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A *p*-DIPHENOL OXIDASE FROM GYMNOSPERMS*

R. C. CAMBIE

Department of Chemistry, University of Auckland, New Zealand

and

SHEILA M. BOCKS

Dyson Perrins Laboratory, Oxford

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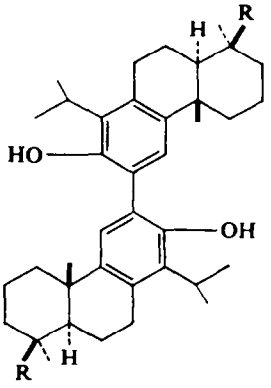
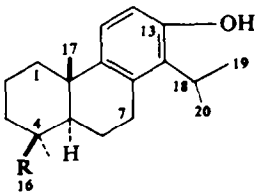
Abstract—A survey of the leaves of thirty species of the family Podocarpaceae and of fourteen other gymnosperms for the presence of a *p*-diphenol oxidase has been carried out. The partially purified enzyme, isolated from the leaves of *Cryptomeria japonica*, has been used for the oxidative coupling of totarol to form podototarin. Taxonomic aspects of the survey are discussed.

RECENTLY we reported[1] the use of an extracellular oxidase from the fungus *Polyporus versicolor*[2,3] for the preparation of the bisditerpenoids podototarin (I, R = CH_3)[4] and 16,16'-dihydroxypodototarin (I, R = CH_2OH)[1] from totarol (II, R = CH_3) and 16-hydroxytotarol (II, R = CH_2OH), respectively. From these results it appears likely that an enzymic coupling of a similar type is involved in the biosynthesis of podototarin and of macrophyllic acid (I, R = CO_2H)[1] in *Podocarpus totara*† and *P. macrophyllus* respectively, and thus it was



(I)



(II)

* A preliminary report of a portion of this work has been published: S. M. BOCKS and R. C. CAMBIE, *Proc. Chem. Soc.* 143 (1963).

† Podototarin has also been isolated from *Podocarpus mannii* (D. A. H. TAYLOR, *J. Chem. Soc.* 1553 (1963)) and *P. macrophyllus* (T. Takahashi, personal communication).

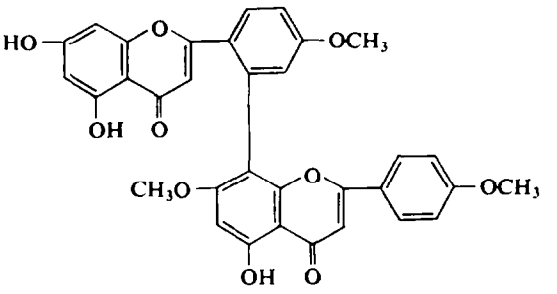
[1] S. M. BOCKS, R. C. CAMBIE and T. TAKAHASHI, *Tetrahedron* **19**, 1109 (1963).

[2] B. R. BROWN and S. M. BOCKS, In *Enzyme Chemistry of Phenolic Compounds* (Edited by J. B. PRIDHAM), p. 129. Pergamon Press, Oxford (1963).

[3] G. BENFIELD, S. M. BOCKS, K. BROMLEY and B. R. BROWN, *Phytochem.* **3**, 79 (1964).

[4] R. C. CAMBIE, W. R. J. SIMPSON and L. D. COLEBROOK, *Tetrahedron* **19**, 209 (1963).

of interest to examine these and related species for the presence of an enzyme capable of oxidative coupling. Furthermore, the occurrence of the biflavonyl kayaflavone (III) in the leaves of three *Podocarpus* species including those of *P. macrophyllus* has been reported,[5,6]



(III)

and it has been suggested that such biflavonyls are probably produced in the plant by oxidative coupling of flavonoid precursors.[6] It was considered that a successful demonstration of the presence of an enzyme catalyzing such coupling reactions in the leaves would support such a suggestion.

