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The C-4 Stereochemistry of Leucocyanidin Substrates for Anthocyanidin Synthase Affects Product Selectivity

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Abstract—Anthocyanidin synthase (ANS), an iron(II) and 2-oxoglutarate (2OG) dependent oxygenase, catalyses the penultimate step in anthocyanin biosynthesis by oxidation of the 2*R*,3*S*,4*S*-*cis*-leucoanthocyanidins. It has been believed that in vivo the products of ANS are the anthocyanidins. However, in vitro studies on ANS using optically active *cis*- and *trans*-leucocyanidin substrates identified cyanidin as only a minor product; instead both quercetin and dihydroquercetin are products with the distribution being dependent on the C-4 stereochemistry of the leucocyanidin substrates.

The anthocyanins **1** are a large class of plant flavonoids that display a variety of biological roles.[1] Their biosynthesis proceeds via the formation of a 2*S*-flavanone **2** from 4*p*-coumaroyl-CoA **3** and malonyl-CoA **4** in a reaction catalysed by a polyketide synthase, chalcone synthase (CHS) (Fig. 1).[2,3] Ring closure to the 'closed-flavonoid' nucleus is then catalysed by chalcone isomerase (CHI).[2,4] A subsequent sequence of oxidation–reduction–oxidation reactions is catalysed by flavanone 3β-hydroxylase (F3H), dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS), respectively.[2] Finally, glycosylation is thought to be mediated by a UDP-glucose:flavonoid 3O-glycosyltransferase (FGT) yielding the anthocyanin products **1**.[2,5,6] ANS, and several other oxygenases of flavonoid biosynthesis including F3H, belong to the iron(II) and 2OG dependent oxygenase family.[2,6] These enzymes typically couple the two-electron oxidation of substrates with the reaction of 2OG and dioxygen to produce succinate and carbon dioxide. The oxidation of substrates is believed to be mediated by a reactive iron oxidising species [Fe(IV) = O ↔ Fe(III)-O·].[7,8]

The direct products of ANS catalysis have been widely assumed to be the anthocyanidins **5**.[2,6,9–11] However, we recently reported that cyanidin **6** represented only a minor product of in vitro incubations of ANS with commercial leucocyanidin (LCD) (a mixture of 2*R*,3*S*,4*S*-*cis*-**7** and 2*R*,3*S*,4*R*-*trans*-**8** epimers).[12] The major product, after quenching, was the four-electron oxidation product, quercetin **9**. Of the observed two-electron oxidation products, *cis*-DHQ **10** (only one enantiomer observed, >90% ee) predominated over the more thermodynamically stable *trans*-DHQ isomer **11** and cyanidin **6**.

Here, we report in vitro studies with ANS using optically active LCD substrates epimeric at the C-4 position. The results demonstrate that the stereochemistry at the C-4 position of the LCD substrate directly affects product selectivity (Figs. 2 and 3).

2R,3S,4R-trans-LCD **8**, prepared by sodium borohydride mediated reduction of the C-4 ketone function of 2*R*,3*R*-*trans*-DHQ **11**, was epimerised at C-4 under mildly acidic conditions, according to Stafford et al.[13] Separation of the C-4 LCD epimers **7**/**8** has been reported on a small scale using paper chromatography and HPLC. Separation of the LCD epimers from the phosphate HPLC buffer was achieved by addition of a C-18E solid-phase extraction purification step.[13] The

