Biosynthesis of (+)-Protolichesterinic Acid in *Cetraria islandica*

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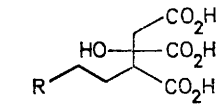
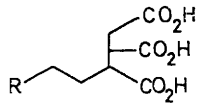
The biosynthesis of (+)-protolichesterinic acid has been studied by use of [1-^{14}C]acetate and [1,4-$^{14}C_2$]succinic acid. The results support the hypothesis that aliphatic lichen acids have common precursors related to the citric acid and fatty acid cycles; however, the extremely low levels of incorporation suggest that the biosynthesis represents very minor metabolic pathways in *C. islandica*. The biosynthesis appears to be inoperative in winter.

We have previously [1] described the derivation of (+)-protolichesterinic acid (PLA) from acetate. Initial succinate experiments gave no incorporation; this was originally interpreted in terms of alternative precursors. Subsequent experiments [2] showed that the lack of incorporation was due to cessation of PLA production in winter.

Aliphatic lichen acids are exemplified by structures (1)—(7); [3] some members exhibit antitubercular and antibiotic properties.[4] No biosynthetic work has apparently been done on any of them, though a theory has been advanced [5] that a reaction related to the citric acid cycle leads to caperatic acid (1), and possibly to

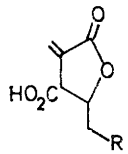


(1) R = $C_{12}H_{25}$ monomethyl ester



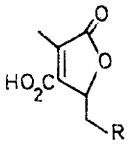
(2) R = $C_{12}H_{25}$ monomethyl ester

(8) R = $C_{14}H_{29}$

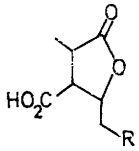


(3) R = $C_{12}H_{25}$ (PLA)

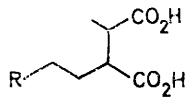
(7) R = $C_{10}H_{21}$



(4) R = $C_{12}H_{25}$ (LA)

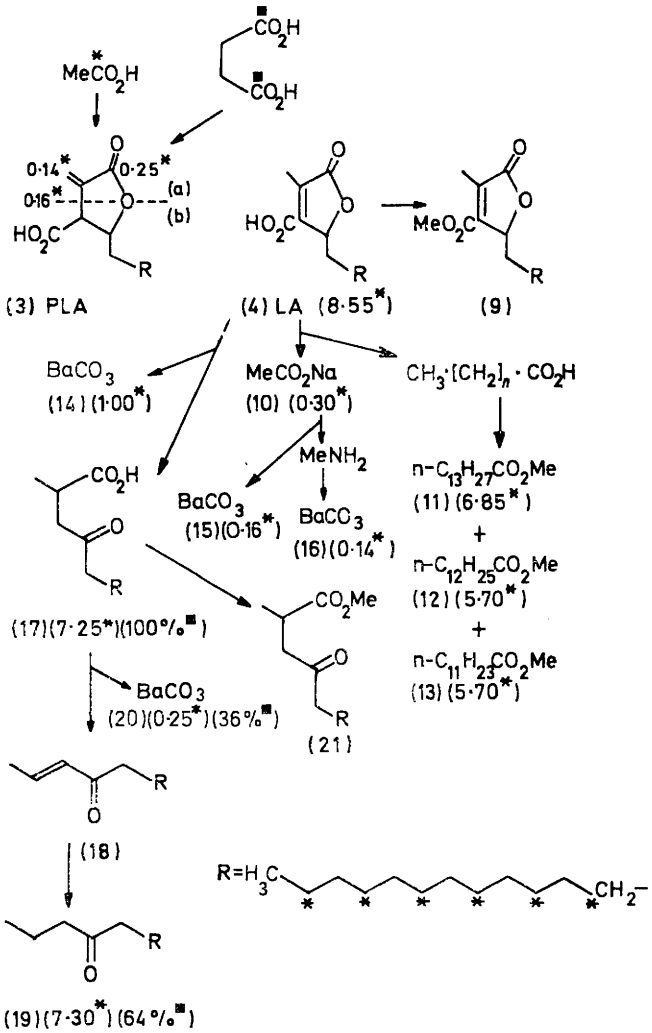


(5) R = $C_{12}H_{25}$



(6) R = $C_{10}H_{21}$

rangiformic acid (2). The remaining structures (3)—(7) could be derived similarly, with loss of one carbon atom. [The *Polyporous* metabolite, agaricic acid (8),[6] is included because of similarities to the other acids.] The intermediacy of fatty acids or their derivatives as biological synthons is supported on structural grounds by the recurrence of chain lengths corresponding to myristic, palmitic, or stearic acid in structures (1)—(8).



