiomycin (NPD) antibiotics have been isolated from the actinomycete *Chainia rubra*[1]. They have unique structures which consist of the chromophore of naphtho[2,3-*b*]pyrane-5,10-dione and three types of side chain bonded to C10a of the chromophore[2,3] (Fig. 1). Type A (NPD-A's) have a branched side chain. The side chain of type B (NPD-B's) is cyclized to form a cyclohexane ring and that of type C (NPD-C's) is cyclized between C7 and C10a of the chromophore to form a 14-membered ring.

The biosynthetic pathway of the NPD's was studied since they have unique structures. Although the side chains of the NPD's are different from each other, their common carbon structures suggest that they may be synthesized from geranyl pyrophosphate of the mevalonate pathway. It seems likely that rings B and C are synthesized from a polyketide precursor.

In this report, we give the results of a preliminary biosynthetic study on the incorporation of [2-^{13}C]acetate and [1,2-^{13}C]acetate into the NPD's.

Materials and Methods

Sodium salts of [2-^{13}C]acetic acid (93% enriched) and [1,2-^{13}C]acetic acid (90% enriched) were purchased from Commissariat à l'Énergie Atomique, France. The former was dissolved in water at a concentration of 70 mg/ml. The latter was dissolved in water at a concentration of 72 mg/ml and diluted with an equal volume of non-labeled acetate solution (72 mg/ml).

^{13}C NMR spectra were recorded at 100 MHz on a JEOL JNM-GX400 equipped with a 5-mm probe. A spectral width of 23 KHz was taken by 32 K sampling points.

Incorporation of ^{13}C-Acetate and Preparation of ^{13}C-Labeled NPD's

A seed culture of *C. rubra* MG802-AF1 was made by the method previously described[1]. Three ml of the seed culture was inoculated into each of several 500-ml Erlenmeyer flasks containing 110 ml of medium (composed of Bacto-Soytone (Difco) 1.0%, galactose 2.0%, corn steep liquor 0.5%, dextrin 2.0%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2%, silicon oil (Shin-Etsu Chemical Industry, KM-70) 0.03%, pH 7.4). ^{13}C-Labeled acetate in 0.5 ml of aqueous solution ([2-^{13}C]acetate 35 mg; [1,2-^{13}C]acetate 18 mg labeled and 18 mg non-labeled) was added to each flask at the beginning of NPD production, viz., after 41 hours cultivation, and the cultures were then incubated further for 30 hours.

^{13}C-Enriched NPD's were isolated from the cultured broth by solvent extraction, silica gel chromatography, Sephadex LH-20 gel filtration, and TLC chromatography as previously reported[1]. In the experiment on [2-^{13}C]acetate incorporation, 9.4 mg of NPD-B1 and 2.0 mg of the mixture of NPD-

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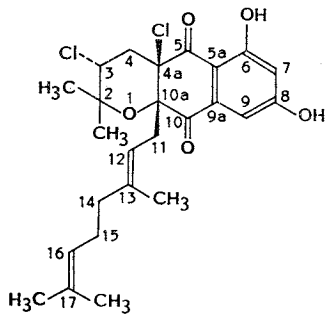
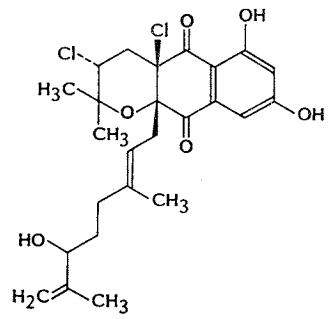
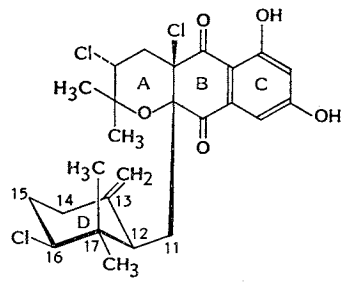
Fig. 1. Structures of napyradiomycins.
[a] Named NPD-A in previous reports[1,2].



