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CHEMICAL AND ENZYMATIC SYNTHESIS OF MONOMERIC PROCYANIDINS (LEUCOCYANIDINS OR 3′,4′,5,7-TETRAHYDROXY-FLAVAN-3,4-DIOLS) FROM (2*R*,3*R*)-DIHYDROQUERCETIN

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Key Word Index—*Pseudotsuga menziesii*; Pinaceae; cell suspension cultures; leucocyanidin; dihydroquercetin; enzymatic reduction; procyanidin biosynthesis.

Abstract—The major product from the reduction of (2*R*,3*R*)-dihydroquercetin with sodium borohydride is the 2,3-*trans*-3,4-*trans* isomer of leucocyanidin [(2*R*,3*S*,4*R*)-3,3′,4,4′,5,7-hexahydroxyflavan] whereas the enzymatic reduction product is the 2,3-*trans*-3,4-*cis* isomer [(2*R*,3*S*,4*S*)-3,3′,4,4′,5,7-hexahydroxyflavan]. The 3,4-*trans* isomer may be partly converted to the 3,4-*cis* isomer under mild acid conditions. The 3,4-*cis* isomer is more acid-labile, and more reactive both chemically with thiols and enzymatically with a diol reductase, than the 3,4-*trans* isomer.

INTRODUCTION

The major reduction product of dihydroquercetin (DHQ, **1**) reductase activity in extracts from cell suspension cultures of Douglas fir needles was shown to be a flavan-3,4-diol, similar to the minor product from sodium borohydride reduction of DHQ (**1**), and was tentatively identified as the 2,3-*trans*-3,4-*trans* isomer (**2**) [1].

The present study shows that the enzymatically produced diol is in fact the 2,3-*trans*-3,4-*cis* isomer (**3**), whereas the major product from sodium borohydride reduction of DHQ is the 2,3-*trans*-3,4-*trans* isomer (**2**), which has been confirmed by ^{1}H NMR and ^{13}C NMR spectroscopy [2] and directly by X-ray crystallography [3], compound **2** crystallizing as a dihydrate from aqueous solution [3]. Conversion of the chemically prepared 3,4-*trans* diol to the 3,4-*cis* isomer, purification of these isomers by paper chromatography (PC), and a comparison of their relative reactivities are discussed.

RESULTS AND DISCUSSION

Sodium borohydride reduction of DHQ

Crude ethyl acetate extracts from the reduction of DHQ with sodium borohydride contained predominantly the 3,4-*trans* diol (**2**). Maximum yields were *ca* 80–85% when analysed by PC or HPLC. Other components present in trace amounts were DHQ (**1**), the 3,4-*cis* diol (**3**) and procyanidin oligomers. The 3,4-*trans* diol is stable in ethyl acetate solution on storage at 4°.

The 3,4-*trans* diol is very sensitive to trace amounts of acid. This often causes batches of diol to produce major amounts of procyanidin polymers on evaporation to dryness, even after passage through a Sephadex LH-20 column in ethanol. Direct evaporation of an ethyl acetate solution to dryness always produced polymer for this reason [2].

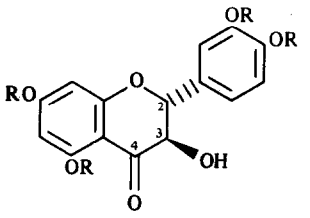
For preparation of small amounts of diol, a satisfactory method is lyophilization of a diol–phosphate buffer mixture (pH 6.8). The product of this process was confirmed to be the 3,4-*trans* diol (**2**) by ^{1}H NMR and ^{13}C NMR spectroscopy. The very small differences between the ^{13}C NMR (see Experimental) and published [2] data were probably due to the presence of phosphate buffer in the solvent.

Larger amounts of 3,4-*trans* diol (**2**) may be prepared by decomposition of the borate complex with pH 4.5 acetate buffer and adsorption of the diol onto XAD-2 resin followed by thorough washing with water to remove the last traces of acid. Crystalline 3,4-*trans* diol may be obtained on evaporation of the ethanol eluate from the resin (ref. [3] and Chan, B. G. and Porter, L. J., unpublished results).