Scheme

Because of its ready availability, we have studied the lactone (3). The degradative pathway is shown in the Scheme, including the previously reported conversions

[1] J. L. Bloomer, W. R. Eder, and W. F. Hoffman, *Chem. Comm.*, 1968, 354.
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[5] Personal communication reported by Y. Asahina and S. Shibata, 'The Chemistry of Lichen Substances,' 1954, p. 215, Japanese Society for the Promotion of Science, Tokyo.
[6] H. Thomas and J. Vogelsang, *J. Pharm. Soc. Japan*, 1940, **60**, 318.

(3) ⟶ (4) [7] and (4) ⟶ (17).[7,8] The inclusion of the reduction (18) ⟶ (19) was necessary as a mixture of (18) and (19) was obtained upon decarboxylation of (17) with copper chromite. Degradation of (4) involved oxidation with permanganate to acetate (10) and a fatty acid mixture, isolated as esters (11)—(13) by g.l.c. Derivatives (9) and (21) were used for cross-checks.

Growth and respiration studies on mycobionts *Cetraria islandica* and *Cladonia papillaria* have been described elsewhere;[9] special apparatus has been developed.[10] Attempts to incorporate acetate into PLA by use of a glucose–nitrate medium[11] failed [in contrast to experiments with the aromatic lichen metabolites usnic acid,[12] gyrophoric acid,[13] lecanoric acid,[14] and atranorin].[14] Feeding problems were solved by stationary hydroponic administration of acetate in 10% glucose solution to whole fresh *C. islandica*. Degradation of (3) thus obtained gave results as shown in the Table. The relative molar activities of (11), (12), and (13) leave little doubt that the aliphatic chain of PLA is derived from head-to-tail linkage of acetate units. Activities of (15), (16), and (20) were not easily interpreted, though the equality of label in (15) and (16) did suggest a symmetrical C_4-intermediate. A subsequent attempt at succinate incorporation failed. Taken together, these two observations were initially interpreted as indicating alternative origin of the three-carbon unit,[1] though positive evidence for this was lacking.

Sample	Relative molar activity (decomp. min.$^{-1}$ mole^{-1} × 10^{-6})
Lichesterinic acid (4)	443·0
Barium carbonate (14) from decarboxylation of (4)	52·0
2-Methyl-4-oxoheptadecanoic acid (17)	376·0
Barium carbonate (20) from decarboxylation of (17)	12·4
Heptadecan-4-one (1)	356·0
Methyl myristate (11)	355·0
Methyl tridecanoate (12)	295·0
Methyl dodecanoate (13)	295·0
Sodium acetate (10)	15·8
Barium carbonate (15) from Schmidt reaction on (10)	7·9
Barium carbonate (16) from oxidation of methylamine	7·0

Further attempts to define the origin of the subunit by using glucose, three-carbon precursors, and an acetate standard failed; the experiments indicated the cessation of PLA production in winter. The non-incorporation of succinate was thus explained on the basis of a seasonal effect, which was verified by successful incorporation of succinate in summer. Severe purification problems necessitated isolation of the acid (17) as the ester (21) by g.l.c.; the ester was then reconverted into (17). Fragment (20) contained a major fraction of label.

EXPERIMENTAL

Instruments used were as follows: Kofler hot-stage apparatus (m.p.s), Perkin-Elmer 137 (i.r. spectra), Beckman DK-2 (u.v. spectra), Varian A60-A (n.m.r. spectra), Aerograph A90-P3 (g.l.c.), and Packard 303 (scintillation counting). Barium carbonate samples were counted by diffusion into Hyamine. Organic substances were counted directly in toluene containing BBOT {2,5-bis-[2-(5-t-butylbenzoxazolyl)]thiophen}. T.l.c. was performed with 100—200 mg. samples on silica gel (Brinkmann GF_{254}; 20 × 20 × 0·2 cm.), with benzene–methanol–acetic acid (85 : 10 : 5) as eluant; recovery was >85% for all compounds reported. Spots were detected under u.v. light. For g.l.c. we used columns of 20% SE-30 on Chromosorb W (60—80 mesh) [4 ft. × ¼ in. (analytical), 8 ft. × ½ in. (preparative)] with >95% recovery for all compounds reported; the apparatus used has been previously described.[15]

Radiochemical degradations were done on the same scale as, and gave essentially the same yields as the following experiments.

