Biosynthesis of the Bufadienolide Ring of Scillirosid in *Scilla maritima*

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Administration of sodium [1,2-$^{14}C_2$]oxaloacetate and sodium [1-^{14}C]acetate to *Scilla maritima* plants yielded labelled scillirosid. A systematic degradation of scillirosid indicated that the α-pyrone ring of the bufadienolide is formed by the condensation of a pregnane derivative (derived from mevalonic acid *via* squalene) and one molecule of oxaloacetic acid.

It has been previously demonstrated that the biosynthesis of plant bufadienolides resembles that of cardenolides just to the formation of a C-21 steroidal precursor. Thus, Tschesche and Brassat [1] determined that pregnenolone is a good precursor of hellebrigenin in *Helleborus atrorubens* whilst we found [2] that the same precursor was incorporated into scillirosid in the plant *Scilla maritima*.

While it has been shown that the building of the lactone ring of cardenolides proceeds through the condensation of acetyl. CoA and a 20-keto-pregnane derivative [3–5], nothing was known about the origin of the three carbon-atoms needed to complete the six membered lactone ring of the bufadienolides.

We have recently reported [6] the results of feeding experiments using different potential precursors of the 3-carbon unit such as propionic acid, pyruvic acid, succinic acid and oxaloacetic acid (labelled at C-4) which were, however, poor precursors of scillirosid.

On the other hand, [1-^{14}C]acetic acid and [1,2-$^{14}C_2$]oxaloacetic acid [7] were incorporated into the bufadienolide in such an extent that they can be considered as precursors of scillirosid.

We wish now to report that degradation of the scillirosid obtained from *S. maritima* bulbs inoculated with [1,2-$^{14}C_2$]oxaloacetate indicates an incorporation of the two labelled atoms of the precursor into C-23 and C-24 of the lactone ring of the bufadienolide pointing to the biosynthetic pathway shown in Fig. 1. The results are complemented with those obtained by similar degradation of the scillirosid derived from a [1-^{14}C]acetate feeding experiment.

Results

Specimens of *S. maritima* were inoculated as indicated in Experimental with [1,2-$^{14}C_2$]oxaloacetic acid and [1-^{14}C]acetic acid in buffered aqueous soln and in separate experiments. The inoculations were done in December, almost one month before the flowering of the plant. It has been reported [8] and confirmed by us that at this time of the year, corresponding to June in the northern hemisphere, the concentration of scillirosid is maximal. The glycoside disappears during the flowering process. The values of tracers inoculated and scillirosid obtained are indicated in Table I. The radioactive scillirosid was isolated by known procedures [9] and degraded as illustrated in Fig. 2. Acetylation of scillirosid (**1**) afforded tetra-O-acetyl-scillirosid (**2**) [10] which was ozonized and the resulting ozonide reduced with zinc and acetic acid [5] yielding compound **3**, glycolic acid from C-23 and C-24, and formic acid from C-21 of the original lactone ring. The glycolic acid was cleaved with sodium periodate giving formaldehyde, collected as the dimedone derivative, and carbon dioxide collected as barium carbonate.

Compound **3** was oxidized with hydrogen peroxide and the resulting intermediate, not isolated, was treated with sodium periodate giving carbon dioxide from C-22 that was collected as barium carbonate. It was found that the bufadienolide derived from [1,2-$^{14}C_2$]oxaloacetate had the same specific activity at C-23 and C-24 whereas C-21 and C-22 were almost devoid of activity. Furthermore, scillirosid was treated with methyl iodide and

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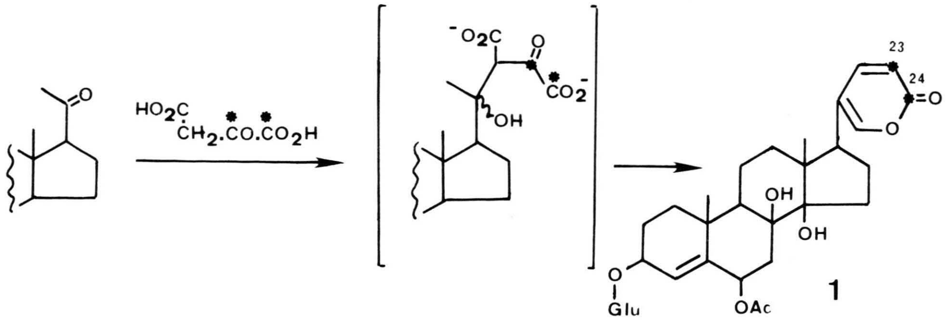

Fig. 1. Possible pathway for the formation of the bufadienolide ring of scillirosid. Labelled carbon-atoms are indicated with heavy dots. Glu: D-glucose; Ac: acetyl group.

