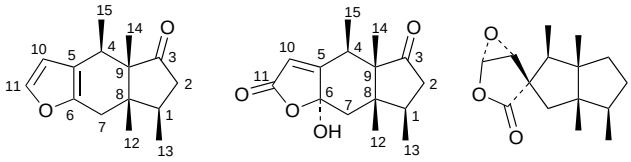
Stable isotope labelling studies on the biosynthesis of pinguisane-type sesquiterpenes in axenic cultures of *Aneura pinguis*

Hiroyuki Tazaki,*[a] Hiroshi Soutome,[a] Takeshi Iwasaki,[a] Kensuke Nabeta[a] and Duilio Arigoni[b]

[a] *Department of Bioresource Chemistry, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, 080 Japan*
[b] *Laboratorium fur Organische Chemie, ETH-Z, 8092 Zurich, Switzerland*

^{2}H and ^{13}C NMR analyses of 6α-hydroxy-3-oxopinguis-5(10)-ene-11,6-olide produced by axcenic cultures of the liverwort *Aneura pinguis* in the presence of ^{2}H- and ^{13}C-labelled mevalonates clarify the biosynthesis of pinguisane-type sesquiterpenes from FPP *via* a 1,2-hydride shift, two 1,2-methyl shifts, a cleavage of the main FPP chain and then recyclization.

Pinguisone **1**[1] and its biosynthetically related compounds have been isolated from many Metzgeriales and Jungermanniale liverworts.[2] They are biosynthetically interesting since the biogenesis of their structures is difficult to explain simply in terms of the isoprene rule. This led us to investigate the structures and biosynthesis of pinguisane-type sesquiterpenes. In previous work, we isolated three new pinguisane-type sesquiterpenes as co-occurring compounds[3] and proposed a biosynthetic pathway to **1** in an axenic culture of gametophytes of *Aneura pinguis* by feeding [2-^{13}C]acetate.[4] As a probe of the biosynthetic pathway of pinguisane-type sesquiterpenes, we administered labelled mevalonates ([4,5-$^{13}C_2$]-, [5-^{13}C]-, [2-$^2H_2$]- and [6-$^2H_3$]-MVA) to the cultured gametophyte of *A. pinguis*. The results revised the previous route.[4] Here we propose the most plausible biosynthetic pathway to form the pinguisane skeleton.



1 **2** **12**

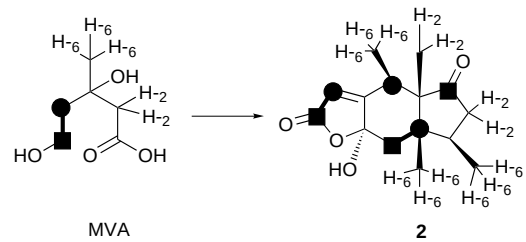
[4,5-$^{13}C_2$]-, [5-^{13}C]-, [2-$^2H_2$]- and [6-$^2H_3$]-MVA were prepared by procedures reported previously.[5] Axenic cultures of gametophyte of *A. pinguis* were grown in Gamborg B5 medium,[6] to which were fed 10.0 mmol of potassium MVA, under continuous light at 20 °C. 6α-Hydroxy-3-oxopinguis-5(10)-ene-11,6-olide **2** was the main sesquiterpene isolated from axenic cultures of *A. pinguis*. Compound **2** was biosynthesized from [4,5-$^{13}C_2$]-, [5-^{13}C]-, [2-$^2H_2$]- and [6-$^2H_3$]-MVA and isolated by HPLC as described previously.[3] Deviations in the levels of ^{2}H and ^{13}C enrichment (1.50–9.45 atom% excess) were observed among the differently labelled MVAs but may have been caused by variations in the incubation periods. Analysis of the resulting ^{2}H and ^{13}C labelled **2** (and its methyl ester) clearly showed the incorporation patterns illustrated in Fig. 1.

