Are anthocyanidins the immediate products of anthocyanidin synthase?

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Anthocyanidin synthase catalyses the *in vitro* conversion of its natural substrate, leucocyanidin, to *cis*- and *trans*-dihydroquercetin, quercetin and a small amount of cyanidin; incubation of *trans*-dihydroquercetin gave quercetin.

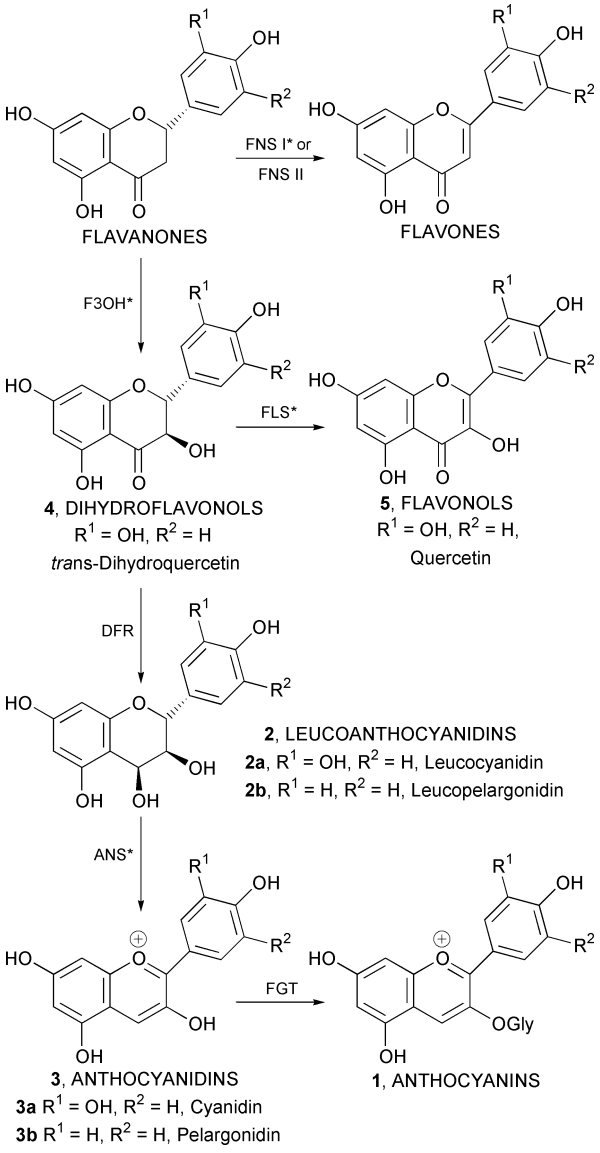
Flavonoids are an important class of plant metabolites whose roles include attracting pollinators, signalling in plant-microbe interactions, protecting plants against radiation and aiding seed dispersal.[1] There is biomedical interest in flavonoids since they inhibit cell proliferation, are antioxidants and display anti-mutagenic, antinflammatory, antithrombic and antihypertensive effects.[2,3] The anthocyanin sub-family of flavonoids cause pigmentation and are used as food colourants.

The penultimate step in the biosynthesis of the anthocyanin family **1** of flavonoids is catalysed by anthocyanidin synthase (ANS) (Scheme 1).[4] Although there are reports on other enzymes involved in anthocyanin biosynthesis, until recently the only reported work on the ANS concerned the identification of its genetic locus and putative gene sequence.[5] The presence of conserved sequence motifs (*e.g.* HXD and RXS motifs, which are involved in iron and co-substrate binding respectively[6,7]) imply ANS belongs to the family of 2-oxogluatarate (2-OG) dependent dioxygenases.[8] Saito *et al.* reported that ANS from *Perrila fructescens* was indeed a 2-OG dependent oxygenase requiring an unusually high level of ascorbate for activity.[9] It was demonstrated that oxidation of leucocyanidin **2a** (R^1 = OH, R^2 = H) and leucopelargonidin **2b** (R^1 = H, R^2 = H) was coupled with conversion of 2-OG to succinate. However, it was noted that the anticipated anthocyanidin products cyanidin **3a** and pelargonidin **3b**, respectively, were only observed in the crude assay incubations subsequent to acidification. The acidified incubations were analysed by HPLC (monitoring at 520 nm) and peaks corresponding to the appropriate anthocyanidin references identified.[9] Here, we report studies on the selectivity of ANS that lead us to question whether anthocyanidins are the immediate products of the ANS reaction.

