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283. The Use of ^{13}C–NMR. Spectroscopy in Biosynthetic Studies. II [1]. Biosynthesis of Narasin, a New Polyether Ionophore[1] from Fermentation of *Streptomyces aureofaciens* [2]

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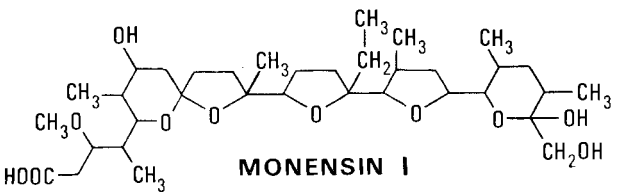
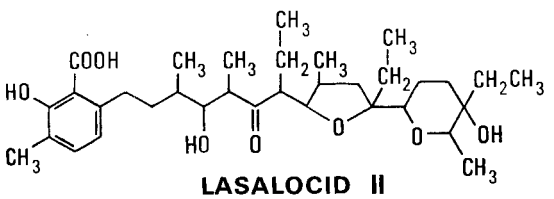
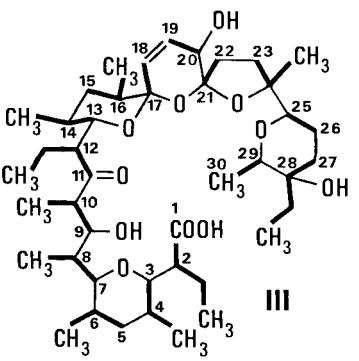
Summary. The labeling of the polyether ionophore, narasin, using ^{13}C-labeled acetate, propionates, and butyrates in the course of fermentation of *Streptomyces aureofacies* resulted in the isolation of differently labeled compounds. A careful examination of the ^{13}C-NMR. spectral data indicated derivation of narasin from five acetate, seven propionate, and three butyrate units. The evidence of indirect incorporation of ^{13}C led to the first documented demonstration of conversion of propionate to butyrate in the course of the synthesis of narasin by *S. aureofaciens*.

Since the discovery of monensin [3] (I), the first recognized ionophoric polyether [4], a number of substances of this type have been described [5]. From biosynthetic studies [6] [7] it is evident that these compounds are derived primarily from acetate, propionate, and butyrate units. Experiments using 1-[^{13}C]-butyrate precursor and ^{13}C-NMR. analysis [8] were used to prove that the three ethyl groups of lasalocid (II) (X537A) are derived from butyrate [7]. Attempts to incorporate ^{13}C-labeled acetate to a detectable level into monensin have been less successful [6]. We report here a more complete study of the biosynthesis of the polyether ionophore narasin (III) (A28086), using ^{13}C-labeled acetates, propionates, and butyrates.

In agreement with the proposed structure III [9], the ^{13}C-NMR. spectrum of narasin shows 43 resonances. The chemical shifts of some of these carbon atoms are







[1] An ionophore is a compound with ability to render cations lipid soluble.

quite variable. We have determined that one source of variation is the hydration of the molecule of the product. Chemical shift variations of up to 1.5 ppm were noted when we compared the ^{13}C-NMR. spectra of narasin samples which had been dried under differing conditions. Other experiments showed differences in the spin-lattice relaxation times (T_1) of the carbon atoms which presumably result from the same problem. We, therefore, measured all the spectra reported herein in wet chloroform. Using this technique, chemical shift measurements are quite reproducible.

The assignment of these resonances has proven to be an extremely challenging task. Early efforts to correlate the spectra of structurally related ionophores were only partially successful. Thus, ionophores with identical substructural features do not always show the expected similarities in the ^{13}C-NMR. spectra. This might result from differing conformations, or from factors such as hydration (*vide supra*) or minor impurities. However, through the use of chemical shift theory [10], multiplicity, T_1 values, specific proton decoupling, comparison with the spectra of derivatives and model compounds, and finally biogenetic labeling results, a tentative assignment of resonances was attained. These assignments are offered in Table 1.

The purpose of this communication is to illustrate the utility of labeling experiments in assignments of some frequencies, particularly methyl group resonances. The lack of knowledge of absolute configurations of asymmetric centers makes it difficult to identify specifically methyl groups resonances by more conventional methods.