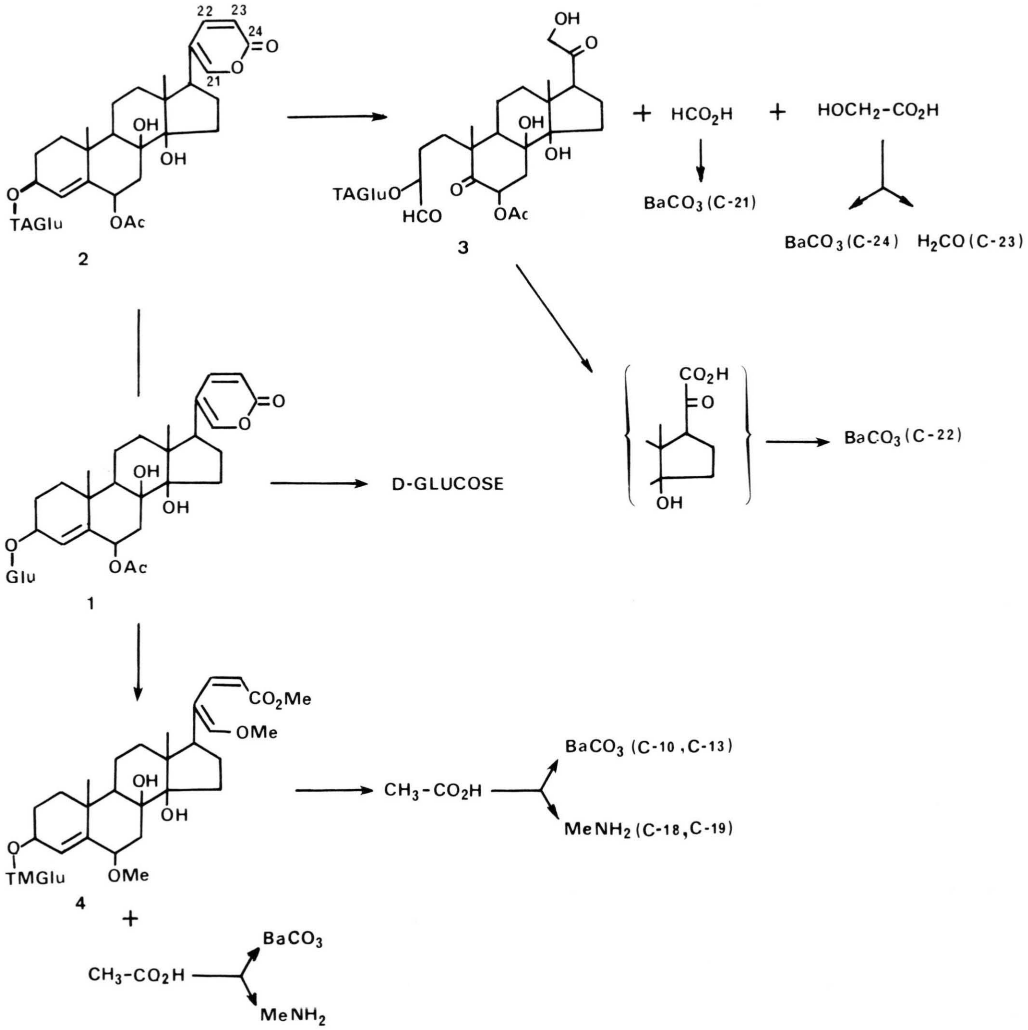

Fig. 2. Degradation of scillirosid (**1**). Glu: D-glucose; TAGlu: tetra-O-acetyl-D-glucose; TMGlu: tetra-O-methyl-D-glucose.

Table I. Administration of tracers to *S. maritima* plants.

Precursor fed	Wt [mg]	Spec. act. [dpm/mmol]	Wt [mg]	Scillirosid Spec. act. [dpm/mmol]	Incorp.[a] [%]
Sodium [1-^{14}C] acetate	2.1	1.3×10^{11}	80	1.0×10^{7}	0.040
Sodium [1,2-^{14}C$_2$] oxaloacetate	63.2	3.6×10^{8}	715	4.07×10^{4}	0.032

[a] Incorporation is defined as the total radioactivity present in the isolated scillirosid divided by the total radioactivity fed to the bulb.