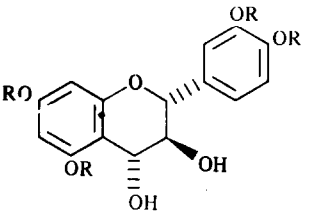
Epimerization of the 3,4-trans *diol to the* 3,4-cis *isomer*

The ready epimerization of oxygen substituents at the benzylic C-4 position of flavanoids is well known, the most recent study being that of Brown and co-workers [4] who have summarized the relevant literature. The rate of this interconversion is promoted by electron-releasing substituents at C-5 and/or C-7 on the A-ring [4]. The products are an equilibrium mixture of pseudo-axial and pseudo-equatorial products, the ratio being the same regardless of whether the axial or equatorial compound is the starting material. The pseudo-axial product is often the thermodynamically more favoured product and therefore usually predominates, but the observed ratio of epimers depends on the system [4]. As would be predicted, epimerization of the 3,4-*trans* diol (**2**) proceeds with great facility to a mixture of itself and the pseudo-axial product (**3**). Epimerization was attempted at several pH values ranging from 2.0 to 7.5 at 30–50°; incubation in

‡To whom correspondence should be addressed.



1 R = H 2*R*, 3*R* dihydroquercetin (DHQ)
8 R = Me



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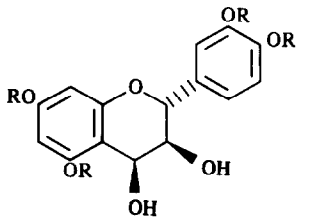
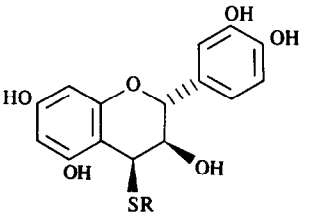
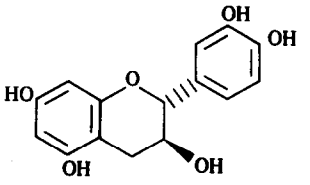


3 R = H
4 R = Me



7 R = CH_2CH_2OH .ME ;
R = $CH_2CHOHCH_2SH$. DTT



6 Catechin

a citrate–phosphate buffer (pH 2.6) at 40° gave the best yields.

Although the instability of the enzymatic reduction product, **3**, from DHQ has prevented us from accumulating enough material to establish its identity directly, the acid epimerization product was identified as the 3,4-*cis* diol (**3**) as follows.

The 3,4-*trans* diol was synthesized from DHQ and treated with citrate–phosphate buffer (pH 2.6) at 40°. The diols were adsorbed from solution onto XAD-2 and eluted with ethanol. The ethanol solution was reduced to a small volume and treated with ethereal diazomethane and methanol. The products were separated by TLC (silica gel) and the 3,4-*trans* and 3,4-*cis* diols were identified by co-chromatography and identity of their ^1H NMR spectra with those of authentic samples of 2,3-*trans*-3,4-*cis*- (**4**) and 2,3-*trans*-3,4-*trans*- (**5**) ′,4′,5,7-tetramethoxyflavan-3,4-diols isolated elsewhere [2].

The epimerization was also monitored directly by 200 MHz ^1H NMR spectroscopy by observing a dilute solution of the 3,4-*trans* (**2**) diol at the position of the H-3 signal (δ 3.92) in D_2O/CD_3COOD_3 at pH ~ 2.5 and 20°. The H-3 signal of the 3,4-*cis* diol appeared as a quartet (*dd*) with the outer signals appearing inside the outer signals of the H-3 quartet of the 3,4-*trans* diol, due to the smaller value of $J_{3,4}$ (3.0 Hz) for the 3,4-*cis* diol. The relative concentration of 3,4-*cis* (**3**) to 3,4-*trans* (**2**) diol was 7:13 after 30 min and this ratio was unchanged after 1.5 hr, but thereafter the signals broadened due to polymerization.

The above ratio of 3,4-*cis* (**3**) to 3,4-*trans* diol (**2**) is apparently the equilibrium concentration, as independent conversions in citrate–phosphate buffer (pH 2.6) and measurement of the relative concentrations of the diols by PC and Prussian Blue analysis also showed that the ratio of **3** to **2** was *ca* 1:2.