The ANS gene from *Arabidopsis thaliana* was over-expressed in *Escherichia coli* BL21 (DE3) using the Novagen pET-24a vector and ANS was purified to *ca.* ~90% purity (by SDS-PAGE analysis) *via* anion-exchange Q-Sepharose and gel filtration chromatography.†

The natural substrates for ANS are relatively difficult to synthesise in enantiomerically pure form and are unstable in solution. Therefore, we investigated the use of a dihydroflavonol, *trans*-dihydroquercetin **4**, as an alternative substrate. Photodiode array (PDA) HPLC analysis identified the product of *trans*-dihydroquercetin incubation as the flavonol quercetin **5**. This result was confirmed by NMR and mass spectrometric analyses. Selected data for the incubation product: (^{1}H NMR 500 MHz; CD_3OD) δ: 4.60 (C-OH, 1H, s br), 6.18 (1H, d, J = 2.0), 6.38 (1H, d, J = 2.0), 6.88 (1H, d, J = 8.5), 7.73 (1H, d, J = 2.0), 7.63 (1H, dd, J = 2.0, J = 8.5); *m/z* (negative ion ESI MS) 301.4 Da.

Using a stopped UV assay the ANS catalysed conversion of *trans*-dihydroquercetin to quercetin was shown to be dependent on both 2-OG and iron(II) and to require a high concentration of ascorbate for significant turnover (40 mM), consistent with the results for ANS from *Perilla fructescens*.[9] Analyses were complicated by precipitation of the flavonoids and their iron



Scheme 1 Later stages of anthocyanin biosynthesis. FNS I , FNS II, flavone synthase I or II; F3OH = flavanone-3β-hydroxylase; FLS = flavonol synthase; DFR = dihydroflavonol-4-reductase; ANS = anthocyanidin synthase; FGT = flavonoid 3-*O*-glucosyltransferase. Asterisked enzymes are 2-OG dependent dioxygenases.[4]

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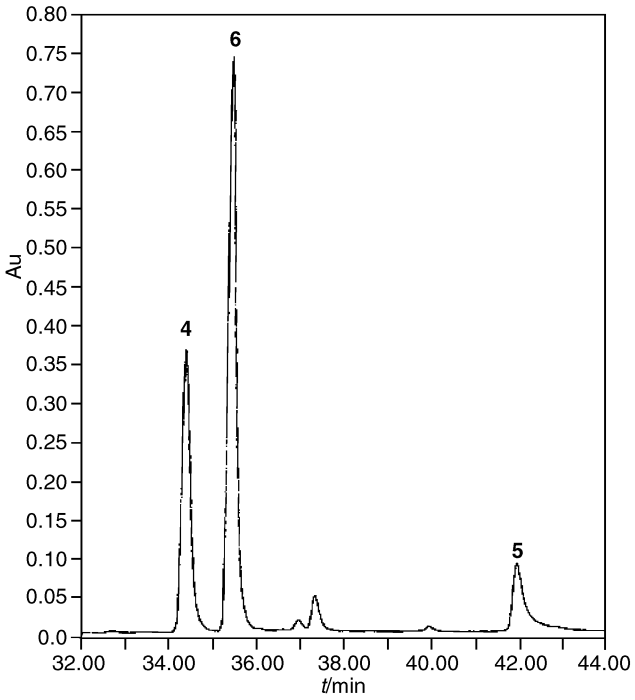
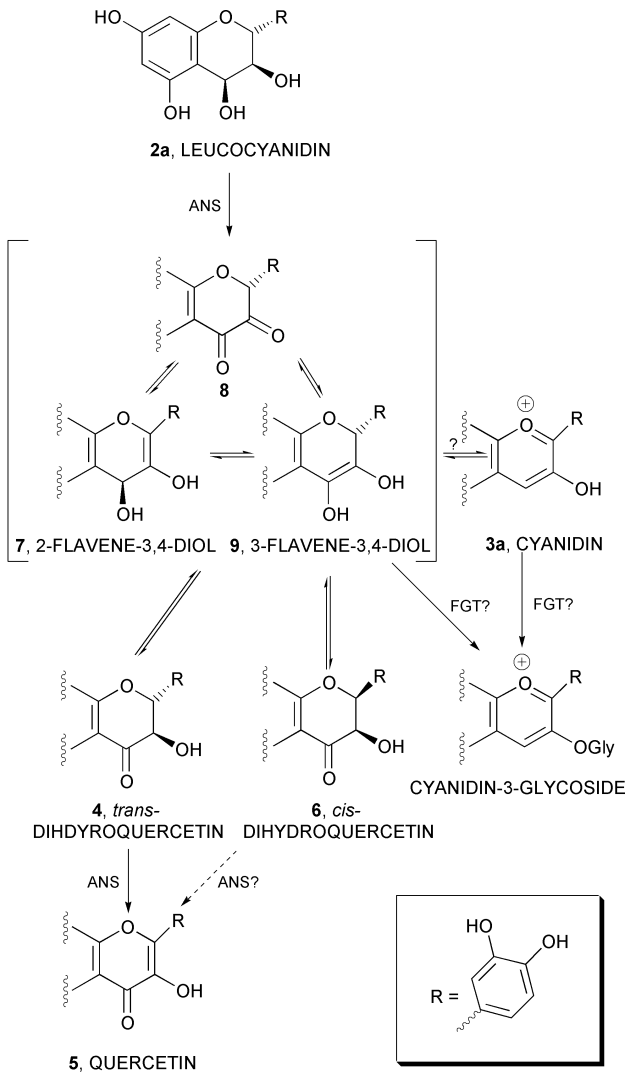