Table II. Degradation products of scillirosid.

Product	Derived from [1,2-^{14}C$_2$]oxaloacetate		Derived from [1-^{14}C]acetate	
	Spec. act. [$\times 10^4$ dpm/mmol]	RSA[a] [%]	Spec. act. [$\times 10^4$ dpm/mmol]	RSA[a] [%]
Scillirosid	4.07	100	208	100
Tetra-O-acetyl scillirosid (**2**)	4.02	99	208	100
Compound **3**	3.75	92	201	97
Glycolic acid	0.33	8.1	6.24	3
Formaldehyde dimedone (C-23)	0.17	4.1	1.50	0.7
Barium carbonate (C-24)	0.16	3.9	5.40	2.6
Barium carbonate (C-22)	0.0081	0.2	0.21	0.1
Barium carbonate (C-21)	0.0082	0.2	0.21	0.1
Compound **4**	3.08	76	202	97
Acetate at C-6	0.94	23	4.10	2
Barium carbonate (carboxylic carbon of acetate at C-6)	0.79	19.4	3.50	1.7
N-methylbenzamide (methyl carbon of acetate at C-6)	0.15	3.8	0.21	0.1
Barium carbonate (C-10 and C-13)	0.24	5.9	11.8	5.7
N-methylbenzamide (C-18 and C-19)	0.036	0.9	0.81	0.4
D-glucose	0.45	11.1	79	38

[a] Relative specific activity.

barium oxide in dimethylformamide yielding the permethylated compound **4** [6] and acetic acid derived from the acetate group located at C-6. A Schmidt degradation on this acetate gave carbon dioxide and methylamine allowing the determination of the activity at both carbons. A Kuhn-Roth oxidation of compound **4** afforded acetic acid derived from the angular methyl groups (C-18 and C-19) and their adjacent carbons. A Schmidt degradation on this acetate gave the original carboxylic carbon as barium carbonate (C-10 and C-13) and the methyl groups as methylamine, collected as N-methylbenzamide. The activities of the degradation products are recorded in Table II.

Moreover, catalytic hydrogenation of scillirosid followed by acid hydrolysis of the reduced product [6] gave D-glucose whose activity value is also indicated in Table II.

Discussion

From the knowledge of the biosynthetic pathway to cardenolides, that is, via a pregnane derivative (C_{21}) to which one molecule of acetate is attached to form the butenolide ring, and the experimental evidences that a pregnane intermediate – like pregnenolone – is also precursor of plant bufadienolides, we assumed as a working hypothesis that the α-pyrone ring of scillirosid should be derived from a nucleophilic condensation – at the carbonyl group of a 20-keto-pregnane derivative – of a small molecule that would provide the 3-carbons needed to complete the ring. Looking for an appropriate molecule for this nucleophilic attack, we thought of pyruvate, succinate, oxaloacetate and even acetate via acetoacetate.

Experiments with pyruvate, succinate, and [4-^{14}C]oxaloacetate gave incorporations into scillirosid in very low proportions which prohibited any degradation of the bufadienolide [6]. On the other hand, [1-^{14}C]acetate and [1,2-$^{14}C_2$]oxaloacetate gave acceptable incorporation values considering that the compounds inoculated are biologically very active and should be involved in a large number of metabolic pathways. Besides its remarkable versatility, oxaloacetate results toxic to plant cells when present in concentrations higher than 20–40 molecules per mitochondria [11] and accordingly it should be rapidly degraded via direct decarboxylation [12, 13] or through Krebs cycle [14]. Further, the oxaloacetate would also follow known metabolic pathways to monosaccharides through phosphoenolpyruvate [15–17], and to acetate which would enter in the mevalonate route to steroids. This was confirmed by the activity values found in the acetate at C-6, and that derived from the angular methyl groups, and in the D-glucose attached at C-3. The figures (Table II) for C-10, C-13, C-18 and C-19 indicate a non-random incorporation of oxaloacetate through acetate into the steroid nucleus, comparable to the values obtained for the [1-^{14}C]acetate feeding experiment. The same conclusion was reached by Schmidt degradation of the acetate located at C-6 which possessed almost all the activity at the carboxylic carbon-atom indicating a metabolic double decarboxylation of oxaloacetate to acetate – via pyruvate – prior its incorporation into scillirosid by acetylation of the possible intermediate with a free hydroxyl group at C-6. It is interesting to remark the striking difference in the total activity of this acetate group according to the labelled source inoculated. The dissimilar values obtained could be attributed to the possibility for a fair amount of oxaloacetate to reach the site of biosynthesis before its transformation into acetate-diminishing the dilution that suffers the labelled acetate inoculated *per se*, but after the formation of the 6-desacetoxy-scillirosid which should be the last intermediate in the formation of scillirosid. In other words, if a portion of the oxaloacetate is transformed into acetate in the presence of the 6-hydroxy-bufadienolide, the labelling of the acetate attached to C-6 should be heavier than in other positions of the final scillirosid. The presence in *S. maritima* of scillirubrosid, a bufadienolide similar to scillirosid but lacking the allylic acetoxy group at C-6 [18], would support the hypothesis that the last step in the biosynthesis of scillirosid should be the introduction of the acetoxy group at C-6.

