Cyclisation of Farnesyl Pyrophosphate to γ-Bisabolene in Tissue Cultures of *Andrographis paniculata*

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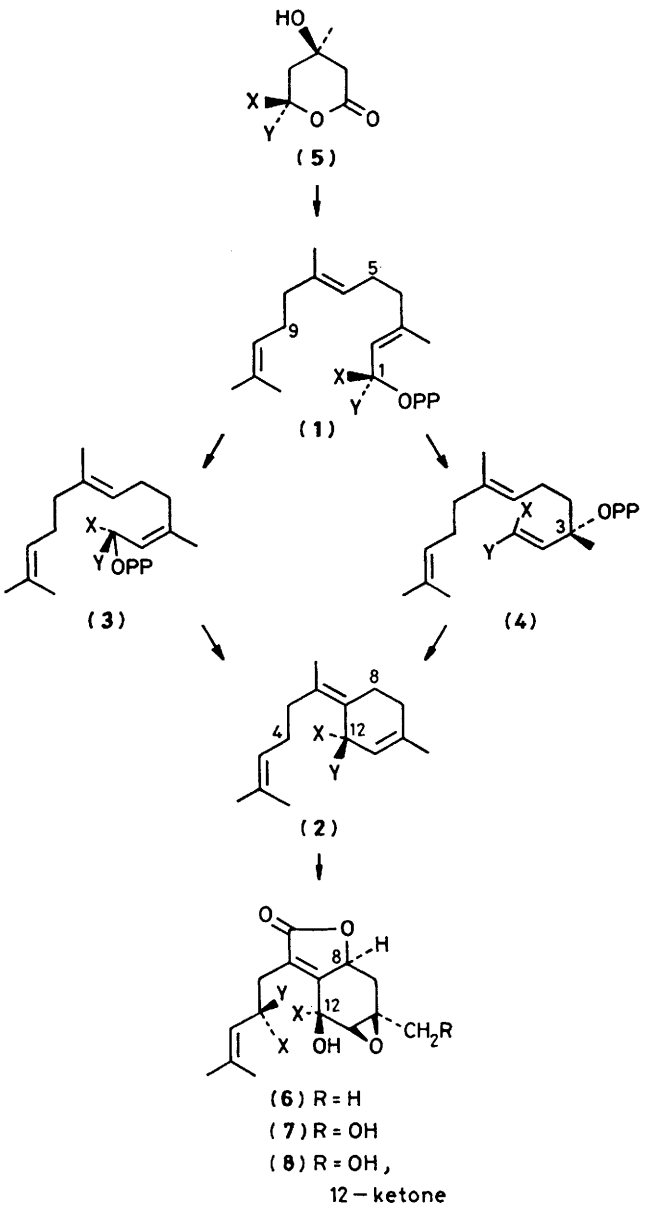
Tissue cultures of *Andrographis paniculata* transform *trans,trans*-[1-$^3H_2$; 12,13-$^{14}C_2$]farnesyl pyrophosphate, and (3*R*,5*R*)- and (3*R*,5*S*)-(5-^{3}H)-mevalonolactones into *Z*-γ-bisabolene without tritium loss; the absolute configuration of paniculide B (**7a**) has been determined by an *X*-ray crystal structure determination of its bis-*p*-bromobenzoate.

The cyclisation of allylic pyrophosphates to cyclohexenes, as exemplified by the conversion of *trans,trans*-farnesyl pyrophosphate (**1a**) into *Z*-γ-bisabolene (**2a**), is a major natural path for the construction of cyclic terpenoids. Formally, cyclisation must occur *via* either *cis,trans*-farnesyl pyrophosphate (**3a**) or nerolidyl pyrophosphate (**4a**). The actual biosynthetic intermediate may approximate to the delocalised ion pair accessible from (**1a**), (**3a**), or (**4a**).[1] We have previously shown[2] that a cell-free system from *Adrographis* tissue cultures converts *trans,trans*-farnesol into *cis,trans*-farnesol with loss of the *pro-S* and retention of the *pro-R* hydrogen atom at C-1 of *trans,trans*-farnesol. We now report that the *same* cell-free preparation will also convert *trans,trans*-[1-$^3H_2$,12,13-$^{14}C_2$]-farnesyl pyrophosphate (**1c**) into γ-bisabolene (**2c**) *without loss of tritium label from C-1*.

