Phytochemistry, 1976, Vol. 15, pp. 121–123. Pergamon Press. Printed in England

BIOSYNTHESIS OF PTEROSIDE B IN *PTERIDIUM AQUILINUM* VAR. *LATIUSCULUM*, PROOF OF THE SESQUITERPENOID ORIGIN OF THE PTEROSIDES*

HIROSHI HIKINO, TOSHIO MIYASE and TSUNEMATSU TAKEMOTO

Pharmaceutical Institute, Tohoku University, Aoba-yama, Sendai 980, Japan

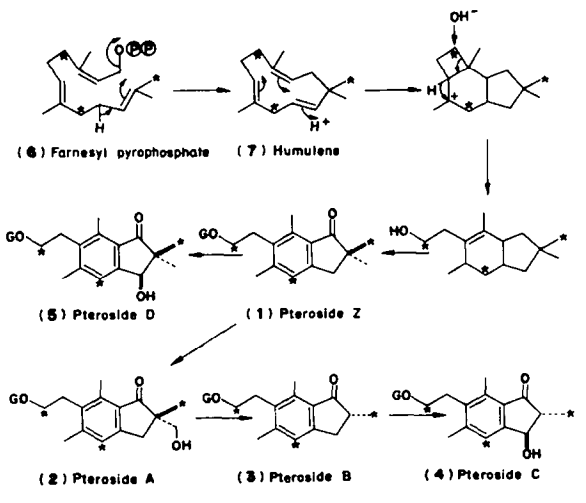
(*Received* 29 *May* 1975)

Key Word Index—*Pteridium aquilinum*; Dennstaedtiaceae; pteroside B; Sesquiterpenes; mevalonate; biosynthesis.

Abstract—Pteroside B was isolated in radioactive form after administration of [2-^{14}C]mevalonate to *Pteridium aquilinum* var. *latiusculum*, demonstrating that the biosynthesis of the aglycone proceeds through the ordinary pathway to sesquiterpenoids. Kuhn–Roth oxidation of the radioactive aglycone was carried out to examine the distribution of the radioactivity among the 3 methyls of the aglycone. The biosynthetic implications of these results are discussed.

INTRODUCTION

Recently we [1] have shown the occurrence of a series of glycosides, pteroside A–D and Z (Scheme 1) in the underground part of the Japanese bracken, *Pteridium aquilinum* Kuhn var. *latiusculum* Underwood. Further 1-indanone derivatives have later been isolated from the same plant [2]. After the structural elucidation of these glycosides had shown them to have formulae **1–5**, the biosynthesis of the aglycone moiety possessing the unique carbon skeleton shown became a subject of interest. Although many different hypotheses can be put forward to account for their origin, it was thought probable that they are sesquiterpenoids in origin. Accordingly we have examined the incorporation of mevalonic acid into the aglycones to see whether they are of terpenoid origin.

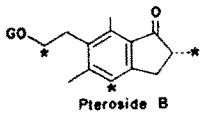
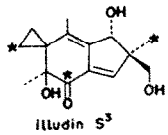


* Part 13 in the series on Biochemical Synthesis. This paper also forms Part 51 in the series on Sesquiterpenoids.

RESULTS

DL-[2-^{14}C]Mevalonolactone (5·85 mCi/mmol, 1·26 × 10^7 dpm) was administered late in August into rhizomes of the bracken ferns which were growing in the herbal garden. The plants were allowed to grow for 1 month further and then harvested. Extraction of the rhizomes with boiling methanol gave an extract (9·22 × 10^6 dpm) which was then taken to near dryness and the solid extracted with ethyl acetate to afford a solution (6·74 × 10^6 dpm). Repeated chromatography of this ethyl acetate extract over silica gel furnished a crystalline substance (7·23 × 10^4 dpm) which on TLC gave only a single spot having the same mobility as pteroside B(3), the main glycoside. The isolated pteroside B was subjected to repeated crystallization (6 in all) without lowering the specific activity of the labelled compound (4·34 × 10^6 dpm/mmol). The identity of the radioactive substance with pteroside B was confirmed by acetylation and by hydrolysis which gave the radioactive derivatives corresponding chromatographically to the tetraacetate and the aglycone, respectively. The total incorporation ratio was 0·2% calculated on the basis that only one stereoisomer of DL-mevalonate (*R*(+)-mevalonate) is biologically active. In order to examine the seasonal dynamics of the biosynthesis of the pterosides, DL-[2-^{14}C]mevalonolactone (10·3 mCi/mmol, 1·10 × 10^8 dpm) was also fed early in June to the rhizomes of bracken and pteroside B isolated after 2 weeks cultivation. The pteroside B thus obtained was enzymically hydrolyzed to the aglycone which after dilution with carrier was submitted to repeated crystallization to the constant specific activity (0·98 × 10^6 dpm/mmol). The total incorporation ratio this time was 0·7%. These ratios coupled with the difference of the incubation periods in the 2 experiments indicate that the accumulation of pteroside B in the rhizomes is much more rapid in early than in late summer. The relatively high rate of incorporation of mevalonic acid into pteroside B indicated that the aglycone is most probably of terpenoid origin.