The examination of the proposed structure of narasin leads to the conclusion that **this molecule would be derived from five acetate, seven propionate and three butyrate units** (III). Visual comparison of the ^{13}C-NMR. spectra of samples **A**, **B** (Fig. 1) and **C** (Fig. 2) with that of **N**, the unenriched ionophore (Fig. 1 and Table 2) supports this assumption. Thus, the spectra of samples **A** and **B** contain seven peaks of enhanced intensity, while that of **C** contains only three. The spectra of **D** (Fig. 3) and **E** (Fig. 4), however, are more complex, containing ten and nine resonances, respectively, which appear to be enhanced in intensity. A more detailed examination of these spectra reveals additional and unexpected complexities.

In Table 2 chemical shifts and enrichment ratios of different carbon atoms of biogenetically enriched samples of narasin have been summarized. Enrichment ratios (ER.) [8] obtained by dividing the normalized intensity of a resonance in the spectrum of a labeled molecule by the normalized intensity of the same peak in unlabeled material, are tabulated for samples **A** through **E**. Following a precedent established in earlier work with ionophores [7], we assumed that any peak showing an enrichment factor of at least 2.0 represented a significantly enriched carbon atom. In sample **A** (Fig. 1), only six resonances meet this criterion, and one additional resonance (29.0 ppm) comes very close (1.91). This last resonance is partially overlapped with a resonance due to an unenriched carbon atom, which might be expected to lead to inaccurate measurement of its intensity. Because the peak at 29.0 ppm seems to have a significantly higher enrichment ratio than the overall average (*ca.* 1.1), we believe it represents a biogenetically labeled carbon atom. This leads to the conclusion that seven carbon atoms of A28086 are derived directly from C(2) of propionic acid, in agreement with expectation.

Table 1. *Assignment of* ^{13}C *resonances*[a])

Carbon	δ[b])	Biogenesis[c])	T_1[d])	Other Bases for Assignment
1	178.4	Bu(1)	2.4	Chemical shift
2	49.3(e)	Bu(2) ✓	0.18	Chemical shift
CH_2(2)	24.0(f)	Bu(3) ✓	0.08	
CH_3(2)	13.2(g)	Bu(4) ✓	0.71	
3	73.9	Pr (1)	ca. 0.2	Ionization shift; specific decoupling
4	28.0(h)	Pr (2) ✓	0.24	
CH_3(4)	19.0(i)	Pr (3) ✓	0.19	
5	36.2(j)	Pr (1)	0.11	
6	29.0(h)	Pr (2) ✓	0.18	
CH_3(6)	18.0(i)	Pr (3) ✓	0.70	
7	75.1(k)	Pr (1)	ca. 0.2	Elimination; specific decoupling
8	41.1(l)	Pr (2) ✓	0.20	
CH_3(8)	15.7(i)	Pr (3) ✓	0.43	
9	72.0(k)	Pr (1)	0.21	Elimination; specific decoupling
10	49.9	Pr (2) ✓	0.17	Chemical shift; comparison to lasalocid
CH_3(10)	7.0(i)	Pr (3) ✓	0.89	
11	216.5	Bu(1)	2.2	Chemical shift
12	56.1(e)	Bu(2) ✓	0.26	Chemical shift; comparison to lasalocid
CH_2(12)	16.4(f)	Bu(3) ✓	0.08	
CH_3(12)	12.1(g)	Bu(4) ✓	0.77	
13	78.4	Pr (1)	ca. 0.2	Specific decoupling
14	32.9	Pr (2) ✓	0.19	
CH_3(14)	12.1(i)	Pr (3) ✓	0.77	
15	35.5	Pr (1)	0.10	
16	36.6(l)	Pr (2) ✓	0.22	
CH_3(16)	13.0(i)	Pr (3) ✓	0.66	
17	99.6	Ac(1)	4.4	Acetylation shift
18	122.0	Ac(2)?	ca. 0.14	Acetylation shift
19	132.0	Ac(1)	ca. 0.14	Acetylation shift
20	67.6	Ac(2)?	0.21	Specific decoupling
21	106.5	Ac(1)	2.8	Acetylation shift
22	38.7	Ac(2)?	0.07	
23	30.5(j)	Pr (1)	0.18	
24	88.5	Pr (2) ✓	2.1	Chemical shift
CH_3(24)	26.1	Pr (3) ✓	0.35	
25	68.5	Ac(1)	0.24	Comparison to lasalocid; specific decoupling
26	21.8	Ac(2)	0.11	
27	29.4(j)	Bu(1)	ca. 0.2	
28	70.8	Bu(2) ✓	1.7	Comparison to lasalocid; multiplicity
CH_2(28)	30.9	Bu(3) ✓	ca. 0.2	
CH_3(28)	6.3	Bu(4) ✓	0.82	
29	77.1	Ac(1)	ca. 0.2	Comparison to lasalocid; specific decoupling
30	14.3	Ac(2)	0.71	Elimination