In spite of these different pathways which lead to the distribution of activity recorded in Table II, the labelled carbons 1 and 2 of oxaloacetate were incorporated into C-23 and C-24 of the α-pyrone ring of scillirosid in equal proportions pointing to the incorporation of the precursor as a unit; the almost negligible incorporation values at C-22 and C-21 in addition to the previous negative result obtained with [4-^{14}C]oxaloacetate [6] would confirm the mechanism proposed in Fig. 1.

Our results from the [1-^{14}C]acetate feeding experiment are in agreement with the previous conclusions because they discard the formation of the α-pyrone ring directly from acetate through mevalonate; if this were the case, we would expect to find the activity at C-23 higher than the activities at C-22 and C-24 which was not the case. Moreover, if acetate would be incorporated in the α-pyrone ring after conversion into oxaloacetate, this could just be through Krebs cycle and in that case oxaloacetate should be labelled at carbons 1 and 4, and therefore, C-24 should have higher activity than C-23 and C-22; this, in fact, was found to be the case.

Furthermore, the results recorded in Table II discard the hypothesis of acetoacetate – derived from [1-^{14}C]acetate – condensing with the pregnane intermediate to form the six-membered lactone ring of scillirosid.

Hence, bufadienolides are formed in plants by a mechanism analogous to the one encountered for cardenolides with the sole difference that the role played by acetate in cardenolide biosynthesis is in this case played by oxaloacetate. It is interesting to remark that animal bufadienolides are apparently biosynthesized by a different pathway [19].

Experimental section

General

Mps are uncorrected. TLC was carried out using Si gel G (Alugram Sil G/UV_{254}, Macherey-Nagel) or cellulose (DC Fertigplatten cellulose F, Merck). ^{1}H and ^{13}C FT-NMR at 100 MHz and 25.2 MHz respectively. Mass spectra at 70 eV by direct inlet. Radioactivity was measured by liquid scintillation counting as previously described [20].

Radiochemicals

Sodium [1-^{14}C]acetate was purchased from New England Nuclear Corp.; [1,2-$^{14}C_2$]oxaloacetic acid was prepared as already described [7].

Administration of tracers and isolation of scillirosid

Bulbs of *S. maritima* L. (red variety) growing in soil were inoculated in December with phosphate buffer soln of [1,2-$^{14}C_2$]oxaloacetic acid (63.2 mg, 3.6×10^8 dpm/mmol) and sodium [1-^{14}C]acetate (2.1 mg, 1.3×10^{11} dpm/mmol) in separate experiments. The tracer soln were administrated in four portions by filling up cavities (15 cm deep × 3 mm diameter) done in the bulbs by means of a borer and pluged afterwards with the same vegetal tissue. The operation was repeated during four consecutive days. After 7 days the bulbs, weighing 4.5 and 1.4 kg respectively, were harvested, dried and ground. The dried material (1.23 and 0.32 kg respectively) was extracted with MeOH and the extract was processessed as previously described [9] for the isolation of scillirosid (**1**). The amount and activity of the isolated scillirosid are indicated in Table I. Scillirosid (**1**) was acetylated (acetic anhydride-pyridine) affording tetra-O-acetyl-scillirosid (**2**) of mp 199–200 °C; ^{1}H NMR ($CDCl_3$): δ 0.82 (3 H, s, Me-18), 1.22 (3 H, s, Me-19), 1.92 (3 H, s, Me–CO), 1.97 (3 H, s, Me–CO), 2.00 (3 H, s, Me–CO), 2.30 (6 H, s, Me–CO), 4.20 (3 H, m, H-3 and CH_2–O), 4.54 (1 H, d, $J = 8$ Hz, HC–O), 4.70–5.30 (4 H, m, sugar protons), 5.41 (1 H, t, $J = 1$ Hz, H-6), 5.90 (1 H, bs, H-4), 6.16 (1 H, d, $J = 8$ Hz, H-23), 7.10 (1 H, d, $J = 2$ Hz, H-21), 7.76 (1 H, dd, $J = 8$ and 2 Hz, H-22); ^{13}C NMR ($CDCl_3$): δ 18.23 (C-19), 19.07 (C-18), 20.69, 20.83, 21.40, 21.60 and 22.22 (CH_3–CO), 37.20 (C-10), 49.40 (C-13), 62.05 (C-6′), 84.59 (C-14), 99.30 (C-1′), 115.43 (C-23), 122.83 (C-20), 130.22 (C-4), 141.81 (C-5), 146.75 (C-22), 148.44 (C-21), 162.40 (C-24), 168.29, 169.48, 169.60, 170.45 and 170.79 (CH_3–$\underline{C}$O); MS (m/z): 457 ($C_{26}H_{33}O_7$, aglycone-$O^{+\cdot}$), 331 ($C_{14}H_{19}O_9$, tetra-O-acetyl-glucosyl).