Table 1. Sp act of acetic acid obtained by Kuhn–Roth oxidation

Parent substance (dpm/mmol)	Acetic acid Found (dpm/mmol) and ratio	Acetic acid Calculated (dpm/mmol) and ratio
 Pteroside B (3·15 × 10⁶)	0·496 × 10⁶ (1·41/9)	0·350 × 10⁶ (1/9)
 Illudin S[5] (2·12 × 10⁵)	0·236 × 10⁵ (1·01/9)	0·233 × 10⁵ (1/9)

Accepting that the aglycones of the pterosides are true terpenoids, a possible metabolic pathway may be considered for the biosynthesis of the pterosides from mavalonic acid *via* farnesyl pyrophosphate (**6**) and humulene (**7**) as depicted in Scheme 1. No definite assumption can be made, however, as to the stages when oxidation at C-1 and the formation of the glucoside linkage occur.

In order to obtain support for this hypothesis, the radioactive aglycone was subjected to Kuhn-Roth oxidation [3] furnishing acetic acid which was characterized as the *p*-bromophenacyl ester. Comparison of the specific activities of the aglycone and the resulting acetic acid revealed that the specific activity of the latter is 1·41/9 of that of the former (Table 1). Since only one of the 3 methyl groups in the aglycone would be labelled from [2-^{14}C]-mevalonolactone, the specific activity of acetic acid generated by Kuhn–Roth oxidation would be expected to be 1/9 of that of the parent substance. Taking into account the fact that the conversion rate of an aromatic methyl into acetic acid by Kuhn–Roth oxidation is rather low, the ratio (1·41/9) of the specific activities above obtained is considered to indicate the C-10 aliphatic methyl carbon is labelled. This finding further provides evidence for the postulated pathway (Scheme 1). Of the 2 geminal dimethyls in pteroside Z (**1**) the α-oriented methyl is enzymatically oxidized to give pteroside A (**2**), which on biogenetic reverse aldol condensation releases the hydroxymethyl forming pteroside B (**3**). The above observation coincides with that in illudin S which is assumed to be formed in *Clitocybe illudens* Schw. through a similar biosynthetic pathway, one third of the total radioactivity being present at the methyl situated at C-10 in the α-configuration in the labelled illudin S produced by feeding *C. illudens* with [2-^{14}]-mevalonic acid[4] (Table 1).

The pterosides thus provide rare examples of sesquiterpenoid glycosides. Since the bracken fern contains pairs of antipodal 1-indanone derivatives [2], the stereochemical multiplicity of the enzymes participating in the biosynthesis of the sesquiterpenoids in this plant is indicated.

EXPERIMENTAL

TLC was performed on Si gel G plates. Radioactivity was measured on a Packard Tri-Carb Liquid scintillation spectrometer, model 3380, equipped with absolute activity analyzer, model 544.

Administration of radioactive mevalonic acid to Pteridium aquilinum *var.* latiusculum *early in autumn.* DL-[2-^{14}C]Mevalonolactone (5·85 mCi/mmol) in H_2O was injected late in August to the rhizomes of *Pteridium aquilinum* var. *latiusculum* grown in soil at a total dose of 1·26 × 10^7 dpm.

Isolation of radioactive pteroside B. One month after the treatment, rhizomes were harvested and extracted 8 × with refluxing MeOH for 4 hr (each extraction). The combined MeOH soln was concentrated to yield an extract (9·22 × 10^6 dpm), which on continuous extraction with AcOEt and evaporation gave a residue (6·74 × 10^6 dpm). Repeated chromatography of the residue over Si gel and elution with C_6H_6–EtOAc (1:2) furnished a pteroside B fraction. After removal of β-sitosteryl glucoside as an insoluble precipitate by treatment with cold MeOH, the MeOH soluble portion was again subjected to Si gel chromatography. Elution with $CHCl_3$–MeOH (10:1) afforded pteroside B as crude crystals (139 mg, 7·23 × 10^4 dpm). Pteroside B thus obtained (2·07 × 10^5 dpm/mmol) was recrystallized 4× from MeOH and MeOH–AcOEt (saturated with H_2O) to give the radioactive pteroside B having a sp act of 4·34 × 10^4 dpm/mmol, which showed on TLC only a single spot corresponding to pteroside B by coloration (revealed by H_2SO_4) as well as by scanning for radioactivity. On further recrystallizations from MeOH and MeOH–AcOEt (saturated with H_2O), there was no change in sp act 4·10 × 10^4 dpm/mmol after the 5th crystallization from MeOH and 4·34 × 10^4 dpm/mmol after the 6th crystallization from MeOH–EtOA (sat. with H_2O).