a) All spectra collected in wet $CDCl_3$

b) Chemical shifts are measured relative to internal TMS.

c) Indicated biogenetic sources of each carbon, as predicted from inspection. Cases where the biogenesis has been confirmed are marked with a check (✓). The number in parenthesis designates the site of the ^{13}C label.

d) T_1 values are in seconds.[b]

e–l) Resonance so indicated can be interchanged in assignment.

Of the seven carbon atoms derived from this source, one has been assigned in Table 1 to C(24). Again, this is in agreement with the biogenetic scheme of narasin (III). The enriched resonance at 49.9 can be assigned on the basis of its chemical shift to one of the α-carbon atoms (C(2), C(10) or C(12)); from the present experiment and the proposed biogenetic scheme we can assign this resonance to C(10). The remaining enriched resonances are predicted to be due to C(4), C(6), C(8), C(14), and C(16). The observed chemical shift range (41.1–28.0 ppm) observed for these resonances is consistent with the environments of these carbon atoms in the proposed structure.

Table 2. *Enrichment ratios*[a]) *of biogenetically enriched samples of narasin*

δ	A Pr(2)	B Pr(3)	C Bu(2)	D Bu(3)	E Bu(4)	F Ac(2)
216.5	–	–	–	–	–	–
178.4	1.02	1.06	0.95	1.34	0.81	0.87
132.0	1.08	1.49	1.20	1.55	1.20	0.92
122.0	1.06	1.46	2.00°	0.99	1.69	1.68
106.5	1.23	1.27	1.44	1.71	1.31	0.94
99.6	0.98	1.05	0.99	1.39	1.26	0.95
88.5	2.03*	0.95	0.89	7.76°	1.11	0.90
78.4	1.03	1.21	0.97	1.01	0.95	1.08
77.1	1.00	1.38	0.90	1.56	0.96	0.89
75.1	1.22	1.34	1.03	1.04	1.06	1.21
73.9	0.96	1.08	1.05	1.49	0.92	0.83
72.0	1.01	1.28	1.36	0.98	1.14	1.13
70.8	0.94	1.49	17.8*	0.68	0.77	1.15
68.5	1.22	1.43	1.53	1.05	1.29	1.20
67.6	1.02	1.50	2.01°	1.09	1.72	1.63
56.1	0.88	2.68°	19.6*	0.64	0.98	1.34
49.9	2.39*	1.11	1.17	9.35°	1.19	1.34
49.3	1.01	2.91°	21.1*	0.92	1.03	1.39
41.1	2.46*	1.02	1.24	8.82°	1.08	1.29
38.7	1.16	1.44	2.54°	1.06	1.25	1.13
36.6	2.38*	1.00	1.24	9.43°	1.11	1.28