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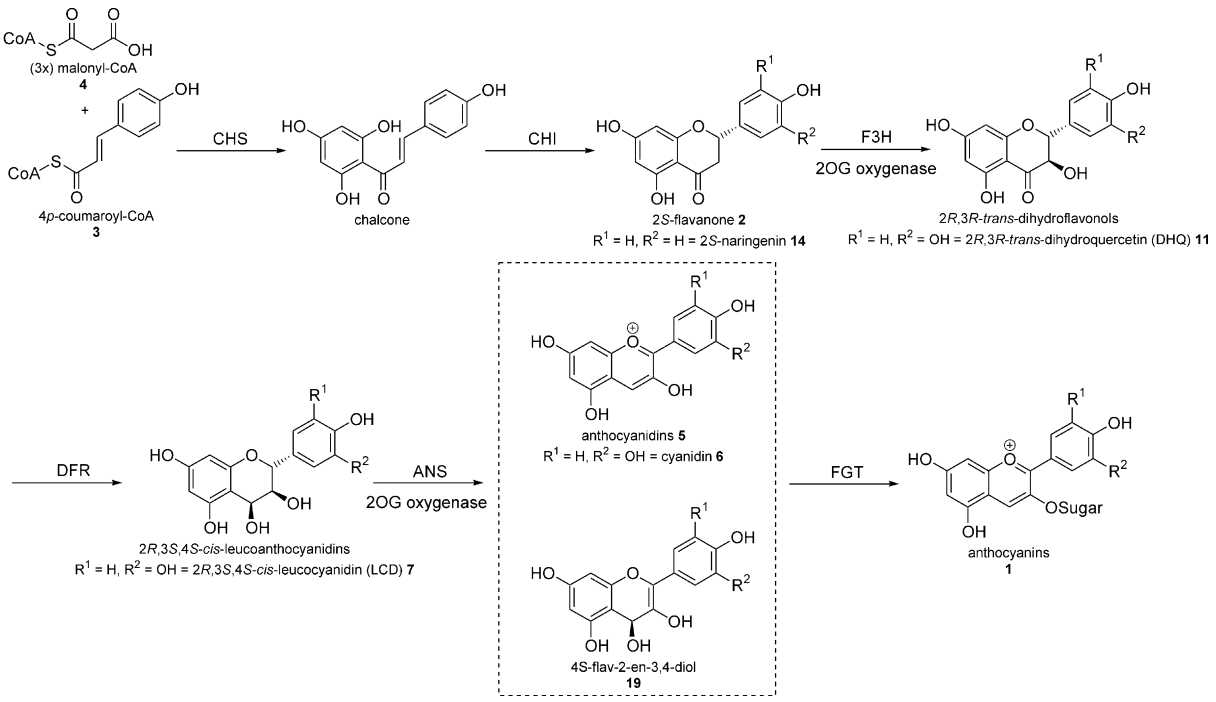

Figure 1. Anthocyanin **1** biosynthesis. Both the previously proposed in vivo products of ANS, anthocyanidins **5**, and the nascent in vivo product proposed in this work, a 4*S*-flav-2-en-3,4-diol **19**, are shown.

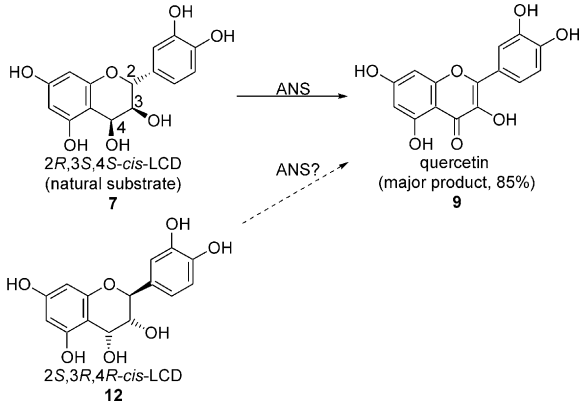

Figure 2. Quercetin **9**, is the major product formed on incubation of 2*R*,3*S*,4*S*-*cis*-LCD **7** with ANS (minor products, include 2*R*,3*S*-*cis*-**10** DHQ and 2*R*,3*R*-*trans*-**11** DHQ and cyanidin **6**). It cannot be ruled out that 2*S*,3*R*,4*R*-*cis*-LCD **12** is a poor substrate for ANS.

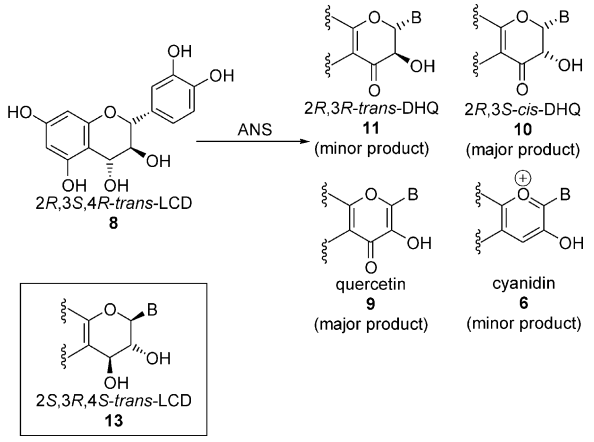

Figure 3. Products formed on incubation of 2*R*,3*S*,4*R*-*trans*-LCD **8** with ANS. It cannot be ruled out that 2*S*,3*R*,4*S*-*trans*-LCD **13** is a poor substrate for ANS and may form DHQ products enantiomeric with those shown above.