In attempts to isolate purified 3,4-*cis* diol by epimerization of the 3,4-*trans* diol in citrate–phosphate buffer, only a 15% yield (or *ca* half that theoretically available) was obtained. Losses occurred during the conversion and subsequent purification via PC and were mainly due to the formation of a series of procyanidin oligomers that showed up both as a continuous streak and as several discrete bands upon PC. The conversion mixture was stable when stored at 4° in anhydrous ethyl acetate. Major losses of 3,4-*cis* diol occurred when lyophilization was attempted under conditions satisfactory for the initial 3,4-*trans* diol preparation. Purification was also attempted via HPLC on C_{18} columns; while the separation was satisfactory, the subsequent extraction step with ethyl acetate to remove the diol from the methanol–phosphate solvent mixture led to poor yields. Purification by PC was the best method of separation, probably because it involved chromatography over a long distance. TLC on either C_{18} or cellulose plates gave poor separations; silica gel was better but streaking occurred. Although the two diols in the ethyl acetate extract of the conversion mixture were effectively separated by one-dimensional PC in BP solvent (see Experimental) elution of the diols from paper in water, buffer or 70% methanol, followed by concen-

tration by evaporation at 40°, also led to serious losses.

The best method so far to purify the two diols for use as enzymatic substrates was to cut out the two bands containing the diols obtained by PC in BP solvent and to store them as small squares in a desiccator. Weighed portions were analysed by the Prussian Blue test-tube method and other portions were used as paper-bound sources of substrates for chemical and enzymatic syntheses by submersion directly in the incubation mixture. Since the paper was present during incubation, a high ratio of diol to paper was necessary. After storage, however, only *ca* 65% of the Prussian Blue positive material could be eluted when compared with analyses made immediately after chromatography. The instability of the 3,4-*cis* diol prevents an accurate measure of its purity.

Enzymatic conversion of the 3,4-cis *diol to catechin* (**6**) *by diol reductase*

Brief reports have been made of the presence of a NADPH-dependent reductase activity in extracts from cell suspension cultures of Douglas fir needles that converts either the chemically or enzymatically prepared 3,4-*cis* diol to catechin (**6**) [5, 6]; a more complete report has been submitted for publication [7]. After incubation at pH 7.4 for 3 hr in the presence of 50 μg of 3,4-*cis* paper-bound diol (plus NADPH and its regenerating system), *ca* 10 μg of catechin was produced. The latter was identified by its positive reaction with the vanillin reagent [1], its behaviour during PC with several solvents, and by its co-chromatography and elution as a single symmetrical peak from a C_{18} column via HPLC with two different methanol–phosphate buffer solvents. The 3,4-*trans* isomer, however, produced only trace amounts of catechin; this could be due either to a weak activity with this isomer or to conversion of it, during incubation, to the enzymatically active 3,4-*cis* isomer.

Non-enzymatic conversion of 3,4-cis *diols to possible thioether compounds, X-1 and X-2*

The 3,4-*cis* diol (**3**), prepared either chemically or enzymatically, was converted non-enzymatically at pH 7.4 to a major Prussian Blue positive product (X-2). The only requirement besides the diol substrate was a thiol agent such as 2-mercaptoethanol (ME) or dithiothreitol (DTT). The 3,4-*trans* diol (**2**) may also form the same product, but in a much smaller amount; this could be due to a slight activity by this isomer directly or only after its conversion first to the 3,4-*cis* diol. The reaction was time-dependent; after a 4–6 hr incubation approximately a *ca* 1:1 mixture of the starting diol and X-2 product was present. A minor amount of a second Prussian Blue positive spot (X-1) was also produced; it had a similar R_f value to that of X-2 in BP solvent, but migrated further in 5% acetic acid. The major product, X-2, gave a positive red colour with the butanol–hydrochloric acid reagent [1] and therefore, was a leucocyanidin or procyanidin; in addition, it formed a vanillin adduct as expected of such compounds [1]. The minor unknown, X-1, was present in too small an amount to determine whether it was also a leucocyanidin or procyanidin. Phosphate buffer was just as effective as Tris, but less product was obtained in Hepes buffer. Similar reaction products were obtained at pH 7 and 8; no other pH values were used.