δ	A Pr(2)	B Pr(3)	C Bu(2)	D Bu(3)	E Bu(4)	F Ac(2)
36.2	0.97	1.45	1.82	0.89	1.55	1.52
35.5	0.93	1.22	1.14	1.01	1.11	1.04
32.9	2.30*	1.00	1.12	8.49°	1.03	1.30
30.9	1.12	1.26	0.81	16.6*	0.63	0.94
30.5	1.19	1.42	1.30	(–)	1.23	1.19
29.4	1.09	1.22	0.84	1.29	0.97	0.98
29.0	1.91*	0.92	1.04	7.88°	0.92	1.11
28.0	2.28*	1.11	1.09	9.60°	1.13	1.31
26.1	1.33	9.37*	1.62	1.23	11.7°	1.55
24.0	1.03	1.24	0.75	18.5*	0.64	0.95
21.8	0.96	1.46	1.76	1.11	1.61	1.59
19.0	1.26	8.94*	1.55	0.98	10.8°	1.36
18.0	1.25	9.29*	1.50	1.04	11.6°	1.63
16.4	1.02	1.33	0.79	16.2*	0.67	0.88
15.7	1.31	9.83*	1.69	1.32	12.1°	1.60
14.3	0.96	1.41	1.68	0.99	1.69	1.54
13.2	1.16	3.49°	1.33	0.84	18.1*	1.53
13.0	1.18	8.41*	1.45	1.04	12.0°	1.47
12.1 12.1	1.04	5.71*	1.36	0.94	14.2*°	1.38
7.0	1.36	9.81*	1.78	1.17	12.6°	1.61
6.3	0.95	2.14°	0.92	0.70	17.4*	1.22

a) Peak heights of each spectrum were normalized to the peak at 216.5 ppm. Enrichment ratios were then calculated by dividing the normalized intensity of each peak by the normalized intensity of the same peak in unriched narasin.

* Carbon labeled through direct incorporation of biogenetic precursor into narasin.

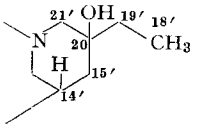
° Carbon labeled through indirect incorporation of biogenetic precursor into narasin.

The ^{13}C-NMR. spectrum of sample **B** (Fig. 1) contains eleven resonances with ER. exceeding 2.0. The intensities of ten of these resonances fall into two groups: six resonances have enrichment factors of 8.4–9.8, while four others average about 2.8. We conclude that the six most highly enriched carbon atoms are those which are directly derived from C(3) of propionic acid, while the four resonances (56.1, 49.3, 13.2, and 6.3 ppm) with enrichment ratios of less than 3.5 represent carbon atoms derived from this source through some less direct process (*vide infra*). The peak at 12.1 ppm has an intermediate enrichment ratio of 5.71. Such an enrichment ratio would result from the overlap of a resonance of high enhancement (ER. ≈ 9) with a resonance of lower enhancement (ER. ≈ 3). The data can thereby again be interpreted to indicate that seven propionate units are incorporated into A28086. All of these seven intensified resonances have T_1 values characteristic of methyl groups, as required by the biogenetic scheme shown in III. One of the resonances of this group is significantly deshielded relative to the others, and is accordingly assigned to the quaternary methyl attached to C(24) [10].

In the ^{13}C-NMR. spectrum of **C** (Fig. 2), three resonances are obviously strongly enhanced. Two of these are assigned on the basis of their chemical shifts to carbon atoms adjacent to carbonyl groups. This is consistent with the proposed biogenesis, which predicts carbon atoms C(2) and C(12) to be derived from C(2) of butyric acid. The third intensified resonance (70.8 ppm) has been assigned to C(28), a result which is again consistent with the proposed biogenesis. In addition, three other peaks (122.0, 67.6, and 38.7 ppm) show significant, though much smaller, enhancement. Again, this must result from some less direct biogenetic process.

The ^{13}C-NMR. spectrum of **D** (Fig. 3) shows evident enrichment of ten carbon atoms. These fall into two major groups: three peaks with enrichment ratios averaging 17.1, and seven with an average ER. ≈ 9. The most intensified peaks have chemical shifts (30.9, 24.0, and 16.4 ppm) which are reasonable for methylene carbon atoms. The most deshielded is very similar in chemical shift to C(18′) of indole-indoline alkaloid vinblastine [11] (IV) which is substituted similarly to the methylene attached to C(28) of A28086. Of the other two methylene groups derived from C(3) of butyrate, one is relatively shielded, possibly as a result of the adjacent ketone [10]. This resonance (16.4 ppm) is accordingly assigned to the methylene group attached to C(12). These assignments, and the assignment of the resonance at 24.0 ppm to CH_2–C(2) by default, are of course tentative, and based primarily on the biogenetic evidence.