RESULTS AND DISCUSSION

In order to carry out a survey of species for the presence of a coupling enzyme it has been found convenient to examine the leaves rather than other parts of the plants since this enabled the examination of many more species than would otherwise have been possible if, for example, heartwoods were employed as the source material. As yet, bisditerpenoids have not been isolated from the leaves nor biflavonyls from the heartwoods of any gymnosperms, [6]and the above approach suffers from the disadvantage that results gained from an examination of leaf tissues need not necessarily have a bearing on biosynthetic sequences in other parts of a plant. However, while little direct evidence as to the mode of coupling of diterpenoids to form bisditerpenoids would be gained from an examination of the leaves, the establishment of a correlation between the presence in the same plant of both a coupling enzyme and biflavonyls could be of chemotaxonomic significance.

By using 2,6-dimethoxyphenol as the test substrate,* buffered aqueous extracts of the fresh leaves of thirty species of the Podocarpaceae and of fourteen other gymnosperms were examined for the ability to catalyse the formation of 3,5,3′,5′-tetramethoxydiphenoquinone. Of these species twenty were found to contain such an enzyme and the detailed results are presented in Table 1. If the fresh extracts were filtered through Hyflo-Supercel no activity was detected in the filtrates of those extracts which initially gave positive tests, indicating that the enzyme was attached to particulate fractions and was not present as a soluble enzyme in the leaf cells. However, if the solutions were kept at 0° for a few days activity was then observed in filtered solutions. The activity of fresh preparations was enhanced by the addition of calcium chloride indicating that the enzyme was probably present in the mitochondria.[7] In

* The advantages of using 2,6-dimethoxyphenol as an assay substrate have been detailed by Brown and Bocks.[2]

[5] T. SAWADA, *J. Pharm. Soc. Japan* **78**, 1023 (1958).
[6] W. BAKER and W. D. OLLIS, In *Recent Developments in the Chemistry of Natural Phenolic Compounds* (Edited by W. D. OLLIS) p. 152. Pergamon Press, Oxford (1961).
[7] W. B. TAPLEY, *J. Biol. Chem.* **222**, 325 (1956).

all cases where *p*-diphenol oxidase activity was observed tests indicated that the enzyme solutions did not require added hydrogen peroxide for their action and peroxidase[8] was thus assumed to be absent.

TABLE 1. TESTS FOR *p*-DIPHENOL OXIDASE

No.	Species	Presence of enzyme	No.	Species	Presence of enzyme
	Podocarpus			Dacrydium	
1	*P. acutifolius* Kirk	+	22	*D. biforme* Pilger	+
2	*P. alpinus* R. Br.	+	23	*D. bidwillii* Hook.	+
3	*P. andinus* Poeppigg	+	24	*D. colensoi* Hook.	+
4	*P. dacrydioides* A. Rich.	−	25	*D. cupressinum* Lamb.	−
5	*P. elatus* R. Br.*	+	26	*D. "farnellii"*†	+
6	*P. falcatus* R. Br.	−	27	*D. franklini* Hook.	−
7	*P. ferrugineus* G. Benn.	−	28	*D. intermedium* Kirk	+
8	*P. gracilor* Pilger	−	29	*D. kirkii* F. Muell.	+
9	*P. hallii* Kirk	−	30	*D. laxifolium* Hook.	+
10	*P. henckelii* Stapf.	−			
11	*P. latifolius* R. Br.	−		Miscellaneous	
12	*P. macrophyllus* D. Don	+	31	*Agathis australis* Salisb.	−
13	*P. milanjianus* Rendle	+	32	*Agathis robusta* Masters	−
14	*P. montanus* Loddiges	+	33	*Araucaria araucana* K. Koch	−
15	*P. nagi* Zoll. et Moritz	+	34	*Araucaria bidwillii* Hook	−
16	*P. nivalis* Hook.	+	35	*Cedrus libani* Lond.	−
17	*P. nubigenus* Lindley	−	36	*Cephalotaxus drupacea* S. et Z.	−
18	*P. salignus* D. Don	−	37	*Cryptomeria japonica* D. Don	+
19	*P. spicatus* R. Br.	+	38	*Cupressus lawsoniana* Murray	−
20	*P. totara* G. Benn.	+	39	*Cunninghamia sinensis* R. Br.	+
21	*P. usambarensis* Pilger	−	40	*Libocedrus chilensis* Endl.	−
			41	*Libocedrus decurrens* Torrey	−
			42	*Sciadopitys verticillata* S. et Z.	−
			43	*Taxus baccata* Linn.	−
			44	*Torreya nucifera* S. et Z.	−