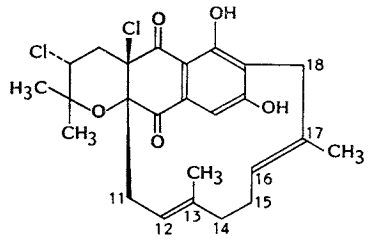
NPD-A1[a]



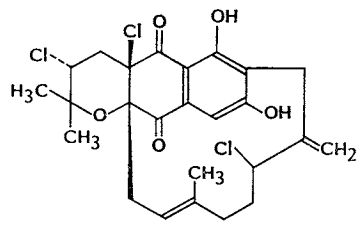
NPD-A2



NPD-B1



NPD-C1



NPD-C2

C1 and -C2 (1 : 1) were isolated from 950 ml of cultured broth. NPD-A1 was not obtained in sufficient quantity to be analyzed by ^{13}C NMR. In the experiment of [1,2-^{13}C]acetate incorporation, 1,080 ml of the cultured broth was purified and NPD-A1, -A2, -B1 and -C1 were obtained in amount of 2.0, 3.2, 14.1 and 5.6 mg, respectively.

Results and Discussion

Assignment of ^{13}C and ^{1}H NMR Signals

Assignment of the ^{13}C NMR signals of NPD's was achieved by reference to previous reports[2,3], but the identity of some signals remained uncertain. Therefore, ^{1}H-^{13}C shift correlation spectrum (^{1}H-^{13}C COSY) and long-range ^{1}H-^{13}C COSY of NPD-B1 were carried out to make certain of the assignments. Consequently, it was proved that several previous assignments should be exchanged:

C2 for C4a, C6 for C8, and C11 for C14. In addition, the signals at 1.61 ppm (br d) and 2.64 ppm (dd) were assigned to H11A and H11B and 1.99 ppm (br d) to H12. The signals at 194.0 and 193.5 ppm were confirmed by ^{13}C-^{13}C couplings to be C5 and C10, respectively.

The signals of NPD-A1, -A2 and -C1 were assigned rationally by the results of the incorporation of [1,2-^{13}C]acetate. Thus, we exchanged some previous assignments as follows: C2 for C4a (NPD-A1), C6 for C8 (NPD-A1) and C4 for C11 (NPD-C1)

Incorporation of [2-^{13}C]Acetate of NPD's

The ratios of intensity of signals on ^{13}C NMR analysis of labeled NPD's to those of non-labeled ones were calculated and are shown in Table 1. The positions of ^{13}C enrichment of NPD-B1 and -C1 are shown in Fig. 2. Those of NPD-C2 were the same as for NPD-C1. Maximal enrichment ratios were up to 5.4, 3.6 and 5.2 for NPD-B1, -C1 and -C2, respectively.

These data suggest that acetate is incorporated into rings B and C by the way of a polyketide intermediate. The labeling patterns of ring A and the side chain are consistent with biosynthesis, involving mevalonate.

Table 1. ^{13}C Chemical shifts (δ) and enrichment ratio of [2-^{13}C]acetate-labeled NPD's.