trans,trans-[1-$^3H_2$,12,13-$^{14}C_2$]Farnesyl pyrophosphate (**1c**) (2.63 μmol, 2.43 μCi ^{14}C μmol^{-1}; ^{3}H/^{14}C of diphenyl urethane recrystallised to constant activity 3.26 ± 0.07, atomic ratio 2:2)[3] was incubated with the 75 000 g supernatant (3 ml; 7.5 mg protein)[2a] from *Andrographis* cultures. The incubation was terminated after 17 h by addition of ethanol and then kept at 0 °C for 30 min. The denatured protein was removed and the supernatant extracted with n-hexane. Labelled γ-bisabolene was recovered from the extract after addition of carrier bisabolene (9 mg, regenerated from the trihydrochloride, m.p. 78—79 °C, with Li_2CO_3–LiBr in dimethylformamide; 2 major + 1 minor g.l.c. peaks were observed on OV-1 at 100 °C) by preperative t.l.c. (ethyl acetate–hexane, 1:3, R_f 0.8) (incorporation 0.23%), and converted into the trihydrochloride (dissolution in dry ether, HCl gas for 1 h at 0 °C). This was mixed with the carrier (18 mg, m.p. 78—80 °C) and crystallised (×3) from methanol to constant isotopic ratio (^{3}H/^{14}C 3.44 ± 0.28). The crystalline trihydrochloride (m.p. 78—80 °C) from the combined mother liquors had ^{3}H/^{14}C 3.61 ± 0.10.

The total retention of the tritium label parallels recent results concerning the *in vivo* formation of trichodiene from *trans,trans*-[1-$^3H_2$,12,13-$^{14}C_2$]farnesyl pyrophosphate by a cell-free system from *Trichothecium roseum*[3a] and of coccinol from [5-$^3H_2$,2-^{14}C]mevalonate by whole cells from *Fusidium coccineum*.[4] In both cases the initial cyclisation of *trans,trans*-farnesyl pyrophosphate to the γ-bisabolenyl cation occurs with complete retention of the tritium label. Extensive work by Croteau[5] suggests an analogous situation in the C_{10} series.

The incorporation of both hydrogen atoms at C-1 of *trans,trans*-farnesyl pyrophosphate into γ-bisabolene conflicts with our previous findings[6,7] that 1/6 of the tritium activity was apparently lost when the same cell-free system converted



Scheme 1. **a**; X = Y = H
b; X = T, Y = H
c; X = Y = T
d; X = H, Y = T

[5-$^3H_2$,2-^{14}C]mevalonate into γ-bisabolene.† This discrepancy has now been resolved and further insight gained into the detailed mechanism of the cyclisation step by additional experiments using (5*S*)- and (5*R*)-[5-^3H]mevalonates as substrates.

Thus (3*R*,5*R*)-[5-^3H][8a]-(**5d**) and (3*RS*)-[2-^{14}C]mevalonolactones were mixed ($^3H/^{14}C$ 1.08 ± 0.02; atomic ratio 1:1) and a portion was converted into the diphenylmethylamide[9] of constant m.p. 98—99 °C and $^3H/^{14}C$ 1.05 ± 0.02. The doubly labelled lactone was incubated (K salts; 3 μCi ^{14}C) with the cell-free system (4 ml) and the γ-bisabolene recovered (incorporation 0.1%), derivatised, and the trihydrochloride crystallised to constant isotopic ratio as above: $^3H/^{14}C$ 2.09 ± 0.03 [only (3*R*)-mevalonate metabolised]; atomic ratio 2.99:3, corresponding to 99.7% retention of tritium label.

(3*R*,5*S*)-[5-^3H][8b]-(**5b**) + (3*RS*)-[2-^{14}C]mevalonate (K salts; 0.6 μCi ^{14}C, $^3H/^{14}C$ 7.01; atomic ratio 1:1) was divided into two equal portions. One was incubated with the cell-free system, as for the (3*R*,5*R*)-isomer above. The recovered γ-bisabolene (incorporation 0.18%) afforded the trihydrochloride of constant isotopic ratio at $^3H/^{14}C$ 13.49, atomic ratio 2.89:3, corresponding to 96.3% retention of the tritium label. The other portion was incubated with callus tissue (dry weight 50 g), grown in suspension for 21 days following transfer from solid medium,[2,10] and the paniculides A, B, and C were harvested after 6 days by solvent extraction and purified by preparative t.l.c. (5% MeOH–$CHCl_3$). Crystallisation to constant isotopic ratio afforded paniculide A (**6b**),‡ m.p. 118—120 °C, $^3H/^{14}C$ 1.92:3,§ incorporation of ^{14}C 0.05%; paniculide B (**7b**),‡ m.p. 146—148 °C, $^3H/^{14}C$ 1.16:3,§ incorporation 2.28%; and paniculide C (**8b**),‡ oil, purified by preparative t.l.c. (5% MeOH–$CHCl_3$) had $^3H/^{14}C$ 0.93:3,§ incorporation 0.15%. Oxidation of paniculide B (MnO_2–benzene) afforded the oily paniculide C. Purified as above this had ^1H n.m.r. and mass spectra identical with those of the natural material and $^3H/^{14}C$ 1.01:3. The shortfall of tritium label in paniculide B ($^3H/^{14}C$ 1.16:3 instead of 2:3)§ is unexplained but consistent. Thus, with [5-$^3H_2$,2-^{14}C]mevalonate as the precursor, the biosynthesised paniculide B (**7c**) had $^3H/^{14}C$ 2.38:3 (instead of 3:3) and the paniculide C (**8c**) obtained from it with MnO_2 had $^3H/^{14}C$ 2.06:3.