* Sample from Oxford Botanic Gardens. A second sample from Kew Botanic Gardens gave a negative test.
† Putative hybrid between *D. laxifolium* and *D. intermedium*.
Samples 2, 3, 5, 6, 8, 11, 13, 18, 27, 33, 34, and 43 were obtained from authenticated species grown in the Oxford Botanic Gardens; samples 4, 10, 14, 17, 21, and 35 from the Royal Kew Botanic Gardens; samples 12, 15, 36, 39, and 44 from the Government Forest Experiment Station, Meguro, Tokyo; samples 9, 16, and 20 from the N.Z. Forest Survey, Westland, New Zealand; samples 37, 40, 41 and 42 from Bagley Wood, Oxford; and samples 1, 7, 19, 22, 23, 24, 25, 26, 28, 29, 30, 31, 32, and 38 from the Auckland or Central areas of the North Island, New Zealand.

Attempts were made to isolate the enzyme from the leaves of selected species, viz. *Podocarpus totara*, *P. nagi*, *P. macrophyllus*, and *Cryptomeria japonica*, which were available in larger quantity and extracts of which showed relatively high enzymic activity. Where possible, solutions of the crude enzyme were examined along lines used for the study of the enzyme from *Polyporus versicolor*.[2,3] The enzyme from the gymnosperns differed from that obtained from *P. versicolor* and from laccase of the laquer trees *Rhus vernicifera* and *Rhus succedana*[3,9] in being considerably more unstable under the conditions employed and only a partial purification was achieved. In other respects, however, including its action on

[8] K. FREUDENBERG, H. REZNIK, H. BOESENBERG and D. RASENACK, *Ber. Chem.* **85**, 641 (1952).
[9] G. BERTRAND, *Compt. Rend.* **120**, 266 (1895); **122**, 1132 (1896); D. KEILIN and T. MANN, *Nature* **143**, 23 (1939).

phenolic substrates it is similar to these latter enzymes being aerobic in action and its activity being strongly inhibited by the addition of sodium azide.[3] All activity was destroyed on boiling solutions for five minutes but dialysis against sodium acetate buffer (0·001 M; pH 4·0) had no adverse effect. From a spectroscopic examination of the products from its action on substrates such as catechol, 2,6-dimethoxyphenol, pyrogallol, and *p*-cresol it appears that the enzyme has a range of specificity similar to that of the enzyme from *Polyporus versicolor* and is a copper oxidase[10] of the type described as a *p*-diphenol oxidase.[11,*]

A study of the effect of pH on the activity of the crude enzyme from two species, *Podocarpus nagi* and *Cryptomeria japonica*, showed that the optimum pH for coupling of 2,6-dimethoxyphenol was about pH 4·0. This value lies outside the physiological range and in this respect and in its pH profile the leaf enzyme resembles the fungal enzyme[3] rather than laccase.[12] Surprisingly, extracts of *Cryptomeria japonica* were found to readily attack tyrosine. Further examination revealed that the pH optimum for this activity, viz. pH 6–8, differed from that of the *p*-diphenol oxidase and thus it was concluded that tyrosinase[13] was also present in the leaves.