The R_f values of X-2 in BP, 5% acetic acid and BAW solvents were so similar to those of catechin (**6**) that the two compounds were not resolved. Elution from a C_{18} column on HPLC in methanol–0.05 M phosphate buffer (pH 6.8), however, indicated a V_E for X-2 between catechin and DHQ, while the minor product, X-1, was eluted just ahead of X-2. Re-chromatography in BP of X-2 eluted from PC sometimes gave rise to a compound similar to or identical to the original diol, an indication of a possible reversal of the ME-dependent reaction.

The major unknown, X-2, was much more stable in 5% acetic acid than the original diol substrate. This characteristic was easily demonstrated by comparing the pattern of Prussian Blue positive spots in 2-dimensional PC in BAW and 5% acetic acid run in reversed sequence. The pattern was similar in either sequence when X-2 or X-1 was chromatographed. The two diol isomers, on the other hand, showed considerable conversion to higher MW forms when the 5% acetic acid solvent preceded BAW. These polymeric products did not migrate, or did so only slightly, in the BAW solvent, but had similar R_f values to those of the diols in 5% acetic acid.

Our tentative conclusion is that the major product X-2 is a thioether (**7**) resulting from a Michael addition of RSH to C-4 in the heterocyclic ring via a quinone methide intermediate [8]. Since the R_f values for both these thioethers were similar, it is necessary to argue that the second thiol group of DTT was not involved due to the dilute concentration of the diol substrate, and that the rest of the molecule did not noticeably alter the mobility of the addition product in the solvents used. The R_f values are about what would be predicted from the products of reaction between a catechin carbenium ion and toluene-α-thiol [9], where two isomers are formed, the 3,4-*cis* isomer predominating. The thioether would be expected to be converted to the carbocation and then oxidized to cyanidin under acid conditions, thus explaining the procyanidin-positive reaction [9].

Tables 1 and 2 summarize the major characteristics of the two diols, and compares their chromatographic characteristics with some related compounds.

Significance for procyanidin synthesis and biosynthesis

There is currently no definitive explanation for the almost exclusive production of the 3,4-*trans* diol (**2**) on DHQ reduction with sodium borohydride, and the 3,4-*cis* isomer on enzymatic reduction, but the following observations may be made.

Considering the chemical reduction first, sodium borohydride reduction of DHQ tetramethyl ether (**8**) at 0° [10] or in boiling methanol [11] produces a mixture of the 3,4-*cis* (**4**) and 3,4-*trans* (**5**) isomers in a ratio of *ca* 13:7, the C-4 axial product therefore being favoured. Reduction of **8** with lithium aluminium hydride [12] gave the 3,4-*cis* isomer (**4**) as the only reported product, which may be rationalized on the basis that the much more bulky solvated H-transfer reagent attacks at the less hindered *re*-face of DHQ tetramethyl ether.

In the case of reduction of DHQ (**1**) itself, the fact that sodium borohydride transfers almost exclusively a hydride ion to the *si*-face of the carbonyl carbon can possibly be explained by the sodium borohydride ion pair forming a complex with the 3- and 5-hydroxyl groups and consequently the hydride ion transfer being directed for delivery to this face.

Table 1. Chromatographic behaviour of the 3,4-*trans* (2) and 3,4-*cis* (3) diols compared with related compounds

	DHQ (1)	2	3	Epicatechin	Catechin	Procyanidin B3*	X-1	X-2
PC (R_f values)								
BP	0.86	0.69	0.55	0.55	0.72	0.48	0.76	0.72
BAW†	0.70	0.53	0.51		0.55	0.27	0.55	0.55
5% HOAC‡	0.29	0.44	0.43	0.27	0.43	0.43	0.60	0.43
HPLC§								
1:4	25	9.5	7.4	>25	13	8.5	15	24
3:17		22	13		26			

*Catechin-(4α → 8)-catechin.

†Correct for diols only if BAW precedes 5% acetic acid in two-dimensional paper chromatography (see text).

‡All R_f values for 5% acetic acid calculated from two-dimensional paper chromatography with 5% acetic acid as the second solvent.

§Elution volume (V_E) in ml. Ratio of solvent in terms of methanol–phosphate buffer.