Sample **E** (Fig. 4) gives a ^{13}C-NMR. spectrum in which nine resonances are obviously enhanced. Two (13.2 and 6.3 ppm) show enrichment ratios exceeding 17, six have enrichment ratios between 10.8 and 12.6. The two carbon resonance at 12.1 ppm shows an enrichment ratio which is the approximate average of these two sets of resonances. On this basis we believe that resonances at 13.2, 12.1, and 6.3 ppm represent carbon atoms directly derived from C(4) of butyric acid. Carbon atoms coming into resonance at 26.1, 19.0, 18.0, 15.7, 13.0, 12.1, and 7.0 ppm appear to be labeled through a secondary, less direct, process.



IV

The methyl resonance at 6.3 ppm is assigned to an ethyl group attached to a quaternary center which bears a hydroxyl group [10] [11] as in C(28). The chemical shifts and relaxation times of the remaining highly labeled carbon atoms are consistent with their assignments to CH_3–C(2′) and CH_3–C(12′), as would be required by the biosynthetic scheme of Fig. 2.

Due to the absence of obvious enrichments, the ^{13}C-NMR. spectrum of narasin precursed with 2-[^{13}C]-acetate has not been reproduced (**F**). On the basis of the assignments above, one would have anticipated in **F** enrichment of the resonances at 122.0 and 67.6 ppm. It is interesting that these resonances show enrichment ratios which are among the highest of **F**. As previously observed [6], however, significant enrichment with acetate in these fermentations is relatively difficult to achieve, and is probably due to a multiplicity of sources of acetate which dilutes the enriched precursor.

Secondary Metabolic Processes. In the above discussion, several instances of secondary or indirect derivation of isotopically labeled A28086 were mentioned. Such results suggest that metabolic interconversions of butyrate, propionate, and acetate may be the source of complication in the interpretation of biogenetic results

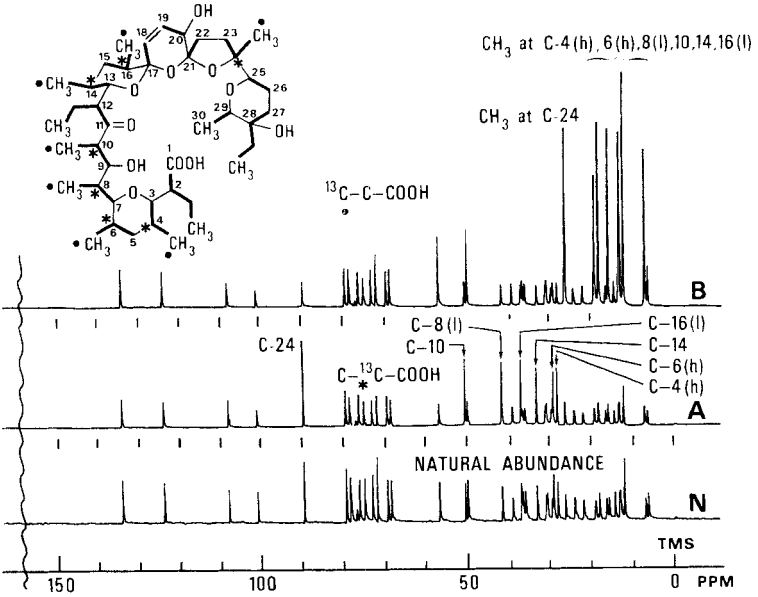


Fig. 1. N Using compounds in natural abundance. – A Using 2-[^{13}C]-propionate. – B Using 3-[^{13}C]-propionate.