The presumed locations of ^{2}H at the C-12, C-13 and C-15 positions in **2**, originating from C-6 of MVA, and at the C-2 and C-14 positions in **2**, originating from C-2 of MVA, were confirmed by feeding [2-$^2H_2$]- and [6-$^2H_3$]-MVA. Deuterium incorporation in the methyl groups at the C-12 and C-15 positions shows that these originate from the C-6 of MVA and provided evidence for 1,2-methyl migrations from C-9 to C-8 and C-5 to C-4 in the formation of the pinguisane skeleton. On the other hand, feeding [2-$^2H_2$]-MVA revealed that, in contrast to previous route,[4] the methyl group at the C-14 originates from the C-2 of MVA.

In the ^{13}C NMR spectrum of **2** incorporating [4,5-$^{13}C_2$]-MVA, ^{13}C–^{13}C coupling was observed between C-7 and C-8 ($J_{C\text{-}7,C\text{-}8}$ = 35.4 Hz), and between C-10 and C-11 ($J_{C\text{-}10,C\text{-}11}$ = 64.7 Hz). The enhanced singlets at C-3 (δ_C 217.3, 1.47 atom% excess) and C-4 (δ_C 32.2, 2.27 atom% excess) originate from C-5 and C-4 of MVA, respectively. The presumed location of ^{13}C at the C-3 position in **2**, originating from C-5 of MVA, was confirmed by feeding [5-^{13}C]-MVA. These results indicated that a cleavage of the C-9–C-10 bond of FPP took place in the formation of pinguisane skeleton.

On the basis of these data, the biosynthetic pathway shown in Fig. 2 is proposed for the pinguisane-type sesquiterpenes of axenic cultures of *A. pinguis.* A postulated cyclized cation (**3**) may be formed *via* the formation of a C-6–C-11 bond from FPP without elimination of diphosphate. Cation **3** is converted to diphosphate **4** with the migration of a 1,2-methyl and a 1,2-hydride shift. Formation of the C-3–C-10 bond in **4** with the elimination of diphosphate gives the bicyclic cation **5**. Rupture of the C-9–C-10 bond of **4** and recyclization to form a cyclopentane ring in **5** gives the indane cation **6** which is further converted to the pinguisane skeleton by a 1,2-methyl shift. An alternative sequence, *i.e.* FPP → cation **13** → **5**, is excluded by the following reasons. The formation of **13** implies an unprecedented *cis*-addition to the isopropylidene double bond. The migration of the H-ligand in **13** would leave the positive charge on a forbidden bridgehead position. Furthermore, the migration of a 1,2-hydride shift from C-9 to C-8 in **13** would be predicted to result in the wrong stereochemistry for the secondary methyl group in the cyclopentane ring.

Recently, compound **11** has been isolated from the liverwort *Dicranolejeunea yoshinagana* (Hatt.) Mizut.[7] Compound **11** is most probably a biogenetic byproduct of a pinguisane-type sesquiterpene formed from cation **6** by a 1,2-vinyl shift. On the other hand, β-monocyclonerolidol **7** isolated from *Ptychanthus striatus* (Lehm. st Lindenb) Nees[8] which co-occurrs with striatene **8** and ptychanolide **12**, and the oxygenated sesquiterpene ricinocarpin A **9** from *Ricinocarpus natans*[9] is postulated to be derived from cation **3**. Similarly, striatene **8** may be also derived from the cyclohexene diphosphate **4**. In addition,