Carbon	B1		C1		C2	
	δ (ppm)	Enrichment ratio*	δ (ppm)	Enrichment ratio*	δ (ppm)	Enrichment ratio*
2	78.8	1.0	79.1	1.0	79.1	1.0
2-$CH_{3\ ax}$	22.4	2.9	22.4	1.8	22.3	1.8
2-$CH_{3\ eq}$	29.0	4.0	29.2	2.4	29.1	1.9
3	58.8	3.1	58.9	1.8	58.6	2.1
4	42.8	1.0	42.0	***	42.0	***
4a	80.9	4.4	77.8	3.6	77.7	3.6
5	194.0	0.9	194.3	0.6	195.2	1.3
5a	108.6	3.6	111.1	2.0	111.2	3.6
6	165.5	0.8	164.6	0.5	162.6	0.7
7	109.4	5.4	123.7	2.0	119.7	2.8
8	164.1	0.9	**		**	
9	108.4	5.0	107.7	2.7	107.5	2.8
9a	135.1	0.7	132.4	0.6	**	
10	193.5	2.9	195.5	1.7	195.4	5.2
10a	84.3	1.3	84.8	1.0	84.8	0.7
11	35.0	1.1	42.2	0.9	41.2	0.5
12	45.9	3.5	118.4	1.9	116.7	1.6
13	145.4	0.9	139.6	0.4	140.9	0.8
13-CH_3 or CH_2	110.2	2.8	13.5	1.3	14.5	1.9
14	35.6	3.9	39.8	***	38.1	2.4
15	34.6	1.0	23.0	0.7	39.8	***
16	70.7	3.5	122.2	1.6	64.0	1.6
17	41.8	1.1	133.2	0.4	145.4	0.6
17-CH_3 or CH_2	15.5_{ax}	3.0	18.3	1.6	116.9	2.4
17-CH_3	26.4_{eq}	4.5				
18			31.3	1.5	29.4	1.8

Each sample was dissolved in $CDCl_3$ and chemical shifts were shown with reference to $CDCl_3$ as 77.0 ppm. Spectra of NPD-C1 and -C2 were measured from their mixture (1 : 1).

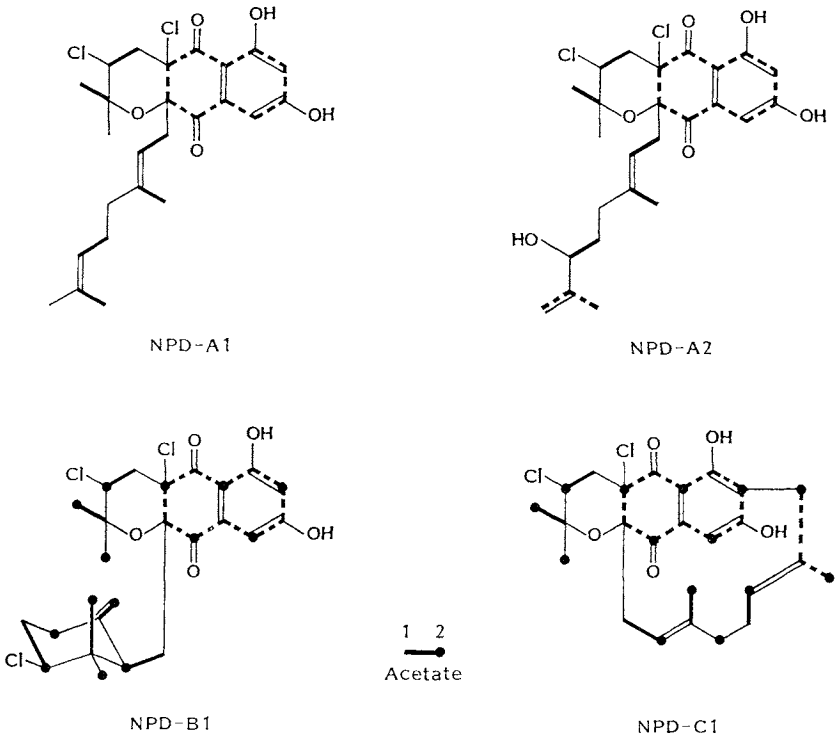
* Enrichment ratios were relative to the C2 signal as 1.0.

** Intensity was too small to calculate.

*** Signal was overlapping with others.

Fig. 2. Labeling patterns of NPD's from [2-^{13}C]- and [1,2-^{13}C]acetates.

Closed circles indicate the carbons enriched by [2-^{13}C]acetate and thick bars indicate ^{13}C-^{13}C couplings observed by the incorporation of [1,2-^{13}C]acetate. Duplicate couplings are shown by broken lines.