Use was made of the partially purified *p*-diphenol oxidase from *Cryptomeria japonica* for the coupling of totarol (II; $R=CH_3$) to form podototarin (I; $R=CH_3$). Incubation of the enzyme with a 10% ethanolic solution of totarol in sodium acetate buffer at pH 4·0 and 30°, followed by the addition of further enzyme at forty-eight hour intervals over a period of two weeks, gave a 58 per cent yield of podototarin which was isolated and identified by comparison with a natural sample. The appearance of a peak in the ultraviolet spectra at 254–256 mμ, associated with a biphenylic system, indicated that other derivatives of totarol, e.g. 16-hydroxytotarol (II; $R=CH_2OH$), also underwent coupling.

Several enzymes and cell-free extracts of higher plants have hitherto been found to catalyse the coupling of phenols and of amines.† Both water soluble and cell-bound insoluble enzymes, described as redoxases, have been reported as being present in the cell layer of the wood which lies directly under the bark of the gymnosperm, *Araucaria excelsa*.[8] However, most of the coupling studies concerned with phenols have been carried out with hydrogen peroxide catalysed by peroxidase.[14] At present there appears to be no previous example in the literature of a single carbon–carbon oxidative coupling through the action of an enzyme from a higher plant alone, the oxidative coupling of totarol with that from *Cryptomeria japonica* providing the first example, Little is known, as yet, about the mechanism of this reaction. The oxidase from *Polyporus versicolor* is believed to function by the production of aryloxy radicals which can then undergo homolytic coupling from oxygen to *o*- or *p*-carbon atoms or from *o*- to *o*-, *p*- to *p*-, or *o*- to *p*-carbon atoms, followed by further changes depending

* It has recently been shown that contrary to previous reports,[12] laccase catalyses the oxidation of monophenols such as *p*-cresol and 2,6-dimethoxyphenol, and it has been suggested that in earlier purification procedures adopted for the preparation of laccase this activity was destroyed by pre-incubation with acetone.[3]

† The oxidative coupling of phenolic compounds including enzymic oxidative coupling has recently been reviewed.[14]

[10] Cf. D. KEILIN and T. MANN, *Nature* **145**, 304 (1940); B. G. MALSTRÖM, R. MOSBACH and T. VÄNNGÅRD, *Nature* **183**, 321 (1959).

[11] *Report of the Commission on Enzymes of the International Union of Biochemistry*, p. 81. Pergamon Press, Oxford (1961); see also W. D. BONNER, *Ann. Rev. Plant Physiol.* **8**, 427 (1957).

[12] C. R. DAWSON and W. B. TARPLEY, In *The Enzymes* (Edited by J. B. SUMNER and K. MYRBACK) (1st Ed.), Vol. 2, Part 1, p. 484. Academic Press, New York (1951).

[13] J. B. SUMNER and G. F. SOMERS, *Chemistry and Methods of Enzymes* (2nd Ed.), p. 236. Academic Press, New York (1947).

[14] A. I. SCOTT, *Quart. Rev.* **19**, 1 (1965); see also J. R. LEWIS, *Chem. & Ind. (London)* 1672 (1964); and H. MUSSO, *Angew Chem. Intern. Ed. Engl.* **2**, 723 (1963).

on the chemical reactivity of the adducts so formed.[2,3] From its similarity to the fungal enzyme that from the gymnosperms probably functions in the same manner.

It is noteworthy that the leaves of *Podocarpus totara*, which contains podototarin in the heartwood,[4] gave a positive test for the presence of a *p*-diphenol oxidase while the closely related *P. hallii*, containing no podototarin, gave a negative test. The leaves of both *P. macrophyllus* and *P. nagi* contain kayaflavone (III) while those of *Cryptomeria japonica* and *Cunninghamia sinensis* also contain the biflavonyls, sciadopitysin, sotetsoflavone, and isoginketin.[5,6] Each of these species gave a positive test for the presence of a *p*-diphenol oxidase and their extracts showed relatively high enzymic concentrations. Contrarywise, fresh leaves of *Sciadopitys verticillata*, *Torreya nucifera*, *Cephalotaxus drupacea*, and *Taxus baccata*, which have been reported to contain biflavonyls,[6] all gave negative tests. The presence of biflavonyls in the leaves of these species was confirmed during the present work by thin-layer chromatography. From these results it would appear that no correlation exists between the presence of biflavonyls and a positive test for a *p*-diphenol oxidase under the conditions as conducted in the present study. However, in view of the instability of the gymnosperm enzyme and the preliminary nature of the present survey, insufficient examples have as yet been studied to decide whether or not the presence of a *p*-diphenol oxidase is of diagnostic or taxonomic importance. Also, the existence of biflavonyls in *S. verticillata*, *T. nucifera*, *C. drupacea*, and *T. baccata* may well be due to the presence of peroxidase in these species.