Table 2. Summary of characteristics of 3,4-*trans* (2) and 3,4-*cis* (3) diols

	2	3
DHQ product		
$NaBH_4$ reduction yield (%)	75–85	10–15*
Reductase yield	0	10–20%
Stability		
Phosphate buffer, pH 6.8	More stable	Less stable
5% HOAc	More stable	Less stable
Enzymatic conversion to (+)-catechin	Trace	Major
Non-enzymatic conversion to thioether	Trace	Major

*After acid conversion of 2.

The enzymatic reduction, which exclusively transfers a hydride ion to the *re*-face of DHQ to form the 3,4-*cis* diol (3), may possibly be explained if the active site of the enzyme contains a metal-ion cofactor such as Zn^{II}, as in alcohol dehydrogenase, which would complex on the *si*-face to the carbonyl oxygen and 3-hydroxyl group, thus directing *re*-face hydride ion transfer from NADPH [13]. Whether or not this system requires a metal-ion cofactor merits further work.

The considerable difference in acid stability between diols 2 and 3 may be readily rationalized on the basis of current knowledge. The major difference between the two isomers is the fact that the 4-hydroxyl group in the 3,4-*trans* isomer adopts a pseudo-equatorial orientation, whereas in the 3,4-*cis* isomer it is pseudo-axial. As first pointed out by Clark-Lewis and Mortimer [14] an axial hydroxyl is ideally aligned to stabilize the developing carbenium ion by overlap of the vacant orbital with the π-electrons of the A-ring, this argument being used to explain the large difference in reactivity of melacacidin and isomelacacidin towards acid-catalysed reactions.

X-Ray crystallography shows that the 3,4-*trans* diol (2) adopts a conformation intermediate between a half-chair and C(3)-sofa in the solid-state [3], and it is reasonable to assume that its conformation in water will be similar. Moreover, other solid-state crystal structures of catechin [15] and epicatechin [16, 17], or their derivatives, show that the heterocyclic ring is quite labile and may adopt conformations at least in the range half-chair C(3)-sofa, the C-2 phenyl ring generally adopting a pseudo-equatorial orientation. The 3,4-*cis* diol (3) is therefore ideally set up to react under mildly acidic conditions as the 4-hydroxyl group is oriented at 80° to the A-ring plane in the conformation adopted by 2, and only a small twist about the C(3)–C(4) bond is required to orient the 4-hydroxyl orthogonally, which is achieved by orientation to a C(3)-sofa conformation.

The next step must be modified from the original proposal of Clark-Lewis and Mortimer [14], as recent work by Brown and co-workers [18] has shown that a 4-hydroxyl in a 5- or 7-hydroxyflavan will be eliminated under either acidic or alkaline conditions to form a quinone-methide intermediate, rather than a carbenium ion directly. However, the effect is the same, viz. that the hydroxyl is ideally oriented to participate in what is the equivalent of a β-diaxial elimination to form a double bond.

It is therefore now virtually certain that condensation to procyanidin oligomers proceeds through a quinone-methide formed in this case from 3, or by prior epimerization of 2 to 3. The quinone-methide route (Fig. 1) probably proceeds in the usual way by a Michael-type addition [19], the first step probably being formation of the protonated intermediate at step (ii), equivalent to the intermediate carbenium ion in an SN_1 mechanism, followed by addition of the flavanoid nucleophile at step (iii)—although obviously the steps could be reversed. All factors favour such a route including the presence of strong electron-donating groups in the A-ring, the possibility of forming both *o*- and *p*-quinone-methides, and the fact that the reaction is proceeding in a solvent with high dielectric constant (water) thereby aiding the stabilization of charge separation in step (i).

The fact that the enzymatic reduction exclusively produces the 3,4-*cis* diol (3) is therefore highly significant as it is the diastereomer most suited to procyanidin formation.