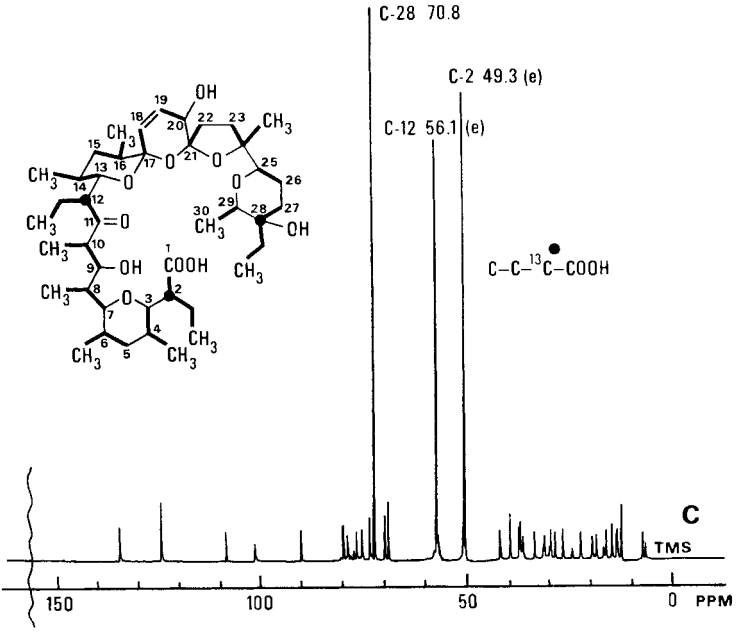


Fig. 2. C Using 2-[^{13}C]-butyrate

Fig. 1–4. *^{13}C-NMR. Spectra*[a]) *of different samples of narasin synthesized*

[a]) As indicated in III, C(1) and C(11) come from C(1) of butyrate and represent COOH and C=O the choice of our precursors, these carbon atoms could not be enriched in the course of labeling,