EXPERIMENTAL

Preparation of Leaf Extracts

Fresh leaves (~5 g, wet weight) were washed with distilled water, macerated with distilled water (5 ml) at 0° in a Waring blendor, and then separated from fibrous material. When only small amounts of leaves were available the extract was prepared by grinding the leaves in a pestle with distilled water and fine glass beads. A portion of the crude extract was then filtered through Hyflo-Supercel and both the original and filtered materials were assayed for enzyme activity, all operations being carried out at 0°.

Enzyme Assay

Portions (2·0 ml) of crude extracts and filtered solutions were each incubated with a 0·1 % aqueous solution of 2,6-dimethoxyphenol (1·0 ml) and 0·01 M-sodium acetate buffer (4·0 ml) at pH 5·0 and 30° for 1 hr. The solutions, coloured orange-red for a positive coupling test, were extracted with chloroform (2 ml) and the u.v. spectrum was determined for the presence of 3,5,3′,5′-tetramethoxydiphenoquinone (λ_{max} 468 mμ). Boiled extracts of the leaves gave no quinone formation under these conditions.

Preparation of the p-*Diphenolase from* Cryptomeria japonica

The aqueous extract, from the leaves of *Cryptomiera japonica*, prepared as above, was kept at 0° for 3 days, filtered through Hyflo-Supercel, and the filtrate was dialysed against 0·001 M-sodium acetate buffer of pH 4·0 in the cold, with three changes of buffer, for 48 hr. The enzyme was precipitated by the addition of ammonium sulphate, separated by centrifugation, redissolved in distilled water, and stored at 0° until required for use. Repetition of the dialysis and precipitation procedures resulted in considerable loss of activity and, in general, with succeeding extracts no further attempt at purification was made.

Solutions of the enzyme were prepared from the leaves of *Podocarpus totara*, *P. nagi*, and *P. macrophyllus* in a similar manner.

Oxidation of Monophenols

In general, the catalysis of monophenols, e.g. 2,6-dimethoxyphenol, *p*-cresol, catechol, 2,6-dimethylphenol, and pyrogallol by the gymnosperm enzyme was carried out by the experimental procedures already reported,[3] except that where possible products were identified spectroscopically. Identical results were obtained for the four species (see above) from which partially purified extracts of the enzyme were prepared.

For the enzymes from *C. japonica* and *P. nagi* the pH optimum for the conversion of 2,6-dimethoxyphenol to 3,5,3′,5′-tetramethoxydiphenoquinone was determined spectroscopically on solutions of the phenol (0·0065 M) in 0·01 M sodium acetate buffer, the optical density at 468 mμ being recorded at various pH values, 10 min after mixing.

Coupling of Totarol

The partially purified enzyme solution from *Cryptomeria japonica* was added in portions (10 ml) at 48 hr intervals to an incubated (30°) solution of totarol (100 mg) in 10% aqueous ethanol buffered with sodium acetate to pH 4·0. The formation of a biphenylic system was indicated by the appearance of a peak at 254–256 mμ in the u.v. spectrum. When the peak had reached a maximum E value ($\sim$2 weeks) the solution was continuously extracted with ether, the solvent removed, and the residue chromatographed on alumina (100 g). Fractions eluted from the column with light petroleum, gave podototarin (58 per cent), m.p. and mixed m.p. 225° (correct u.v. and i.r. spectra).

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