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BIOSYNTHETIC STUDY AND RADIOISOTOPE LABELING OF INOSTAMYCIN

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ABSTRACT.—To obtain radioactively labeled inostamycin, the biosynthesis of inostamycin was studied by feeding experiments with ^{13}C-labeled precursors. From the ^{13}C-nmr spectrum of inostamycin enriched from incorporation of [1-^{13}C]propionate, [1-^{13}C]butyrate, and [1-^{13}C]acetate, it was shown to be derived from six propionate and five butyrate units. Radioactively labeled inostamycin was prepared biosynthetically with [1-^{14}C]propionate. As a result, [^{14}C]inostamycin of 2.97 mCi/mmol was prepared. Using this labeled inostamycin, its accumulation in NRK cells was examined.

Inostamycin is a novel polyether compound isolated from the culture broth of *Streptomyces* sp. MH816-AF15 as an inhibitor of CDP-DG:inositol transferase (1,2). It inhibited phosphatidylinositol turnover *in situ* and induced cellular morphological change (3,4). Also, inostamycin circumvented multidrug resistance in cancer cells, and its effect was long lasting (5,6). Therefore, radio-

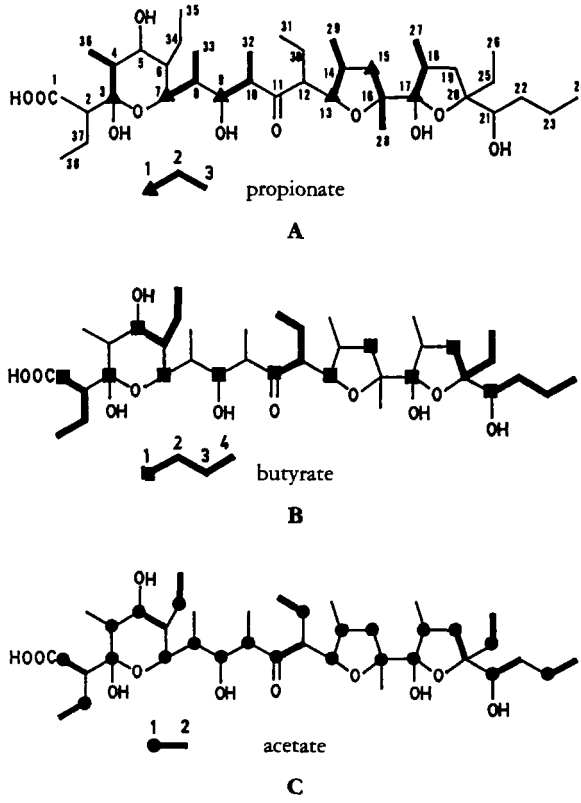

FIGURE 1. Incorporation of carbon-13 to inostamycin. Inostamycin was labeled with [1-^{13}C]propionate (A), [1-^{13}C]butyrate (B), or [1-^{13}C]acetate (C). ^{13}C-Incorporated positions are marked from assigned signals in each ^{13}C-nmr spectrum.

TABLE 1. Incorporation of [1-^{13}C]propionate, [1-^{13}C]butyrate, and [1-^{13}C]acetate into Inostamycin.

Carbon	^{13}C Chemical shift ppm[a]	Mult.[b]	Enrichment ratio[c]		
			[1-^{13}C]propionate	[1-^{13}C]butyrate	[1-^{13}C]acetate
1	181.3	s	1.4	26.1	6.2
2	56.0	d	—[d]	—	0.6
3	100.7	s	17.3	13.6	2.4
4	38.2	d	—	1.5	2.6
5	71.1	d	0.5	21.2	4.6
6	37.5	d	—	—	—
7	74.8	d	22.1	19.9	3.2
8	32.3	d	—	1.1	2.2
9	76.6	d	19.9	12.7	1.8
10	47.4	d	—	1.0	2.1
11	215.1	s	—	18.6	4.1
12	55.1	d	—	—	0.6
13	83.7	d	24.8	18.2	2.9
14	34.8	d	0.3	0.7	2.2
15	42.7	t	24.3	21.7	3.4
16	86.3	s	—	1.1	1.7
17	108.3	s	20.3	15.7	2.5
18	38.4	d	—	1.1	2.0
19	37.5	t	—	19.3	4.0
20	87.3	s	0.9	—	0.5
21	70.0	d	0.7	25.0	5.3
22	34.8	t	0.3	0.7	—
23	20.3	t	0.8	2.4	7.6
24	14.4	q	1.0	1.0	1.0
25	31.1	t	—	1.6	4.1
26	7.2	q	—	—	0.9
27	14.7	q	0.9	0.8	1.1
28	24.0	q	0.7	0.7	0.7
29	15.6	q	0.6	1.1	0.8
30	15.0	t	—	2.1	7.0
31	12.5	q	1.0	0.9	1.0
32	12.9	q	0.7	1.0	0.8
33	5.3	q	—	1.2	1.0
34	18.4	t	0.8	2.0	6.5
35	10.8	q	0.6	1.0	0.8
36	13.2	q	1.1	1.0	1.0
37	20.1	t	0.7	1.8	6.4
38	12.4	q	0.7	—	0.8

[a]Chemical shifts are shown with reference to $CDCl_3$ as 77.0 ppm.
[b]Multiplicities determined from DEPT spectrum.
[c]Enrichment ratio was relative to the C-24 signal as 1.0.
[d]Signals were hidden in the noise.

actively labeled inostamycin should be useful for studying the mechanism of its action.