purified LCDs were characterised by 1-D and 2-D ^{1}H NMR. The identity of the separated C-4 epimers was demonstrated, in part, by analysis of the C-3,C-4 ^{1}H coupling constants ($J = 3.5$ Hz for *cis*-LCD **7** and $J = 7.0$ Hz for *trans*-LCD **8**).[13] In addition to preparing optically active 2*R*,3*S*,4*S*-*cis*-LCD **7** and 2*R*,3*S*,4*R*-*trans*-LCD **8**, racemic *cis*-LCD **7**/**12** and *trans*-LCD **8**/**13** substrates were also synthesised.

HPLC analysis of incubations of ANS with either optically active 2*R*,3*S*,4*S*-*cis*-LCD **7** or racemic *cis*-LCD **7**/**12** demonstrated that quercetin **9** was the major product (Table 1 and Fig. 2). Minor products observed were *cis*-DHQ **10**, *trans*-DHQ **11** and cyanidin **6**.

HPLC analysis of incubations of ANS with either optically active 2*R*,3*S*,4*R*-*trans*-LCD **8** or racemic *trans*-LCD **8**/**13** gave a more even product distribution, with *cis*-DHQ clearly being the major product (Table 1 and Fig. 3). These results imply that most (if not all) of the *cis*-DHQ **10**, *trans*-DHQ **11** and cyanidin **6** produced in ANS incubations of commercial LCD **7**/**8** arises from 2*R*,3*S*,4*R*-*trans*-LCD **8**.

A 2OG decarboxylation assay was used to measure the specific activity of ANS with the different LCD substrates (Fig. 4). A reduced rate of $^{14}CO_2$ formation was observed in the presence of racemic *cis*-LCD **7**/**12** and *trans*-LCD **8**/**13** substrates relative to the chirally pure substrates **7** and **8** (Table 2).

Table 1. Observed product distributions from ANS incubations (20 min) with 2*R*,3*S*,4*S*-*cis*-LCD **7** and 2*R*,3*S*,4*R*-*trans*-LCD **8** after quenching with MeOH (percentage product distributions calculated by analysing HPLC peak area against standards)[13,14]

Substrate	% *cis*-DHQ	% *trans*-DHQ	% Cyanidin	% Quercetin
	10	**11**	**6**	**9**
2*R*,3*S*,4*S*-*cis*-LCD **7**	10	3	2	85
2*R*,3*S*,4*R*-*trans*-LCD **8**	55	11	4	30

LCD **7**/**8** are prone to epimerisation (at C-2 and C-4) and it is possible that some of the minor products arise from 'contaminating' stereoisomers. However, no evidence was obtained by HPLC analysis to indicate that such epimerisations were occurring under the incubation conditions.

Table 2. Rate of $^{14}CO_2$ formation from 1-[^{14}C]-2OG in the presence of ANS

Substrate	Specific activity of $^{14}CO_2$ formation from 1-[^{14}C]-2OG (μkat kg^{-1})	Specific activity of $^{14}CO_2$ formation from 1-[^{14}C]-2OG corrected for uncoupled turnover (μkat kg^{-1})
Racemic *cis*-LCD **7**/**12**	84.1 (±6.9)	41
2*R*,3*S*,4*S*-*cis*-LCD **7**	181.5 (±28.7)	138
Racemic *trans*-LCD **8**/**13**	188.4 (±19.3)	145
2*R*,3*S*,4*R*-*trans*-LCD **8**	385.0 (±31.4)	342

Values are means of three experiments, standard deviation is given in parentheses.

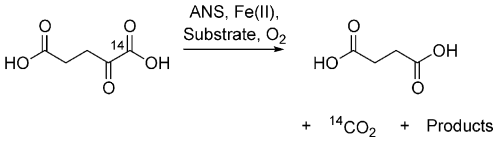

Figure 4. 2OG turnover by ANS.

These results imply that the active site of ANS is selective for LCD substrates with the natural 2*R*,3*S*-stereochemistry; this conclusion is supported by crystallographic evidence.[15] The studies also infer that 2*R*,3*S*,4*R*-*trans*-LCD **8** is more catalytically active under in vitro conditions than the natural 2*R*,3*S*,4*S*-*cis*-LCD **7** stereoisomer.