Ozonolysis of tetra-O-acetyl scillirosid (**2**)

a. *Derived from [1,2-$^{14}C_2$]oxaloacetate*

Radioactive compound **2** (129 mg, 4.2×10^4 dpm/mmol) was dissolved in methylene chloride (50 ml), the soln cooled to −60 °C, and ozone was passed through the soln for 60 min when TLC analysis revealed the complete disappearance of the starting compound. The soln was treated at room temp with zinc dust (400 mg) and 50% aqueous acetic acid (3 ml).

After stirring for 18 h, the zinc was filtered off and the filtrate was washed with water (3 × 10 ml) and with 0.1 N aqueous sodium hydroxyde (10 ml). The combined aqueous extracts were made basic (pH 9) with 1 N aqueous sodium hydroxyde and let aside until pptation of zinc hydroxyde; this was removed by filtration and the filtrate was made acidic with 6 N hydrochloric acid. The soln was distilled adding water to the distillation flask to maintain the volumen at about 30 ml. The distillate was collected over 0.1 N sodium hydroxyde (5 ml) until neutrality in the distillate (about 60 ml), and it was lyophilized. The residue, containing sodium acetate (from the acetic acid added to reduce the ozonide) and sodium formate (from C-21 of scillirosid), was treated with hydrogen peroxide (150 vol, 8 ml) and kept at 4 °C for 2 h. All traces of carbon dioxide were swept out of the soln by means of a stream of pure nitrogen and the system was connected to a set of traps containing aqueous barium hydroxyde; the soln was made acidic with 6 N hydrochloric acid and the liberated carbon dioxide (from C-21) was collected as barium carbonate (24.6 mg, 0.82×10^2 dpm/mmol). The acidic aqueous soln remaining in the reaction flask was lyophilized. The residue, containing glycolic acid, which had been detected in previous assays by preparation of its *p*-bromophenacyl derivative of mp 137–138 °C, was extracted with ether (3 × 8 ml), and the ethereal extract was evaporated to dryness. The residue (7.5 mg) was dissolved in a phosphate buffer (pH 5.8, 2 ml) and a soln of sodium metaperiodate (120 mg) in water (2 ml) was added. After 24 h, nitrogen was blown through the soln and the evolved stream was passed through a set of traps containing saturated aqueous dimedone soln, and aqueous barium hydroxyde soln which were separated by empty traps. The barium carbonate (derived from C-24) was thus collected (33.0 mg, 1.60×10^3 dpm/mmol). The formaldehyde-dimedone derivative (derived from C-23) (12.9 mg) had a spec. act. of 1.67×10^3 dpm/mmol.

The remaining methylene chloride extract was evaporated and the residue (89 mg) was purified by TLC affording compound **3**; ^{1}H NMR ($CDCl_3$): δ 0.92 (3H, s, Me-18), 1.28 (3H, s, Me-19), 2.02, 2.04, 2.08, 2.10 and 2.18 (15 H, five s, Me−CO), 4.20 (3H, m, H-3 and $HOC\underline{H}_2-CO$), 4.56 (1H, d, $J = 8$ Hz, H-1′), 5.40 (2H, t, $J = 1$ Hz, H-6), 9.50 (1H, s, −CHO at C-4); ^{13}C NMR ($CDCl_3$): in comparison with the spectrum of compound **2**, it showed the disappearance of the signals at δ 115.43, 122.83, 130.22, 141.81, 146.75, 148.44 and 162.40, and the appearance of new signals at δ 200.12 ($-\underline{C}HO$ at C-4), 208.21 ($-\underline{C}O-CH_2OH$) and 217.15 (−CO at C-5).