Lichesterinic Acid (LA) (4).—(+)-Protolichesterinic acid (PLA) (400 mg.) was refluxed with acetic anhydride (10 ml.) for 1 hr. The acetic anhydride was removed and the residue was treated with acetone–water (4 : 1) (5 ml.) at reflux for 30 min. Solvent was again removed. Conversion into LA was essentially quantitative (n.m.r.) from pure PLA. Radiochemical runs were always followed by multiple chromatography. In addition to reported[7,8] properties LA exhibited τ 9·13 (3H, t *J* 7 Hz), 7·80 (3H, d, *J* 1·5 Hz, allylic coupling), 4·83 (1H, poorly resolved), (1H, s, variable with concentration), and 8·77 (others). Also PLA exhibited τ 9·13 (3H, t, *J* 7 Hz), 6·39 (1H, d, *J* 6 Hz), 5·20 (1H, dt, *J* 6 and 7 Hz), 3·98 (1H, d, *J* 3 Hz), 3·53 (1H, d, *J* 3 Hz), −0·95 (1H, s, variable with concentration), and 8·74 (others).

2-Methyl-4-oxoheptadecanoic Acid (KA) (17).—Lichesterinic acid (4) (104·3 mg.) was heated at 100° for 2 hr. with N-sodium hydroxide (CO_2-free; 1 ml.) under nitrogen (CO_2-free). The mixture was cooled and 1·2N-hydrochloric acid (1·2 ml.) was added under nitrogen (CO_2-free). Carbon dioxide evolved was collected as barium carbonate (48·8 mg.). The solution was extracted with ether (3 × 5 ml.). The extract was evaporated to dryness and the residue was subjected to t.l.c. The main fraction, R_F 0·50—0·60 (74·6 mg.) yielded KA, m.p. 81—83° (from acetic acid) (61 mg.) which, in addition to reported properties, exhibited $\nu_{max.}$ 2896, 2600—3300, 1690 (with unresolved shoulder), 1460 1400, 1245, 1185, 1142, 1098, 931, 921, 816, and 712 cm.$^{-1}$, τ 9·13 (3H, t, *J* 7 Hz), 7·62 (2H, t, *J* 7 Hz), and 1·45 (1H, s, variable with concentration), 6·8—7·6 (5H, complex), and 8·76 (others).

Decarboxylation of 2-Methyl-4-oxoheptadecanoic Acid (KA).—The acid (68 mg.) was refluxed with quinoline (3 ml.) containing copper chromite (110 mg.) at 265 °C for 1 hr.

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[9] J. L. Bloomer, *Bryologist*, in the press.
[10] J. L. Bloomer, *Appl. Microbiol.*, 1968, **16**, 1426.
[11] V. Ahmadjian, *Bryologist*, 1964, **67**, 87.
[12] H. Taguchi, U. Sankawa, and S. Shibata, *Tetrahedron Letters*, 1966, 5211.
[13] K. Mosbach, *Acta Chem. Scand.*, 1964, **18**, 329.
[14] M. Yamazaki, M. Matsuo, and S. Shibata, *Chem. and Pharm. Bull. Japan*, 1965, **13**, 1015.
[15] J. L. Bloomer and W. R. Eder, *J. Gas Chromatog.*, 1968, **6**, 448.

to give carbon dioxide [as $BaCO_3$ (21·4 mg.)]. The quinoline mixture was filtered, diluted with ether (20 ml.), and extracted with excess of N-hydrochloric acid. The ethereal solution was washed with water until neutral (litmus), and then dried ($MgSO_4$), filtered, and evaporated to give a residue of crude ketones (18) and (19) (47·3 mg.). A solution of the residue in ethanol (20 ml.) was filtered, and palladium–charcoal (5%; 10 mg.) was added. The mixture was hydrogenated for 2 hr., filtered through Celite, and evaporated to yield a brown oil. G.l.c. gave a white solid (19) (17 mg.) as the major component, m.p. 41—42°, n.m.r. and i.r. spectra identical with those of synthetic heptadecan-4-one.[16]