Crystallographic analyses and studies demonstrating C-3 hydroxylation of the unnatural substrate naringenin **14** (Fig. 1) by ANS, support a mechanism for ANS catalysis involving initial C-3 oxidation of the flavonoid nucleus.[15,16] Furthermore, when an LCD substrate labelled with deuterium at C-4 was incubated with ANS, the label was conserved in the cyanidin product. This demonstrates that C-4 oxidation of LCDs does not occur during ANS catalysed cyanidin formation.[16]

For the formation of *trans*-DHQ **11** from incubations of ANS with 2*R*,3*S*-LCD **7**/**8** substrates the relative stereochemistry at the C-2 and C-3 positions is conserved. Formation of this product could thus occur via C-4 oxidation. However, since formation of cyanidin **6** and *cis*-DHQ **10** (see below) almost certainly occurs via reaction at C-3, formation of *trans*-DHQ **11** via reaction at C-3 seems likely.

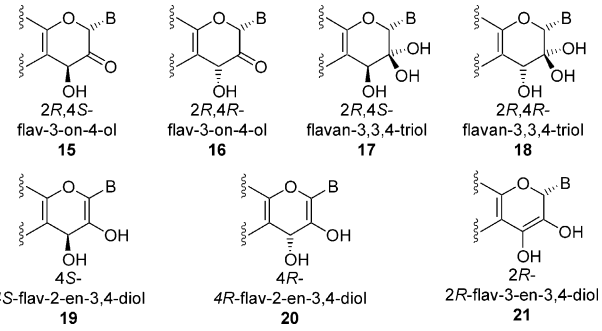

Figure 5. Possible initial oxidation products of 2*R*,3*S*-LCDs **7**/**8**.

In the formation of *cis*-DHQ **10** from either 2*R*,3*S*,4*S*-*cis*-LCD **7** or 2*R*,3*S*,4*R*-*trans*-LCD **8** there is an alteration in the relative C-2 and C-3 stereochemistry. Possible intermediates enabling this are a flav-3-on-4-ol **15**/**16** (from 2*R*,3*S*,4*S*-*cis*-LCD **7** and 2*R*,3*S*,4*R*-*trans*-LCD **8**, respectively), a flavan-3,3,4-triol **17**/**18**, a flav-2-en-3,4-diol **19**/**20** or a 2*R*-flav-3-en-3,4-diol **21** (Fig. 5).

Crystallographic work has implied that direct oxidation at C-2 is unlikely.[15] Thus, oxidation of 2*R*,3*S*,4*S*-*cis*-LCD **7** or 2*R*,3*S*,4*R*-*trans*-LCD **8** at C-3 to give either flav-3-on-4-ols **15**/**16**, respectively, or flavan-3,3,4-triols **17**/**18**, respectively, is the most probable mechanism of reaction.

The elimination of water from flavan-3,3,4-triols **17**/**18** is favoured in a stereo-electronic sense by a diaxial arrangement of the newly introduced C-3 hydroxyl group and the hydrogen involved in elimination. Tautomerisation of flav-3-on-4-ols **15**/**16** is similarly favoured by involvement of a *pseudo*-axial hydrogen by allowing maximal

overlap with the adjacent π orbital of the carbonyl function.

Analysis of the structures of the flav-3-on-4-ols **15**/**16** and the flavan-3,3,4-triols **17**/**18** modelled into the ANS active site implied that the C-2 hydrogen of all these intermediates is *pseudo*-axial (Fig. 6). However, the C-4 hydrogen is *pseudo*-equatorial for the intermediates from 2*R*,3*S*,4*S*-*cis*-LCD **7**, and *pseudo*-axial for the intermediates from 2*R*,3*S*,4*R*-*trans*-LCD **8**. Thus, formation of a 4*S*-flav-2-en-3,4-diol **19** from the initial C-3 oxidation product of 2*R*,3*S*,4*S*-*cis*-LCD **7** is likely to be kinetically favoured, over formation of a 2*R*-flav-3-en-3,4-diol **21** (Fig. 7). In contrast, on C-3 oxidation of 2*R*,3*S*,4*R*-*trans*-LCD **8** formation of both a 4*R*-flav-2-en-3,4-diol **20** and a 2*R*-flav-3-en-3,4-diol **21** may be considered favourable (Fig. 8).