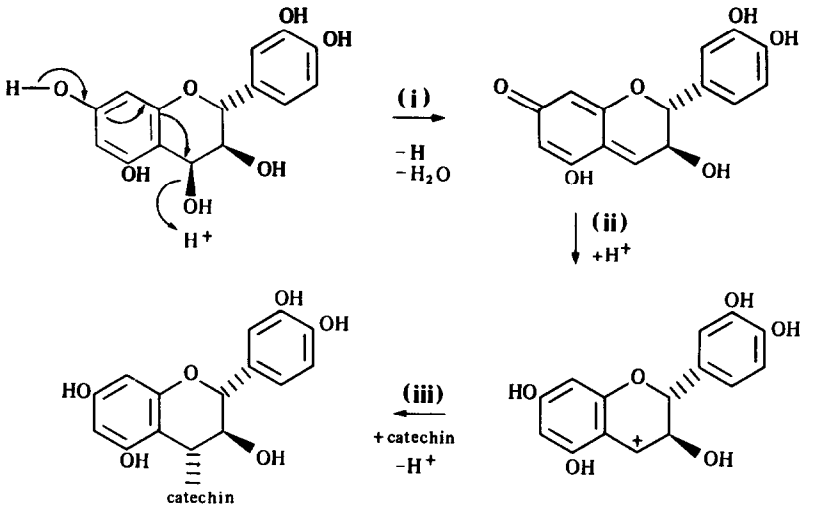


Fig. 1. Route from the 3,4-*cis* diol (3) to procyanidins.

CONCLUSION

Non-enzymatic reduction of (2*R*,3*R*)-dihydroquercetin (1) with sodium borohydride yielded almost exclusively the more acid-stable 3,4-*trans* diol (2), which can be partly converted to the 3,4-*cis* diol (3) by epimerization under mild acid conditions. Enzymatic reduction, on the other hand, yielded only the 3,4-*cis* diol (3). A rationale for these differences has been offered in terms of the differing effects of complexation on the reactive intermediate in both chemical and enzymatic reductions, but confirmation must await further studies.

The failure to isolate flavan-3,4-diols with a 5,7-dihydroxylation pattern has been attributed to their instability [2, 5, 20]. The fact that the enzyme forms the less stable of the two isomers makes it even more unlikely that the enzymatic 3,4-*cis* product will be found free in plant tissues. However, it could be easily stabilized if a reversible formation of a thioether at C-4 occurs *in vivo*. The RSH group could be part of a protein.

EXPERIMENTAL

Analyses by PC. Descending PC was performed with 22.5 × 56.5 cm sheets of Whatman No. 1 with *n*-BuOH saturated with 0.01 M Pi buffer, pH 6.8 (BP), developed to 35 cm in *ca* 17 hr. For 2D PC, the same BP mixture was the first solvent, followed by ascending PC in the short direction in 5% HOAc. 2D ascending PC was done with 23 × 29 cm sheets, developed in BAW (*n*-BuOH–5% HOAc–H_2O, 6:1:2) and 5% HOAc. Aliquots of EtOAc extracts were dried on 2 cm diameter circles. Phenolic spots were visualized with a spray of fresh Prussian Blue reagent mixture of equal vols. of 1% $FeCl_3 \cdot 6H_2O$ and 1% $K_3Fe(CN)_6$ (w/v).

Quantitative determinations for PC. Estimates of the amounts of the diols in Prussian Blue positive areas were made visually or with a densitometer with a red cellophane filter (Dennison's No. 85-022) by comparison with a standard curve made with catechin. More accurate measurements of amounts in unsprayed areas were determined with a Prussian Blue test-tube assay after elution of compounds in spots cut out from the expected R_f areas. Unsprayed spots or portions of bands (0.5–1 cm^2) from freshly dried chromatograms were immersed in 2 ml H_2O for 10 min. After removal of the paper, 0.05 ml each of freshly prepared solns of $FeCl_3 \cdot 6H_2O$ and $K_3Fe(CN)_6$ (w/w) was added and the *A* at 650 nm was determined spectrophotometrically after 10 min. The μg equivalents were based on standards of either the 3,4-*trans* diol or catechin.

HPLC separations were made on a C_{18} Partisil 10 ODS 3 column with either 1:4 or 3:17 solvent mixtures of MeOH–0.05 M Pi buffer, pH 6.8 [1]. Since EtOAc interfered with elution values (V_E), diols present in EtOAc extracts were forced into 0.2 ml 0.01 M Pi buffer, pH 6.8, by evapn of the EtOAc under vacuum at 40° after addition of buffer. The flavanoids were monitored at 280 nm, and peak areas converted to μg equivalents with known standards or by absorption coefficients reported previously [1].