MVA **2**

Fig.1 ^{2}H and ^{13}C labelling of **2**

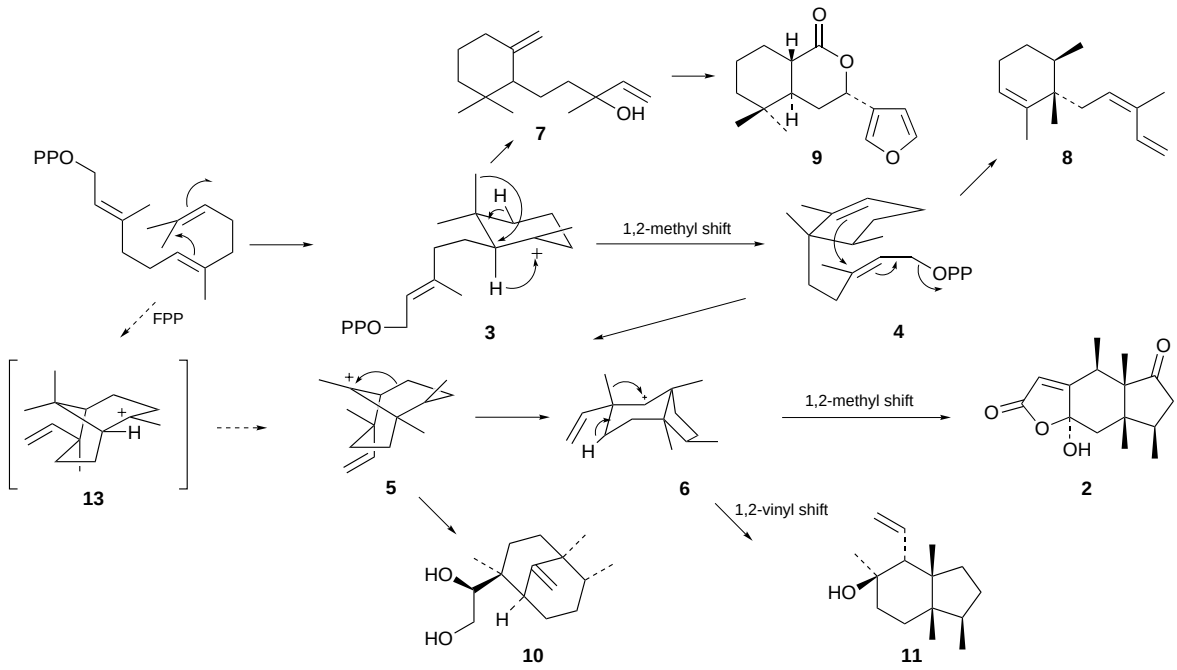


Fig. 2 Proposed biosynthetic pathway of pinguisane type and related sesquiterpenes

trifarienol A **10** isolated from *Cheilolejeanea trifaria* is postulated to be formed from cation **5**. Thus, the biosynthetic pathway illustrated in Fig. 2 seems to be widely occurring in liverworts which produce not only pinguisane-type sesquiterpene but also three other skeletal sesquiterpenes represented by **7**, **8** and **10**.

While the pathway shown in Fig. 2 satisfies the results of labelling studies, the sequence of steps involved need to be proven by enzymatic studies.

This research was supported financially by the Agricultural Chemical Research Foundation of the Japan Society for Bioscience, Biotechnology and Agrochemistry, the Suhara Memorial Foundation, and a Grand-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References

1 V. Benesova, Z. Samek, V. Herout and F. Sorm, *Collect. Czech. Chem. Commun.*, 1969, **34**, 1810.

2 Y. Asakawa, in *Progress in the Chemistry of Organic Natural Products*, ed. W. Hers, H. Griesbach, G. W. Kirby, R. E. Moore, W. Steglich and C. Tamm, Springer, Wein, 1995, vol. 65, pp. 1–618.

3 H. Tazaki, H. Soutome, K. Nabeta, H. Okuyama and H. Becker, *Phytochemistry*, 1996, **42**, 465.

4 H. Tazaki, K. Nabeta, H. Okuyama and H. Becker, *Biosci. Biotechnol. Biochem.*,1995, **59**, 158.

5 K. Nabeta, T. Ishikawa, T. Kawae and H. Okuyama, *J. Chem. Soc., Chem. Commun.*, 1995, 681; (*b*) K. Nabeta, T. Ishikawa and H. Okuyama, *J. Chem. Soc., Perkin Trans. 1*, 1995, 3111.

6 O. L. Gamborg, R. A. Miller and K. Ojima, *Exp. Cell. Res.*, 1968, **50**, 151.

7 M. Toyota, H. Koyama, T. Hashimoto and Y. Asakawa, *Chem. Pharm. Bull.*, 1995, **43**, 714.

8 R. Takeda, H. Naoki, T. Iwashita, K. Mizukawa, Y. Hirose, T. Isida and M. Inoue, *Bull. Chem. Soc. Jpn.*, 1983, **56**, 1125.

9 G. Wurzel and H. Becker, *Phytochemistry*, 1990, **29**, 2565.

10 T. Hashimoto, H. Koyama, S. Takaoka, M. Tori and Y. Asakawa, *Tetrahedron Lett.*, 1994, **35**, 4787.

Received, in Cambridge, UK, 14th March 1997; Com. 7/01784G