Formation of cyanidin **6** from 2*R*,3*S*-LCDs involving loss of the C-4 hydroxyl group from a flav-2-en-3,4-diol **19**/**20** intermediate has been suggested.[5,6,10] The current work is consistent with this proposal. However, in vitro, quercetin **9** is the major product from 2*R*,3*S*,4*S*-*cis*-LCD **7**. A further catalytic cycle, involving C-3 or C-4 oxidation of the intermediate **19** could lead to the major quercetin **9** product (Fig. 7). Modelling studies on the active site of ANS with flav-2-en-3,4-diols (both 4*S*-**19** and 4*R*-**20**) indicated that the C-4 hydrogen of the 4*S*-flav-2-en-3,4-diol **19** is more accessible to C-4 oxidation than that of the 4*R*-flav-2-en-3,4-diol **20**. This difference may rationalise the increased levels of quercetin **9** observed in incubations of 2*R*,3*S*,4*S*-*cis*-LCD **7** relative to 2*R*,3*S*,4*R*-*trans*-LCD **8** (see below).

The increased likelihood of 2*R*-flav-3-en-3,4-diol **21** formation from oxidation of *trans*-LCD **8**/**13** substrates can explain why these substrates give more DHQ **10**/**11** products than observed with 2*R*,3*S*,4*S*-*cis*-LCD **7** (Fig. 8). Tautomerisation in the active site results in preferential (at least) formation of *cis*-DHQ **10**, possibly due to ligation of the C-3 hydroxyl group to the Fe(II) centre (the *cis*/*trans* equilibrium position is ca. 1:9 in solution).[14]

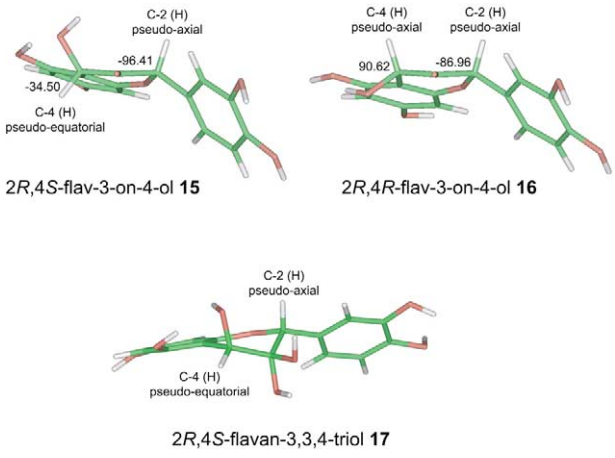

Figure 6. Models of the flav-3-on-4-ols **15**/**16** and 2*R*,4*S*-flavan-3,3,4-triol **17** of LCD in the active site of ANS. Torsion angles for the flav-3-on-4-ols are from the appropriate H to **C-2**/**C-4, C-3, O**.

The preferred formation of quercetin **9** and low levels of two-electron oxidation products observed in vitro from incubations of 2*R*,3*S*,4*S*-*cis*-LCD **7** may reflect the lack of release (or very tight binding) of a 4*S*-flav-2-en-3,4-diol intermediate **19**. The presence of a trapped 'product' in the active site may also be related to the lower specific activity for 2*R*,3*S*,4*S*-*cis*-LCD **7** compared to 2*R*,3*S*,4*R*-*trans*-LCD **8** in vitro with the 2OG turnover assay. The possibility of two substrate binding sites is supported by crystallographic analyses.[15] In vivo, however, it is clear that ANS together with the final enzyme of anthocyanin **1** biosynthesis, a UDP-glucose:flavonoid 3O-glycosyltransferase (FGT) mediate anthocyanin **1** formation. It may be that ANS and FGT interact to allow release of a 4*S*-flav-2-en-3,4-diol **19**, which is the substrate for FGT (Fig. 1). This is supported by studies