Lichesterinic Acid Oxidation.—A solution of LA (4) (57·4 mg.) in ether was added to glass beads (2 mm.; 1·5 g.). The ether was carefully evaporated off to disperse the LA on the surface of the beads and water (3 ml.) was added. The apparatus was connected to a barium carbonate collection system, and provision was made for the addition of permanganate solution (0·1M; 10 ml.) under nitrogen. The system was flushed with nitrogen and heated to 100°; the permanganate solution was then added dropwise during 30 min. Sulphuric acid (10%; 2 ml.) was then added, and the apparatus was swept with nitrogen for 10 min. Sodium sulphite solution (3 ml.) was then added to destroy oxides of manganese. The solution (pH 2) was cooled to 0° and extracted with ether (2 × 15 ml. and 3 × 10 ml.). The ethereal solution was extracted with N-sodium hydroxide (2 ml.) and the aqueous solution (pH 10) of salts was evaporated (octyl alcohol was added to prevent foaming). To the salts were added conc. sulphuric acid (0·1 ml.), butanol (0·5 ml.), and chloroform (5 ml.), and the mixture was transferred to a column (2·5 × 50 cm.) of Celite 545, with 0·5N-sulphuric acid as the stationary phase as previously described.[17]

Elution with chloroform gave fatty acids (41·0 mg.), which were esterified with diazomethane in ether. Preparative g.l.c. (160 °C) gave compounds (9), (11), (12), and (13) (5·4, 10·6, 1·8, and 2·1 mg.).

Elution with 10% butanol–chloroform gave acetic acid, which was titrated with sodium hydroxide (0·1N; CO_2-free); the solution was then evaporated and subjected to Schmidt degradation.[18]

Protolichesterinic Acid Derived from Sodium [1-^{14}C]-*Acetate.*—*C. islandica* (12 g.) was thoroughly washed with distilled water, and 10% glucose solution (150 ml.) containing sodium [1-^{14}C]acetate (100 μc; 0·1 mg.) was added. The mixture was swirled under constant illumination for 5 days, and the glucose solution was then decanted. The lichen was washed with water, (3 × 30 ml.); examination of a portion of the washings indicated that 1% of initial activity remained. The lichen was dried and extracted with ether (Soxhlet; 2 hr.); the ether solution was concentrated to 25 ml. and extracted with saturated sodium hydrogen carbonate (4 × 25 ml.). The latter extracts were shaken with ether (25 ml.), acidified with acetic acid, and extracted with ether. Evaporation of the ether layer yielded crude acids (305·2 mg.), which were taken up in ether (10 ml.). The solution was filtered (Celite) and evaporated to give PLA (195·7 mg.).

Repetition on four samples (12 g.) of lichen with hydroponic administration of glucose (10%; 150 ml.) gave PLA (186·4 mg.) which was combined with that already obtained, recrystallized from methanol (yield 277·9 mg.), and diluted with inactive material (to 577·9 mg.).

Degradation results are given in the Table. Multiple t.l.c. and recrystallization to constant activity were insufficient to give radiochemically pure PLA; results are therefore based on LA, which could be obtained pure by multiple t.l.c. and recrystallization (MeOH), and cross-checked as its methyl ester.

Feeding Experiments at Different Times of Year.—In addition to the preceding experiment with [1-^{14}C]acetate in August, other feeding experiments were done with 100 μC (0·1 mg.) of tracers (hydroponic technique with 10% glucose). In November, [1,4-$^{14}C_2$]succinic acid gave no incorporation, and in February, [1-^{14}C]acetate, [1,4-C_2]-succinic acid, [1-^{14}C]pyruvate, [2-^{14}C]pyruvate, and [1-^{14}C]-glucose (150 mg.; 100 μc fed in 150 ml. of water) all failed to give incorporation. In June, however, all precursors were incorporated; only the succinate run was verified by degradation (as follows).

Methyl 2-Methyl-4-oxoheptadecanoate (21) *derived from* [1,4-$^{14}C_2$]*Succinic Acid.*—Repetition of the hydroponic experiment with lichen (12 g.) and [1,4-$^{14}C_2$]succinic acid (100 μc; 0·1 mg.) gave PLA which was converted into LA as before. In this series, neither LA nor derived (conditions given under 2-methyl-4-oxoheptadecanoic acid) KA was amenable to the purification procedures used in the [1-^{14}C]-acetate series, so KA was esterified with diazomethane in ether. Multiple g.l.c. gave KA methyl ester (5·5 mg.), which was diluted with inactive material (102·7 mg.) and subjected to g.l.c. once more to give material (75·2 mg.), which was reconverted into the acid by heating with sodium hydroxide (10%; 5 ml.) and methanol (1 ml.) (100°; 1·5 hr.). Cooling and acidification (HCl), extraction with ether (5 × 5 ml.), and evaporation gave KA (70 mg.; $1·02 \times 10^6$ decomp. min.$^{-1}$ mol.$^{-1}$; 0·004% incorp.), which was further degraded to give (20) and (19) ($3·60 \times 10^5$ and $5·60 \times 10^5$ decomp. min.$^{-1}$ mol.$^{-1}$, respectively.

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