The biosynthesis of polyether compounds such as monensin (7), lasalocid A (8), and narasin (4-methylsalinomycin) (9) has been reported. Lasalocid A was first shown to incorporate butyrate into the ethyl groups of the antibiotic. Lysocellin, having a similar structure to that of inostamycin, was shown to be derived from one acetate, two butyrate, and eight propionate units (10). As inostamycin is also a polyether compound, it would be expected to be synthesized via the polyketide pathway. In the present report, we describe the biosynthesis of inostamycin and the biosynthetic prepara-

tion of radioactively labeled inostamycin.

The feeding experiment with sodium [1-^{13}C]propionate resulted in enhancement of six carbon signals in the ^{13}C-nmr spectrum at C-3, -7, -9, -13, -15, and -17 (Figure 1A, Table 1). In the case of sodium [1-^{13}C]butyrate, 11 carbon signals were enhanced at C-1, -3, -5, -7, -9, -11, -13, -15, -17, -19, and -21 (Figure 1B, Table 1). Among these 11 carbons, six of them (C-3, -7, -9, -13, -15, -17) gave enhanced ^{13}C-nmr signals similar to those of the propionate (C-1)-incorporated molecule. Many carbon signals were enhanced by feeding sodium [1-^{13}C]acetate, and their enrichment ratios were lower than those of propionate and butyrate (Figure 1C, Table 1). Among them ten carbon signals (C-1, -5, -11, -19, -21, -23, -25, -30, -34, -37), whose enrichment ratios were higher than the mean ratios of enriched signals (3.8), could be assigned to five pairs of head-to-tail condensed acetates. The other enhanced 12 carbon signals (C-3, -4, -7, -8, -9, -10, -13, -14, -15, -16, -17, -18) could be divided into six pairs of an unusually fashioned condensation, in which C-1 of acetate was next to another C-1 of acetate. Although it was originally suggested that butyrate might be converted to propionate via succinyl-CoA and methylmalonyl-CoA (10,11), subsequent studies proposed that butyrate could undergo a rearrangement to isobutyryl-CoA and would be converted to propionate via methylmalonyl-CoA (12,14). Our results would support this latter pathway.

Therefore, we concluded that inostamycin is biosynthesized through condensation of six propionate and five butyrate units (Figure 2).

Since propionate was efficiently incorporated, sodium [1-^{14}C]propionate was used for radioisotope labeling. As a result, [^{14}C]inostamycin with high specific radioactivity (2.97 mCi/mmol, 1.9 mg) was obtained from the supernatant of the culture broth (400 ml). The labeled [^{14}C]inostamycin (2.43 mCi/mmol, 1.1 mg) was also obtained from the precipitate fraction.

The radioactively labeled inostamycin was shown to be stable in DMEM supplemented with 5% calf serum for 24 h (data not shown). For the cellular use of inostamycin, its accumulation in NRK cells was examined. At 1 μg/ml of [^{14}C]inostamycin, the accumulation increased rapidly within 30 min, and then reached the steady-state level (Figure 3A). Furthermore, the accumulation was dose-dependent at 0.5–2 μg/ml of [^{14}C] inostamycin (Figure 3B).

Thus, elucidation of its biosynthetic components led to the preparation of highly radioactive inostamycin. Using this labeled inostamycin, we are now studying the interaction between inostamycin and its cellular target.

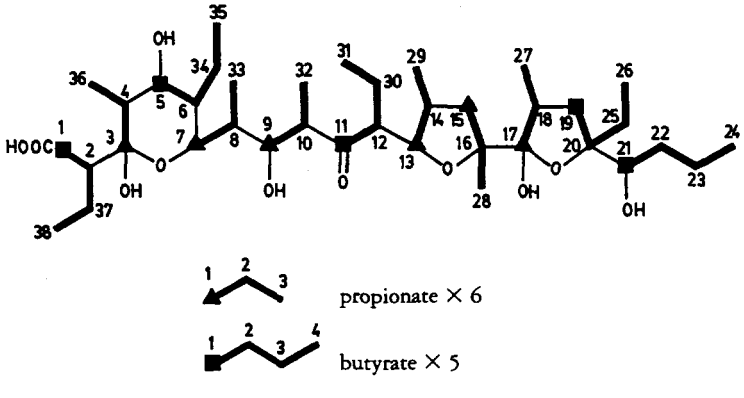

FIGURE 2. Proposed component units of inostamycin.