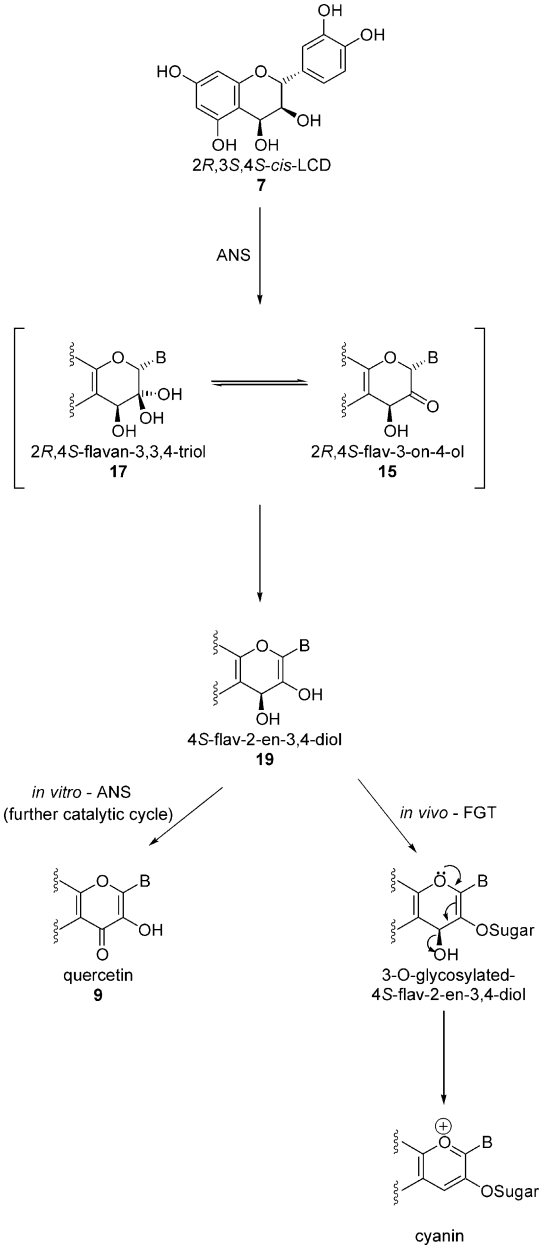

Figure 7. In vitro formation of quercetin **9** by ANS and in vivo formation of cyanin by ANS and FGT.

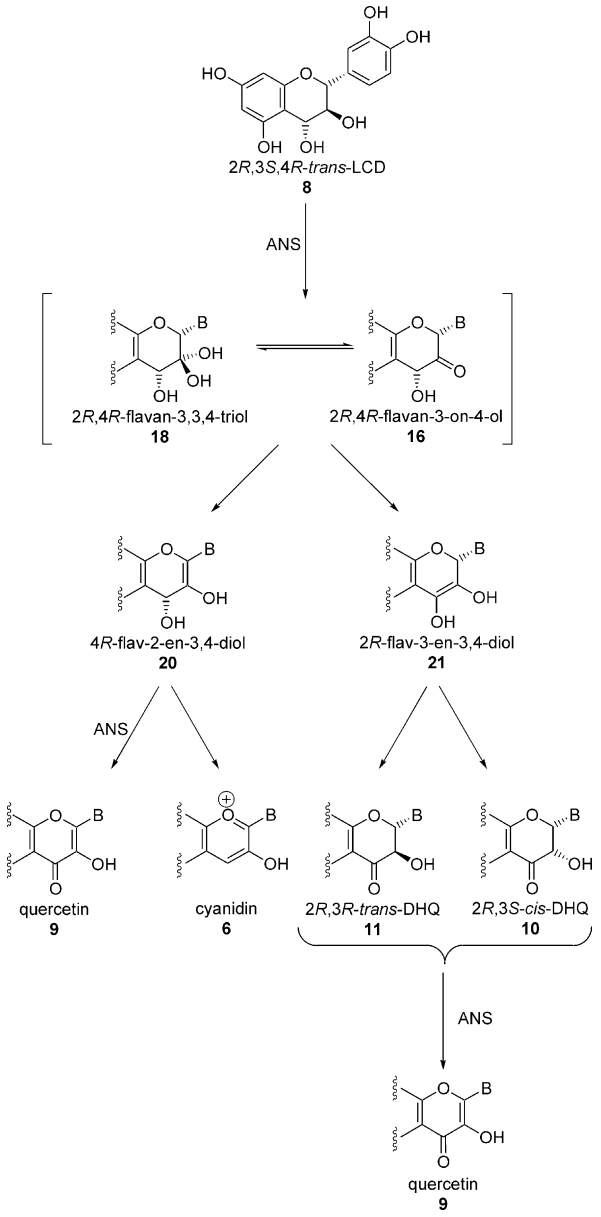

Figure 8. Mechanism of product formation of ANS mediated oxidation of 2*R*,3*S*,4*R*-*trans*-LCD **8**. The 4*R*-flav-2-en-3,4-diol **20** may also be released from the active site of ANS and tautomerise to give a thermodynamic distribution of DHQ, with *trans*-DHQ predominating.

showing that the enzymes of flavonoid biosynthesis form a membrane bound multi-enzyme complex.[17–21]

Acknowledgements

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