Short Communication

Biosynthesis of Pinguisone in an Axenic Culture of the Liverwort Aneura pinguis

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Pinguisone accumulated in cultured gametophytes of Aneura pinguis to a significantly high level. A biosynthetic study on the formation of pinguisone was carried out by feeding [2,13C]-acetate to the cultured gametophytes. Pinguisone was labeled at an adequate level to determine the labeling positions by a 13C-NMR analysis. The labeling pattern indicated two-methyl migration and C–C bond cleavage of the main chain in farnesyl diphasphate in the formation of pinguisone.

Biosynthesis of the lower terpenoids in higher plants has been widely investigated.1) A biosynthetic experiment involving feeding selective 2H- and 13C-labeled precursors such as acetate and mevalonate (MVA) to higher plants, however, proved to be unexpectedly difficult in practice because of the frequently low and non-specific incorporation. In contrast to higher plants, our recent studies using the cell culture technique on liverworts2,3) have demonstrated that lower terpenes formed from exogenously supplied precursors (2H- and 13C-labeled) in cultured cells were labeled at an adequately high level to determine the biosynthetic pathway by GC-MS2) and NMR analyses.3) As an extension of our study, we investigated the biosynthesis of sesquiterpenes of the pinguisone type in axenic cultures of the gametophytes of Aneura pinguis.

Pinguisone (I) and its biosynthetically related compounds have been isolated from liverworts of the Metzgeriales including A. pinguis,4) and the Jungermanniales including Porella platyphylla,5) Tricholepis sacculata,6,7) and Ptychantus striatus.8) The absolute stereochemistry of I has been established by an X-ray analysis of its p-bromo-benzylidene derivative.9) Compound I is biosynthetically interesting since biogenesis of its structure is difficult to be simply explained in terms of the isoprene rule. We report here the production of pinguisone (I) in an axenic culture of the gametophytes of A. pinguis, and the incorporation of exogenously supplied [2-13C]-acetate into compound I.

An intact plant of A. pinguis was collected in Germany and identified by Dr. Mues (University of Saarlandes, Germany). An axenic culture of A. pinguis was induced from the surface-sterilized gametophytes. The cultures were grown in 200-ml flasks with 30 ml of a modified B5 liquid medium (pH 6.0) containing 5 g/liter of sucrose. The flasks were kept under continuous light of 2000 lux at 22°C and subcultured at intervals of 2 months. The incorporation of [2-13C]-acetate (99 atom% 13C) was carried out by feeding the acetate (5 mm) to the gametophytes in 100 × 30 ml of the B5 liquid medium without sucrose. The gametophytes were grown for 35 days, those grown in the medium without acetate (60 g, dry weight) and with acetate (11.5 g) then being harvested by filtration, air-dried and pulverized. The powdered materials were extracted with Et2O in Soxhlet apparatus, each Et2O solution then being concentrated in vacuo to dryness (2.32 g without acetate and 0.89 g with acetate). Non-labeled and 13C-labeled I were purified by chromatography on a Sephadex LH-20 column (25 mm i.d. × 150 cm) with CH3Cl2–MeOH (1:1) as the eluent, and then by vacuum liquid chromatography on a silica gel column (20 mm i.d. × 10 cm) with n-hexane–EtOAc (gradient) elution to give non-labeled (292.8 mg) and 13C-labeled I (50.8 mg). Purified non-labeled I was identified by a direct comparison with an authentic sample of I (1H- and 13C-NMR spectra, and EIMS, [M]+ and mp data), while 13C-labeled I was identified by its 1H spectrum. EIMS of labeled I showed the [M + H]+ ion at m/z 241 (1.4% relative intensity to [M]+ at m/z 232). The 13C assignment of I as indicated in the Table was confirmed by the long-range connectivity observed in the COLOC spectrum.

![Fig. Structure of Pinguisone (I).](image)

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Non-labeled</th>
<th>Incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.7</td>
<td>41.5 (14.8)</td>
</tr>
<tr>
<td>2</td>
<td>41.5</td>
<td>217.6</td>
</tr>
<tr>
<td>3</td>
<td>29.1</td>
<td>117.1</td>
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<tr>
<td>4</td>
<td>147.5</td>
<td>45.4</td>
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<tr>
<td>5</td>
<td>28.1</td>
<td>56.4</td>
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<tr>
<td>6</td>
<td>108.8</td>
<td>180.8</td>
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<tr>
<td>7</td>
<td>108.8</td>
<td>13.5</td>
</tr>
<tr>
<td>8</td>
<td>18.9</td>
<td>8.5</td>
</tr>
<tr>
<td>9</td>
<td>13.6</td>
<td>13.6 (15.3, d)</td>
</tr>
</tbody>
</table>

Figures in parentheses are 13C enrichment (atom% excess).

Table 13C-NMR Data for Natural and Biosynthetically 13C-Enriched Pinguisone (I)
of the non-labeled species.

Pinguisone (1) had accumulated in the cultured gametophytes of *A. pinguis* at a level almost identical to that in an intact plant from a natural source. Table summarizes the levels of $^{13}$C enrichment of each carbon, which were determined by the relative peak areas in $^{13}$C-labeled 1 to those in non-labeled 1. The $^{13}$C-enriched carbons were specifically distributed in the C-2, C-4, C-6, C-8, C-10, C-12, C-13, C-14, and C-15 positions as indicated by 1a in Scheme. The levels of $^{13}$C enrichment (11.3–15.3%) were higher when compared with those found in higher plants. Added acetate, however, had been incorporated at levels similar to those normally associated with fungal cultures. These facts indeed demonstrate that cultured gametophytes of the liverworts provide effective research tools for studying lower terpene biosynthesis.

Labeling pattern 1a conclusively excludes the biosynthetic pathway proposed by Tori *et al.* According to their proposal, pinguisone must be labeled as 1b. Two $^{13}$C–$^{13}$C couplings between C-4 and C-15 ($J_{CC}=35.4$ Hz) and between C-8 and C-12 ($J_{CC}=35.4$ Hz) clearly indicate that two (Me-13 and Me-15) of the four methyl groups had migrated in the formation of pinguisone. To account for the geochemistry and labeling pattern of 1, pinguisone must have been formed via the rupture of at least one C–C bond in the main chain of farnesyl diphosphate. We thus propose the simple but possible biosynthetic pathway to form pinguisone that is indicated in Scheme. A postulated decaline cation (2) may be formed via the formation of the C-3/C-12 (or C-13) and C-6/C-11 bonds in farnesyl diphosphate with the elimination of diphosphate. Decaline cation 2 is further converted to decaline cation 3 with the migration of two 1,2-methyls and a 1,2-hydride shift. Rupture of the C-9/C-10 bond in 3 and recyclization to form a cyclopentane ring give indane cation 4. Ion 4, which is supported by a direct linkage between non-labeled C-3 and C-9 in 1, is prone to further reaction (4 to 1). An alternative sequence to form indane cation 6 via decaline cation 5 may also be possible.

Further study is in progress to obtain direct evidence for the migration of methyl groups and a hydrogen, and for rupture of the C–C bond in the formation of pinguisone by using $^{13}$C- and $^2$H-labeled precursors.

References