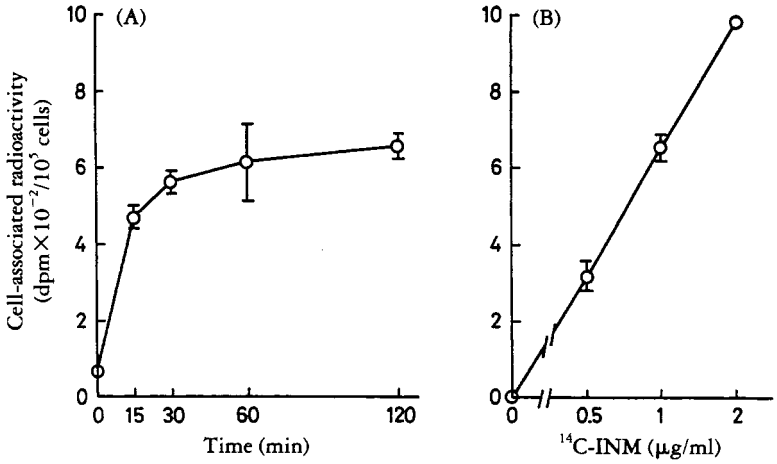

FIGURE 3. Accumulation of inostamycin in NRK cells. NRK cells were incubated with 1 μg/ml of [^{14}C]inostamycin at 37° for the indicated periods (A). The cells were incubated with the indicated concentrations of [^{14}C]inostamycin at 37° for 2 h (B). Values are means of triplicate determinations.

EXPERIMENTAL

LABELED COMPOUNDS.—Sodium [1-^{14}C]propionate (51.0 mCi/mmol) was purchased from New England Nuclear, Boston, MA. Sodium [1-^{13}C]acetate (99% ^{13}C enriched), sodium [1-^{13}C]propionate (99%), and sodium [1-^{13}C]butyrate (99%) were obtained from Aldrich Chemical Co.

FERMENTATION.—*Streptomyces* sp. MH816-AF15 (deposited with the Fermentation Research Institute of the Industrial Science and Technology, Tsukuba, Japan, under the collection number FERM P-10398) was inoculated into 100 ml of seed medium containing 2.0% galactose, 2.0% dextrin, 1.0% Bacto Soytone, 0.5% corn steep liquor, 0.2% $(NH_4)_2SO_4$, and 0.2% $CaCO_3$ (pH 7.4) in a 500-ml Erlenmeyer flask and cultured at 27° for 6 days on a rotary shaker (180 rpm). Three ml of this seed culture were transferred to 100 ml of a production medium containing 2.0% glycerol, 2.0% dextrin, 1.0% Bacto Soytone, 0.3% yeast extract, 0.2% $(NH_4)_2SO_4$, and 0.2% $CaCO_3$ in a 500-ml Erlenmeyer flask and cultured at 27° for 5 days on a rotary shaker (180 rpm). For feeding experiments with ^{13}C- and ^{14}C-labeled compounds, the medium was replaced with synthetic medium consisting of 1.0% glycerol, 1.0% dextrin, 0.2% $(NH_4)_2SO_4$, and 0.2% $CaCO_3$ alone. Each labeled precursor was added in the sterile condition at 3 days after the inoculation, and the cultures were incubated further for 3 days.

PREPARATION OF ^{13}C- AND ^{14}C-LABELED INOSTAMYCIN.—Sodium [1-^{13}C]acetate, sodium [1-^{13}C]propionate, or sodium [1-^{13}C]butyrate was given to each flask at concentration of 0.15 mg/ml, whereas sodium [1-^{14}C]propionate was given at 0.5 μCi/ml. The culture broth was centrifuged at 10,000 rpm for 10 min, and the precipitate was extracted with Me_2CO. The Me_2CO extract was concentrated *in vacuo* and further extracted with EtOAc. The supernatant was extracted with EtOAc and combined with the EtOAc extract from the precipitate, then concentrated *in vacuo*. The dried extract was dissolved in a small amount of $CHCl_3$, applied to a Si gel column, and eluted with $CHCl_3$-MeOH (100:0-100:3). The eluate containing inostamycin was dried and washed with MeCN. The MeCN-insoluble precipitate was dissolved in $CHCl_3$ and evaporated to give a powder of inostamycin. From 500 ml of the culture broth 4.5–12.0 mg of ^{13}C-labeled inostamycin was obtained. The ^{13}C-nmr spectra were taken in $CDCl_3$ on a JEOL JNM-GSX270 NMR spectrometer at 67.5 MHz. ^{14}C-Labeled inostamycin was purified separately from the supernatant and the precipitate of the culture broth.

ACCUMULATION OF INOSTAMYCIN.—NRK cells were plated at 1×10^5 cells/well in 12-well plates (Costar) in 1 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum (Gibco), and incubated at 37° overnight. The cells were washed with Dulbecco's phosphate-buffered saline (PBS) and incubated in serum-free DMEM (50 mM Hepes: pH 7.5) with [^{14}C]inostamycin at 37°. After incubation, the cells were washed with ice-cold PBS, and trypsinized. Then, the cell-associated radioactivity was counted in 3 ml of Atomlight (DuPont).

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