Radioactive compound **3** was dissolved in dioxane (2 ml), treated with hydrogen peroxide (150 vol, 2 ml) and the soln was kept at 0 °C for 30 min; while pure nitrogen was bubbled through the reaction mixture, a soln of sodium metaperiodate (250 mg) in water (0.6 ml) was added and the evolved carbon dioxide (derived from C-22) was collected as barium carbonate (20.1 mg, 0.81×10^2 dpm/mmol).

b. *Derived from [1-^{14}C]acetate*

Radioactive tetra-O-acetyl-scillirosid (93.4 mg, 2.08×10^6 dpm/mmol) was degraded as described above. The radioactivity of the degradation products are indicated in Table II.

Methylation of scillirosid.
Isolation of the acetate at C-6

A soln of active scillirosid (from the oxaloacetate feeding) (79.4 mg, 4.07×10^4 dpm/mmol) in dimethylformamide (0.7 ml) was treated with barium oxide (140 mg) and methyl iodide (0.2 ml) and the mixture was stirred at room temp for 24 h. Chloroform was added and the inorganic salts were filtered off. The filtrate was washed with 5% aqueous sodium thiosulphate soln, with water, dried over magnesium sulphate and evaporated. The residue (89.2 mg, 3.08×10^4 dpm/mmol) was identified as tetra-O-methyl-D-glucosyl-6-O-methyl-scillirosidine (**4**) [6]. The aqueous washings were made alkaline (0.1 N sodium hydroxyde), and extracted with ether which was discarded, and the remaining aqueous soln was lyophilized. The residue, containing the acetate originally located at C-6, was acidified and extracted with ether. The extract was neutralized and evaporated affording sodium acetate (15.1 mg, 0.94×10^4 dpm/mmol).

Schmidt degradation of sodium acetate

The previous sodium acetate (15 mg) dissolved in conc. sulphuric acid (1 ml) was mixed at 0 °C with sodium azide (50 mg). The mixture was warmed to 60 °C and the evolved carbon dioxide was collected as barium carbonate (12.6 mg, 0.79×10^4 dpm/mmol). The contain in the reaction flask was poured into ice-water (100 ml). This was made alkaline by addition of 10% aqueous sodium hydroxyde and distilled. The distillate was collected over 2 N hydrochloric acid and lyophilized. To the residue benzoyl chloride (1 ml) and 1 N aqueous sodium hydroxyde (4 ml) were added, the mixture was shaked vigorously and let aside overnight.

It was extracted with ether (3 × 3 ml) which was washed with water, dried over magnesium sulphate and evaporated. The N-methylbenzamide thus obtained (22.6 mg) was recrystallized from petrol yielding 18 mg of mp. 78−79 °C and spec. act. 1.50×10^3 dpm/mmol.

Similar reaction procedures (methylation and acetate degradation) were carried out with the scillirosid derived from the sodium [1-^{14}C]acetate feeding experiment. The results are shown in Table II.

Kuhn-Roth oxidation of compound **4**

Compound **4** (from the oxaloacetate experiment) (88.1 mg) was added to a soln of chromium trioxide (5 g) in 2 N sulphuric acid (10 ml) and the mixture was distilled. Water was added to the distillation flask to maintain the volumen at about 10 ml. When about 60 ml had been collected it was titrated with 0.1 N sodium hydroxyde and evaporated to dryness.

The residue was crystallized from EtOH-ether affording sodium acetate (19.5 mg). This acetate was submitted to a Schmidt degradation as above described yielding barium carbonate (19.3 mg, 2.40×10^3 dpm/mmol) from C-10 and C-13, and N-methylbenzamide (19.1 mg, 0.36×10^3 dpm/mmol) from C-18 and C-19.

Similar reactions on scillirosid derived from sodium [1-^{14}C]acetate afforded the same degradation products whose activity values are indicated in Table II.

Hydrogenation of scillirosid and hydrolysis of the reduced product

These reactions were conducted as described elsewhere [6]. The radioactivity values of the D-glucose, isolated as its penta-O-acetyl derivative, are shown in Table II.

Acknowledgements

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