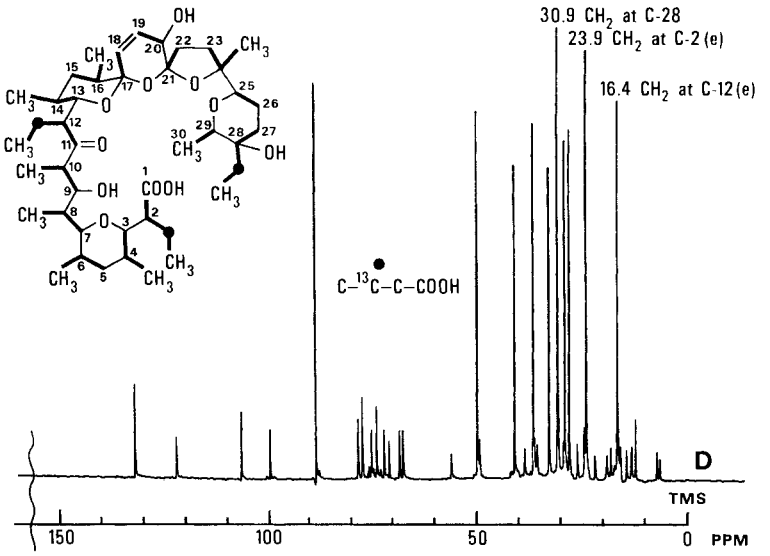


Fig. 3. D Using 3-[^{13}C]-butyrate

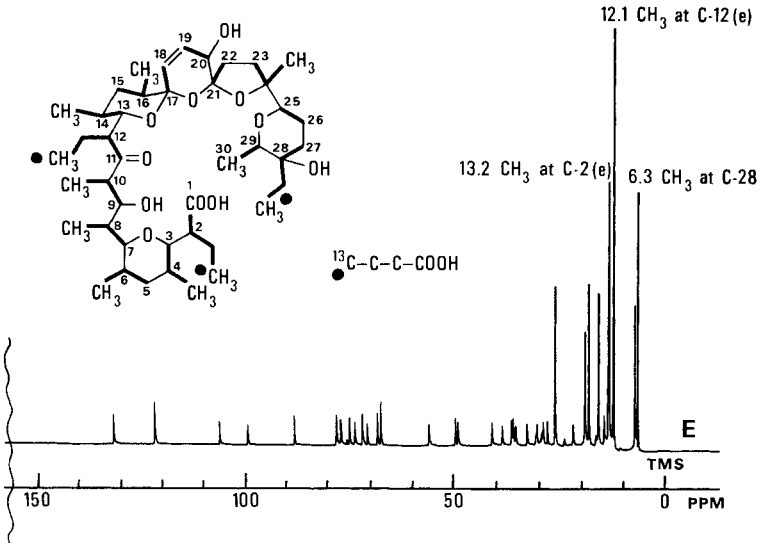


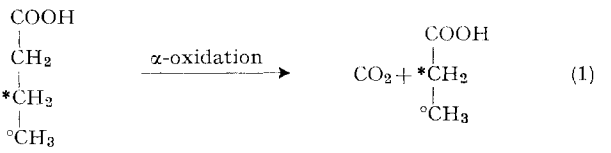
Fig. 4. E Using 4-[^{13}C]-butyrate

bei Streptomyres aureofaciens *under use of differently labeled compounds*

functions. Their chemical shifts are found at 178.4 and 216.5 ppm respectively. In view of therefore the portion of the ^{13}C-NMR. spectra below 150 ppm has been deleted from these figures.

based on ^{13}C-NMR. spectroscopic data. Recent research into the biosynthesis of macrocyclic antibiotics suggests such complications to be especially important when ^{13}C-labeled butyrate is used as precursor [12].

Our results are most clearly defined in the cases of samples **D** and **E**, derived from 3- and 4-[^{13}C]-butyrate, respectively. In **D** the three carbon atoms labeled through direct incorporation of butyric acid into A28086 show enrichment ratios of 16.2 to 18.5. A second set of seven carbon atoms are also enriched, showing an average enrichment ratio of 8.8. The latter carbon atoms are the same as those shown in sample **A** to be derived from C(2) of propionate (*cf.* Table 2). Sample **E** also clearly shows two sets of enriched carbon atoms; the set of carbon atoms showing smaller enrichments (average ER. = 11.8) corresponds exactly to the seven carbon atoms shown in sample **B** to be derived from C(3) of propionate. These results would suggest that α-oxidation is important in this fermentation (see eq. 1).

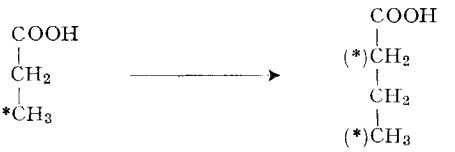


(1)

However, α-oxidation would require that C(2) of butyrate be metabolized to C(1 of propionate. This is not in accord with the results obtained from sample **C**. In samples **C**, **D**, and **E** all carbon atoms derived *directly* from butyrate show comparable enrichment ratios. In **C**, however, there is no set of carbon atoms with intermediate enrichment ratios of 8 to 12, as in **D** and **E**. Instead, three carbon atoms with enrichment ratios of 2 to 2.5 are observed at 122.0, 67.6, and 38.7 ppm. The first two of these resonances have been assigned to carbon atoms which are proposed to be derived from C(2) of acetate. This suggests that β-oxidation is also operative in this case, as was observed in an analogous polyether [7]. The relative enrichment ratios of the *indirectly* derived carbon atoms of **C**, **D**, and **E** suggest, however, that β-oxidation is less important than the process which converts C(3) and C(4) of butyrate to C(2) and C(3), respectively, of propionate.

Sample **B** also shows two sets of enriched carbon atoms. The set of lower enrichment consists of peaks at 56.1, 49.3, 13.2, 12.1, and 6.3 ppm. The first two of these were shown in **C** to be derived from C(2) of butyrate; the last three arise from C(4) of butyrate (*cf.* sample **E**).

The available evidence, therefore, appears to indicate that C(3) of propionate can be incorporated into butyric acid at either the C(2) or C(4) position (see eq. 2).



(2)

The peak at 70.8 ppm, also shown to be derived from C(2) of butyrate, is not labeled. The enrichments in sample **A** are not high enough to determine the fate of C(2) of propionate in secondary metabolic processes.

In view of these results, it is evident that enzymes present in the fermentation of *Streptomyces aureofaciens* are capable of interconversions of acetate, propionate, and butyrate. At the present time, the mechanisms of these interconversions are not clear. Certainly, simple α- and β-oxidation schemes do not account for all the observed interconversions. We believe that the results obtained from sample **B** represent **the first case of documented conversion of propionate to butyrate.** Further studies designed to lead to a better understanding of these secondary processes are presently underway.

Experimental Part

Fermentation and Labeling. – Extensive fermentation studies leading to the production of narasin have been described [13]. In our labeling studies, the following conditions were used.