The enrichment of the carbons of B and C rings was higher than that of the other ones. This may be caused by the difference in how acetate is handled by the two pathways; in the polyketide pathway acetate is used directly *via* acetyl-CoA, while in the mevalonate pathway it is metabolized *via* several steps to form the precursors.

Incorporation of [1,2-^{13}C]Acetate into NPD's

^{13}C NMR chemical shifts of NPD-A1, -A2, -B1 and -C1 labeled with [1,2-^{13}C]acetate and their ^{13}C-^{13}C coupling constants (J_{CC}) are shown in Table 2. Acetate arrangements proved by J_{CC} are shown in Fig. 2. The carbons in rings B and C showed two kinds of coupling with equal signal intensities except for C7 and C9. These coupling constants are coincident with those of adjacent carbons. C7 and C9 might couple with their adjacent carbons at the same coupling constants and therefore had higher signal intensities. From these results, the biosynthesis of the B and C rings may involve two different patterns of acetate arrangement. Scytalone, a fungal metabolite, has the structure of 3,4-dihydro-3,6,8-trihydroxy-1(2*H*)-naphthalenone. Biosynthetic studies on scytalone were made by using [1,2-^{13}C]acetate and two coupling constants were observed as with NPD's[4,5]. It can be presumed that NPD's are produced like scytalone *via* a symmetric intermediate such as 1,3,6,8-tetrahydroxynaphthalene (Fig. 3).

As shown in Fig. 2, the axial methyl carbon of ring A is coupled with C2, and C3 is coupled with C4. The equatorial methyl carbon has no coupling with any other carbons, though it is derived from

Fig. 3. Postulated biosynthetic pathway of B and C rings of NPD's.

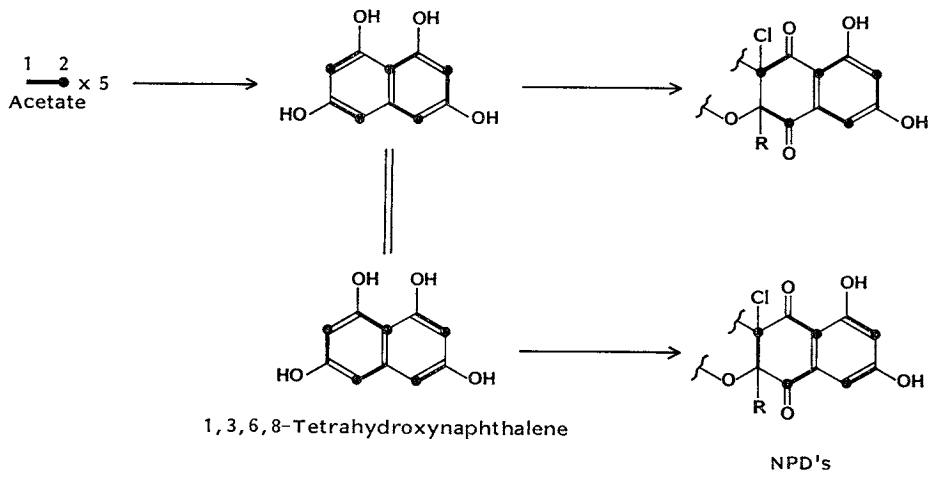


Table 2. ^{13}C Chemical shifts (δ) and J_{CC} of [1,2-^{13}C]acetate-labeled NPD's.