Fig. 1 HPLC trace (λ_{max} 287 nm for **4**, **6**; 372 nm for **5**) of ANS assay with leucocyanidin **2a** after incubation for 20 min. The peaks correspond to *trans*-dihydroquercetin **4**, *cis*-dihydroquercetin **6** and quercetin **5** respectively. Note the **2a** used (Apin) contained contaminating **4** but not **6**.



Scheme 2 Proposed intermediates for ANS and FGT turnover of leucocyanidin.[4] The absolute stereochemistry of products **4** and **6** from **2a** has not been determined.

complexes; the maximal catalytical rate was seen at *ca.* pH 6.0. Selwyn analysis[10] indicated that ANS was unstable under the assay conditions at protein concentrations < 70 μg ml^{-1}.

PDA HPLC (monitoring from 200–800 nm) was then used to study ANS catalysed oxidation of a 'natural substrate' leucocyanidin **2a** (Fig. 1). Comparison with known standards identified products as *cis*-**6**‡ and *trans*-**4** dihydroquercetin, quercetin **5** and cyanidin **3a**. The latter was clearly a minor product.

Time course analysis of the leucocyanidin incubation revealed that as the reaction progressed the relative concentration of *trans*-dihydroquercetin decreased as that of quercetin increased. Incubations using mixtures of *cis*-**6** and *trans*-**4** dihydroquercetin demonstrated that *trans*-**4** rather than *cis*-**6** dihydroquercetin was preferentially converted to quercetin **5**.

The observation that both *cis*-**6** and *trans*-**4** dihydroquercetin are produced from leucocyanidin is consistent with the major nascent product of leucocyanidin incubation being a 2-flavene-3,4-diol **7**, as suggested by Heller and Forkmann or ketone **8**, or a 3-flavene-3,4-diol **9** (Scheme 2).[4] Intermediates **7**, **8**, **9** may be non-enzymatically isomerised to give both *cis*-**6** and *trans*-**4** dihydroquercetin. Under the incubation conditions the production of cyanidin is not favoured and it is possible that *in vivo* aromatisation does not occur until the next step,[4] *i.e.* that catalysed by the flavonoid glucosyltransferase (FGT). Alternatively, it is possible that **6** is the (major) product (and substrate for FGT) with the observed **4** arising by isomerisation of **6**.

ANS catalyses conversion of *trans*-dihydroquercetin **4** to quercetin **5**, *i.e.* the same reaction catalysed by flavonol synthase (FLS) (Scheme 1)[4] may reflect incomplete evolution of substrate selectivity or that the pathway has evolved to maintain/develop redundancy in enzymes selectivities. ANS is an interesting enzyme for study due to the ease of tautomerisation/isomerisation of its products, particularly its intermediate products which have not yet been isolated.

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Notes and references

† The mass and N-terminal protein sequence of the first eleven residues of purified ANS were consistent with the predicted values.[5]

‡ Synthetic **6** reference was obtained by isomerisation of **4** (*trans*:*cis*, 9:1).[11] Leucocyanidin (>95% purity) was from Industrial Research Limited, New Zealand.

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