A lyophile pellet of *Streptomyces aureofaciens* NRRL8092 was used to inoculate 50 ml of vegetative medium of the following composition: glucose, 20 g; nutrisoy flour, 15 g; corn steep liquor, 10 g; $CaCl_2$, 2 g and water, 1000 ml. The pH was adjusted to 6.5 prior to sterilization and dispensed in 250 ml Erlenmeyer flasks. After 48 h incubation at 30° on a rotary shaker (250 rpm), 0.5 ml (1% inoculum) was inoculated to 250 ml Erlenmeyer flasks containing 50 ml of fermentation medium containing the following ingredients: glucose, 15 g; *Stadex* 11 or tapioca dextrin, 30 g; enzyme hydrolysed casein, 3 g; NZ-amine A, 1 g; yeast extract, 2.5 g; $CaCO_3$, 2 g; $MgSO_4 \cdot 7\,H_2O$, 1 g; blackstrap molasses, 15 g; refined soybean oil, 5 ml and water, 1000 ml. Fermentation was carried out on a conventional rotary shaker at 30°. At the 24 h fermentation stage, ^{13}C-labeled substrates (all precursors contained 90 atom % ^{13}C except for 2-[^{13}C] acetate, which contained 52 atom % ^{13}C)[2]) were added in water at a final concentration of 1 mg/ml. After the addition of the precursor, the fermentation was continued for additional 8–9 days. Narasin titers varied from 1.0–1.5 mg/ml when estimated turbidimetrically with *Staphylococcus aureus* NRRLB-314.

Isolation. – The isolation, characterization and properties of narasin have been described [2]. In our experiments, these conditions had to be modified to permit the isolation of small amounts of polyether.

The whole broth (1 l, pH 7.9) was adjusted to pH 3.0 with 1 N HCl and stirred for one h. After the addition of 30 g of Hyflo and filtering, the mycelial cake was extracted in a blender two times with 200 ml of a solution containing 50 g of $NaHCO_3$ in 1 l of methanol. The methanolic solution was evaporated to approximately 100 ml, pH adjusted to 7.5 with 1 N HCl and extracted two times with 200 ml of $CHCl_3$, dried over Na_2SO_4, filtered and evaporated i.V.

In case where 1.0 g of sodium-3-[^{13}C]-butyrate was used for labeling, the residue of 1.3 g was obtained and chromatographed over 130 g of Silica (*Grace* 62) using TLC. (Silica, ethyl acetate, vanillin spray with heating) to monitor the appearance and purity of different factors. Elution was carried out with benzene and benzene/ethyl acetate in 150 ml. fractions. Residues (110 mg) resulting from elution with 3.0 l of benzene, 3.3 l of benzene/ethylacetate 9:1 and 8.9 l of these solvents in 8:2 ratio were discarded. The elution was then continued with additional 9 l

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[2]) All precursors were reagent grade *Merck, Sharp* and *Dohme* of Canada, Ltd, and found to be better than 99% pure by GC.

of the same solvent mixture and afforded 355 mg of the fluffy white mass. This material was dissolved in 20 ml of acetone, filtered, 20 ml of H_2O added, pH adjusted to 3.0 using 1 N HCl and stirred for 1 h. The formed precipitate was filtered and recrystallized from 16 ml of acetone/water 3:1 (*v/v*). After crystallization at RT., 190 mg of narasin were collected. Recrystallization of the acid from 12 ml of acetone/water 3:1 afforded 180 mg of crystalline and homogeneous (TCL. and bioautography) material, m.p. 98–100°, resolidification and melting 195–200°.

Collection of Spectra. – ^{12}C-NMR. spectra were collected on a *JEOL* PFT-100 multinuclear spectrometer interfaced with a *JEOL* EC-100 data system. Narasin samples (*ca.* 50 mg) were dissolved in 0.5 ml $CDCl_3$ to which one drop of water had been added, and placed in cylindrical small volume sample bulbs (*Wilmad* 529-E-10). Spectra were collected into 16 K of computer memory, using a spectrum width of 6.25 KHz and a repetition time of 2 s. Conditions of data collection and transformation would be expected to lead to line broadening of 0.7 Hz.

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