Carbon	A1		A2		B1		C1	
	δ (ppm)	J_{CC} (Hz)	δ (ppm)	J_{CC} (Hz)	δ (ppm)	J_{CC} (Hz)	δ (ppm)	J_{CC} (Hz)
2	78.7	39	78.9	39	78.8	39	79.1	39
2-$CH_{3\ ax}$	22.2	39	22.3	39	22.4	39	22.4	39
2-$CH_{3\ eq}$	28.8	s	28.9	s	29.0	s	29.1	s
3	58.8	37	58.7	37	58.7	37	58.9	37
4	42.7	37	42.6	37	42.7	37	42.1	37
4a	79.0	37, 42	79.2	36, 42	80.9	35, 41	77.8	37, 41
5	193.9	42, 57	193.6	41, 58	193.9	41, 58	194.1	41, 58
5a	110.4	*	109.5	58, 62	108.4	58, 62	111.1	58, 63
6	164.7	62, 70	(165.1)	76, 70	165.4	62, 70	164.5	63, 70
7	109.4	69	109.9	70	109.4	69	124.0	70
8	163.1	*	(164.6)	63, 68	164.4	65, 68	162.1	64, 70
9	107.5	65	108.2	63	108.5	65	107.8	64
9a	135.5	52, 64	134.9	48, 64	135.0	49, 65	132.2	51, 64
10	195.6	42, 51	195.6	41, 48	193.6	41, 49	195.7	40, 51
10a	83.5	36, 41	84.1	37, 41	84.3	35, 41	84.8	37, 40
11	41.2	44	40.2	45	35.0	37	42.2	43
12	114.9	44	116.3	45	45.9	38	118.3	44
13	142.8	42	141.5	42	145.3	75	139.6	41
13-CH_3 or CH_2	16.4	42	16.0	42	110.2	75	13.5	41
14	39.7	s	35.8	s	35.6	s	39.8	s
15	25.9	43	31.9	37	34.5	35	23.0	44
16	123.7	44	75.2	38	70.7	35	122.0	43
17	131.7	42	146.6	42, 72	41.8	38	133.4	38, 44
17-CH_3	17.5 *cis*	42	17.7	42	15.4_{ax}	38	18.3	44
17-CH_3 or CH_2	25.6 *trans*	s	111.6	72	26.4_{eq}	s		
18							31.3	38

Each sample was dissolved in $CDCl_3$ and chemical shifts were shown with reference to $CDCl_3$ as 77.0 ppm.

s: Signal was singlet, so the carbon had no coupling with others.

* Intensity was too small to calculate.

the C2 of acetate as described above. Therefore the unit $(CH_3)_2$C2-C3-C4 may be derived from mevalonate.

As for the side chains, C11-C12, C13-C13-CH_3, C15-C16, and C17-C17-CH_3 are coupled with each other. C14, the *trans*-methyl carbon connected to C17 of NPD-A1, and the equatorial methyl carbon connected to C17 of NPD-B1 has no coupling. But C17 of NPD-A2 has two coupling constants and is coupled with both C17-CH_3 and C17-CH_2. Also C17 of NPD-C1 has two coupling constants coincident with C17-CH_3 and C18. The acetate pattern in the side chain as shown in Fig. 2 is consistent with an origin of two isoprene units. Moreover geranylpyrophosphate is a possibility for the precursor of the side chain of NPD-A1. The side chain of NPD-B1 seems to be synthesized by halogenation and cyclization of NPD-A1. Two coupling constants of C17 seen for NPD-C1 can be interpreted to indicate that the dimethyl carbons of C17 have an equal chance to be linked to C7 of ring A. NPD-A2 also has a similar double coupling constant suggesting that A2 may be synthesized from NPD-A1. Therefore, NPD-A1 may be a precursor of NPD-A2, -B and -C. Recently, we found that NPD-A1 was converted to NPD-B1 and -B3 by a bromoperoxidase. Properties of this enzyme and the reaction mechanism will be reported in detail elsewhere.

Consequently, we suggest that the NPD's are synthesized biologically from five acetate and three mevalonate units. A few isoprenoid antibiotics produced by actinomycetes, such as helvolic acid[6] from *Streptomyces reticuli*, pentalenolactone[7] from *Streptomyces* sp., and terpentecin[8] from *Kitasatosporia griseola*, are known. If labeled mevalonate were incorporated into the NPD's, the pathway would be defined with greater certainty. Unfortunately all attempts to incorporate [5-^{13}C]mevalonate have failed, perhaps because of a lack of permeability of exogenous mevalonate or because of other unknown factors. Interestingly, producers of pentalenolactone also could not incorporate mevalonate[7].

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