Preparation of 2,3-trans-3,4-trans-*diol* (2) *from DHQ* (1). The DHQ used was described previously [6] or was prepared from the inner bark of Douglas fir. DHQ (10 mg) in EtOH (1 ml) was stirred with $NaBH_4$ (5 mg), added in one batch, for 2 hr at room temp., H_2O (10 ml) was added, followed immediately by 20% HOAc (0.35 ml) to pH 3.5. The soln was extracted with EtOAc (1 × 5 ml; 2 × 2 ml) and the latter back-washed with 0.5 M Pi buffer pH 8 (3 × 0.2 ml). This crude EtOAc extract, containing predominantly the 3,4-*trans* diol, was either lyophilized for NMR analyses or was used to synthesize the 3,4-*cis*-isomer.

Lyophilization of 3,4-trans *diol preparation.* The crude EtOAc extract (8 ml) was concd to 2 ml; 1.5 ml 0.01 M Pi buffer, pH 6.8, was added and the rest of the EtOAc evapd. This aq. buffer soln was shell-frozen in a small flask with a solid CO_2–Me cellosolve bath and lyophilized (vacuum at 5–25 mm) to a white powder containing phosphate salts, 3,4-*trans* diol and minor amounts of high MW forms. The powder was dried over P_2O_5 and then stored over $CaCl_2$ in a desiccator at 4°. The product had the following spectral characteristics: ^1H NMR (80 MHz; in Me_2CO-d_6): δ 3.85 (*m*, H-3), 4.59 (*d*, *J* = 10.0 Hz, H-2), 4.96 (*d*, *J* = 7.9 Hz, H-4), 5.84 (*d*, *J* = 2.3 Hz, H-8), 5.96 (*d*, *J* = 2.3 Hz, H-6), 6.8–7.0 (H-2', H-5' and H-6'). ^{13}C NMR (20 MHz; in Me_2CO-d_6–H_2O, 1:1): δ 72.6 (C-4), 74.0 (C-3), 81.0 (C-2), 95.5 (C-8), 97.3 (C-6), 115.7 (C-2'), 115.8 (C-5'), 120.6 (C-6'), 130.6 (C-1'), 145.6 (C-3'), 146.1 (C-4').

Small-scale chemical conversion of the 3,4-trans (2) *to the* 3,4-cis (3) *diol.* The crude EtOAc extract (8 ml) was rotary-evaporated *in vacuo* at < 40° to 2 ml, and then 4 ml 0.05 M citrate–Pi buffer, pH 2.6 was added. After the remaining EtOAc had been evapd,

the mixture was incubated at 40° for 30 min to epimerize the 3,4-*trans* diol. The conversion mixture contained a 2:1 ratio of the *trans* to the *cis* diol.

Purification of the two diols by strip PC. The above conversion mixture was extracted with EtOAc (3 × 2 ml), dried over Na_2SO_4, and applied to one 2 × 20 cm strip for descending PC in BP. Two Prussian Blue positive bands were detected with the $FeCl_3$–$K_3Fe(CN)_6$ spray applied to a narrow vertical strip cut out of the chromatogram; one at R_f 0.53 was the residual 3,4-*trans* isomer, while the lower band at R_f 0.36 was the 3,4-*cis* isomer. The non-sprayed parts of the two diol bands were cut into small squares, portions were analysed by the Prussian Blue test-tube method, and the rest were stored in a desiccator for use as substrates.