As illustrated in Scheme 1 (3*R*,5*S*)-[5-$^3H_1$]mevalonate (**5b**) will lead[11] to *trans,trans*-(1*S*)-[1,5,9-$^3H_3$]farnesyl pyrophosphate (**1b**).‡ This will isomerise without hydrogen loss[12] to *cis,trans*-(1*R*)-[1,5,9-$^3H_3$]farnesyl pyrophosphate (**3b**).‡ Cyclisation, with inversion at C-1, then affords (12*S*)-[4,8,12-$^3H_3$]-γ-bisabolene (**2b**).‡ Alternatively, the nerolidyl pyrophosphate (**4b**) (or its C-3 epimer with T and H interchanged), an intermediate in the above *cis–trans* isomerisation,[12] cyclises by an *anti*-S_N' process[13] to the *same* (12*S*)-[4,8,12-$^3H_3$]-γ-bisabolene (**2b**). Hydroxylation with retention of configuration[14] must then afford paniculides A (**6b**) and B (**7b**), having the (12*S*) configuration, as shown, in which tritium is retained. Tritium *was* retained at C-12 (see above) and the predicted absolute configuration of natural paniculide B [as (**7a**)], previously inferred[15] from the c.d. of the corresponding ketone, has now been confirmed by *X*-ray analysis as follows: paniculide B bis-*p*-bromobenzoate, m.p. (from chloroform–light petroleum) 142—143 °C, was prepared from the diol and *p*-bromobenzoyl chloride in refluxing tetrahydrofuran in the presence of triethylamine and a catalytic amount of 4-dimethylaminopyridine. It was separated from the primary mono-*p*-bromobenzoate, m.p. 178—180 °C, by column chromatography over silica.

Crystal data: $C_{29}H_{26}Br_2O_7$, M = 646.3, monoclinic, a = 14.326(6), b = 4.826(2), c = 19.974(9) Å, β = 102.27(3)°. Space group $P2_1$, Z = 2. Cell dimensions and intensity data were obtained with an Enraf–Nonius CAD-4 diffractometer using monochromated Mo-K_α radiation. The structure was solved by standard techniques and refined to R = 0.041 (R_w = 0.044) for 2101 reflections having $I > 2\sigma(I)$. Hydrogen atoms were included. The data were corrected for absorption. Two structure factor calculations were performed in which the imaginary part of the anomalous dispersion was included for the first time, and in which there was no scale factor refinement. The first calculation using the previously deduced

† These misleading results may be attributable to the low, albeit consistent, counts obtained in these experiments; furthermore, the apparent loss of 1 in 6 tritium atoms per mol corresponds to an error of only 2.78% per labelled site.

‡ Only the isotopic labels relevant to the discussion are shown in Scheme 1.

§ C-8 label lost during lactone formation.

absolute configuration of paniculide B gave $R_w = 0.0522$ whilst the second calculation for the enantiomeric structure gave $R_w = 0.0552$. Using Hamilton's test[16] the *X*-ray results thus confirm the absolute configuration of paniculide B as in (**7a**) with a confidence limit exceeding 99.5%. Refinement of the preferred enantiomer converged at $R = 0.039$ ($R_w = 0.042$).¶ The corresponding factors for the enantiomer were 0.044 and 0.047.

Our experiments thus reveal that the cell-free system from *Andrographis* cultures contains two independent enzyme systems, whose functions partly overlap: (a) a *trans,trans*- to *cis,trans*-farnesol isomerase[2] and (b) a *trans,trans*-farnesyl pyrophosphate isomerase-cyclase.

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¶ The atomic co-ordinates for this work are available on request from the Director of the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW. Any request should be accompanied by the full literature citation for this communication.