Acetylation of radioactive pteroside B. Radioactive pteroside B (1 mg) was acetylated with Ac_2O and Py in the customary manner to furnish pteroside B tetraacetate, TLC (developed by $CHCl_3$–AcOEt (17:3): R_f 0·50 (revealed by I_2) same as the authentic sample). The scan of the plate for radioactivity showed only one peak at R_f 0·50.

Hydrolysis of radioactive pteroside B. Radioactive pteroside B (1 mg) in H_2O (1·5 ml) was treated with a β-glycosidase preparation from *Aspergillus niger* at room temp. for 25 hr. Isolation with EtOAc gave the radioactive aglycone, TLC (developed by $CHCl_3$–MeOH (20:1): R_f 0·82 (revealed by I_2) same as the co-chromatographed sample). No radioactivity was observed other than the peak corresponding to the authentic sample.

Administration of radioactive mevalonic acid to Pteridium aquilinum *var.* latiusculum *early in summer.* DL-[2-^{14}C]Mevalonolactone (10·3 mCi/mmol) in H_2O was injected early in June to rhizomes of *Pteridium aquilinum* var. *latiusculum* grown in soil in a total dose of 1·10 × 10^8 dpm.

Isolation of radioactive aglycone. Two weeks after the administration, rhizomes were harvested and extracted 5 × with hot MeOH. The MeOH extract (4·45 × 10^7 dpm) was continuously extracted with EtOAc yielding an extract (3·36 × 10^7 dpm). Si gel chromatography of the extract and elution with $CHCl_3$–MeOH (20:1) gave a portion which on preparative TLC [developed by $CHCl_3$–MeOH (5:1)] furnished radioactive pteroside B as crude crystals (49 mg, 8·91 × 10^5 dpm). The pteroside B (7·23 × 10^6 dpm/mmol) in H_2O was stirred with the β-glycosidase at room temp. for 20 hr. After extraction with EtOAc, the product was submitted to preparative TLC [developed by $CHCl_3$–MeOH (20:1)] to give the aglycone (15·6 mg, 5·27 × 10^5 dpm). The product (15·56 mg, 7·06 × 10^6 dpm/mmol) was dil with cold aglycone (36·08 mg) and crystallization 4 × from MeOH gave the radioactive aglycone possessing a sp act of 1·03 × 10^6 dpm/mol. The aglycone was further crystallized from the same solvent with no change in specific activity (0·97 × 10^6 dpm/mmol and 0·98 × 10^6 dpm/mmol after the 5th and 6th recrystallizations from MeOH).

Kuhn–Roth oxidation of radioactive aglycone. The radioactive aglycone (0·31 mg, 0·99 × 10^6 dpm/mmol) was dil with cold aglycone (7·77 mg) and treated with CrO_3–H_2SO_4, prepared according to Garbers [3], and H_2O free from CO_2, at 160° to obtain a distillate (*ca* 100 ml). After neutralization with 0·004 *N* NaOH, solvent was distilled off yielding NaOAc as crude crystals. Addition of *p*-bromophenacyl bromide

(28 mg) in EtOH (1·3 ml) and heating under reflux for 45 min gave the product which on preparative TLC (developed by C_6H_6–AcOEt (19:1)) furnished the acetate (20 mg) showing on TLC a single spot, coinciding with the authentic sample. Crystallization from MeOH afforded *p*-bromophenacyl acetate having a constant sp act of $5{\cdot}97 \times 10^3$ dpm/mmol.

REFERENCES

1. Hikino, H., Takahashi, T., Arihara, S. and Takemoto, T. (1970) *Chem. Pharm. Bull.* (*Tokyo*) **18,** 1488; Hikino, H., Takahashi, T. and Takemoto, T. (1971) *Chem. Pharm. Bull.* (*Tokyo*) **19,** 2424; Hikino, H., Takahashi, T. and Takemoto, T. (1972) *Chem. Pharm. Bull.* (*Tokyo*) **20,** 210.
2. Kuroyanagi, M., Fukuoka, M., Yoshihira, K. and Natori, S. (1974) *Chem. Pharm. Bull.* (*Tokyo*) **22,** 2762 and refs. cited therein.
3. Garbers, C. F., Schmid, H. and Karrer, P. (1954) *Helv. Chim. Acta* **37,** 1336.
4. Anchel, M., McMorris, T. C. and Singh, P. (1970) *Phytochemistry* **9,** 2339.