Identification of 3,4-cis *diol* (3) *as the epimerization product from* 2. A sample of 3,4-*trans* diol, 0.5 g, synthesized from DHQ and purified as described elsewhere [2] was treated with 500 ml, 0.05 M citrate–Pi buffer (pH 2.6), for 30 min at 40°. The soln was equilibrated twice with 100 ml MeOH-washed XAD-2 resin (Mallinckrodt) and the resin washed thoroughly with H_2O, dried on a Buchner funnel, and washed 4–5 times with EtOH. The EtOH was dried (Na_2SO_4) and evapd to a small vol., and CH_2N_2–Et_2O soln added with enough MeOH to effect soln. The reactants were stored at 0° for 48 hr and then the soln was evapd to dryness. The product was dissolved in $CHCl_3$ and applied to silica gel prep. TLC plates and developed in C_6H_6–Me_2CO (4:1). The diol tetramethyl ethers were detected either in UV or by fuming with $SnCl_4$–$SOCl_2$ [2]. The 3,4-*cis* diol (R_f 0.24) and 3,4-*trans* diol (R_f 0.18) bands were eluted with Me_2CO, and on evapn the solids were treated with warm MeOH, crystalline material separating from each fraction on standing. The (2*R*,3*S*,4*S*)-3′,4′,5,7-tetramethoxyflavan-3,4-diol (4) was identified by mp 187–188° (lit. value [12] 189–190° and from the ^{1}H NMR (200 MHz; in $CDCl_3$ after D_2O exchange): δ 3.99 (*dd*, H-3), 4.93 (*d*, J = 10.4 Hz, H-2), 5.04 (*d*, J = 4.0 Hz, H-4), 6.13 (AB_q, H-6 and H-8), 3.76 (1 × OMe), 3.87 (1 × OMe), 3.90 (1 × OMe), 3.92.

Direct observation of epimerization of 3,4-trans *diol* (2) *by* ^{1}H *NMR.* Earlier attempts to observe the epimerization at 90 MHz in D_2O or D_2O–Me_2CO-d_6 failed because of the high concn of substrate required to observe a spectrum (*ca* 30 mg/0.25 ml), which promoted polymerization. The expt was therefore repeated at 200 MHz on a dilute soln (0.25 mg/0.5 ml) in D_2O. The spectrum monitored the H-3 signal of the diols, which appeared as a double-doublet (*dd*), J = 9.5 Hz and J = 7.5 Hz in the spectrum of 2 before epimerization. The H-3 signal for the 3,4-*cis* diol (3) also appeared as a *dd*, overlaying the signal of 2, but with a much narrower width, 10.5 Hz (cf. 17.0 Hz for 2) on initiating the epimerization by addition of CD_3COOD.

Use of paper-bound substrates. Weighed portions of squares from dried paper chromatograms were used as sources of diols by suspension directly in incubation mixtures without a separate elution step. The ratio of μg diol to mg paper wt was kept as high as possible. A 1.0–1.7 ratio was satisfactory; a lower ratio involved too much paper to permit stirring during the incubation and during the subsequent extraction with EtOAc.

Enzymatic conversion of the 3,4-*diols to catechin* (6) *by a diol reductase* [5–7]. About 50 μg (uncorrected for purity) of the paper-bound diol isomers, prepared chemically, was added to a 1 ml vol. containing Tris buffer at pH 7.4, 1 μmol of NADPH, a regenerating system of NADPH consisting of 6 μmol of glucose-6-P and 1 unit of glucose-6-P dehydrogenase, and 200–400 μg of protein from a crude enzyme extract from cell suspension cultures of Douglas fir (*Pseudosuga menziesii* needles). The latter was prepared as previously described [1], except that 5 mM ascorbate was substituted for 2-ME [1]. After an incubation period of *ca* 3 hr at 30°, the mixture was extracted with 3 × 1 ml of EtOAc, the latter concd to 0.2 ml under vacuum at 40° and analysed via PC or HPLC.

Enzymatic preparation of 3,4-cis-*diol.* A 5 × scale incubation mixture, comparable to that for diol reductase activity except that DHQ was substituted for the diol substrate, was used to isolate enough 3,4-*cis* diol after 1D PC in BP to use as a paper-bound substrate.

Non-enzymatic conversion of diol to possible thioethers (7), *X*-1 *and X*-2. In a 5 ml vol. of 0.1 M Tris buffer, pH 7.4, containing 6 mM 2-mercaptoethanol or 0.6 mM dithiothreitol, *ca* 50 μg (32 μM) of either of the paper-bound chemically or enzymatically prepared diols (uncorrected for purity) was added with occasional stirring of the paper. After incubation for *ca* 6 hr at 30°, the aq. paper mixture was extracted with successive aliquots of EtOAc, the amount being dependent on the quantity of paper present. The EtOAc extract was analysed via PC or HPLC.

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