CHEMCOMM Communication
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Evidence for oxidation at C-3 of the flavonoid C-ring during anthocyanin biosynthesis

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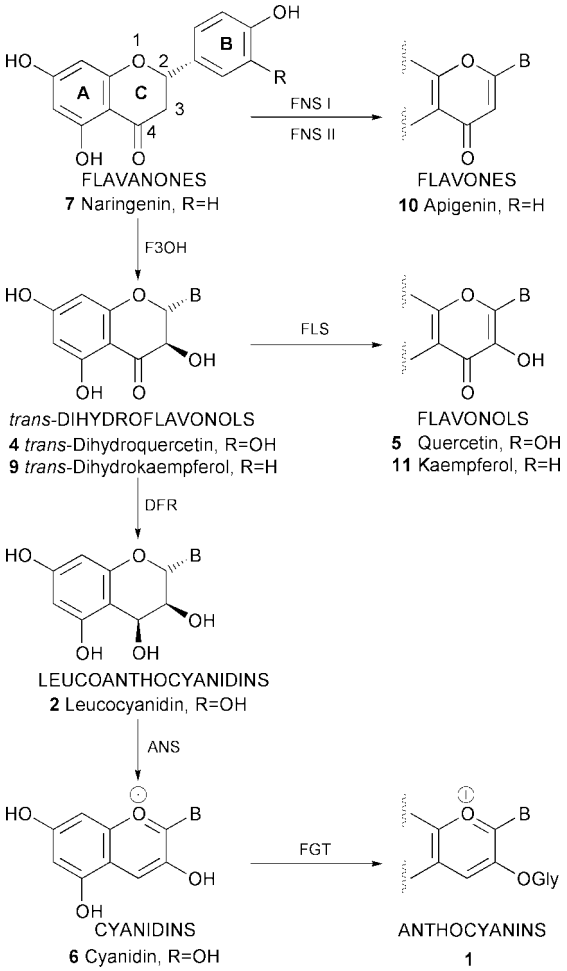
Received (in Cambridge, UK) 26th June 2001, Accepted 8th August 2001
First published as an Advance Article on the web 3rd September 2001

Evidence is presented for initial oxidation at the C-3 position of the flavonoid C-ring and for two bifurcating steps during catalysis by anthocyanidin synthase.

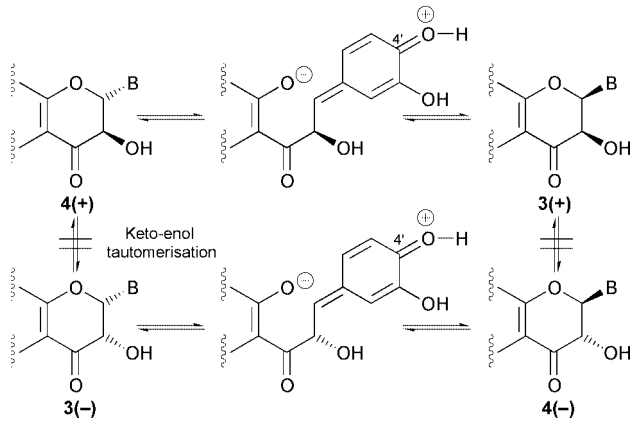
Anthocyanidin synthase (ANS)‡ catalyses the penultimate step in the biosynthesis of the anthocyanin group **1** of flavonoid pigments in plants (Scheme 1).[1] Recent studies have confirmed ANS as a non-haem Fe^{II} and 2-oxoglutarate dependent oxygenase requiring high levels of ascorbate for optimal catalytic activity *in vitro*.[2–4] ANS is one of four 2-oxoglutarate dependent oxygenases that catalyse modification of the central γ-pyran C-ring during flavonoid biosynthesis (Scheme 1). Of the others, flavone synthase I (FNS I) and flavonol synthase (FLS) both catalyse desaturation across the C-2–C-3 bond, whilst flavanone-3β-hydroxylase (F3OH) catalyses stereoselective hydroxylation at C-3 of the flavanones.[5–7] Several mechanisms for the conversion of leucoanthocyanidins to anthocyanidins have been proposed. These include initial C-2 hydroxylation and desaturation across the C-2–C-3 bond.[8,1,2]



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.

Recently, we reported that *in vitro* incubation of ANS (*Arabidopsis thaliana*) with its natural substrate leucocyanidin **2** leads to both *cis*-**3** and *trans*-**4** dihydroquercetin (DHQ) (the chiral purity of these compounds was unassigned), as well as quercetin **5** and the anticipated natural product cyanidin **6**.§ The ratio of *cis*:*trans*-DHQ observed in nascent quenched incubation mixtures was displaced towards the less stable *cis*-isomer **3**, suggesting it was a direct enzymatic product. It was also demonstrated that ANS catalyses conversion of *trans*-DHQ **4** to quercetin **5**.[3] Here we describe results that provide a clearer mechanistic picture for ANS and reveal there are two potential points of bifurcation during catalysis.

The observation of several products, some prone to isomerisation, from the incubations of leucocyanidin **2** with ANS made it desirable to define which are released from the active site and which are produced by subsequent isomerisation. By investigating the chiral purity of the DHQ products, it was hoped inferences could be made as to their origin. Initially we confirmed a report that dihydroflavonol isomerisation occurs predominantly *via* opening of the γ-pyrone C-ring with anomeric assistance from the C-4′ hydroxy group of the B-ring.[9,10] Upon refluxing *trans*-DHQ **4** in D_2O or CD_3OD–D_2O, no detectable (< 5%, by ^1H NMR analyses) deuterium was incorporated at C-3 of the C-ring, implying isomerisation does not predominantly occur *via* keto–enol tautomerisation (Scheme 2).



Scheme 2 Mechanism for *cis–trans* isomerisation of dihydroquercetin.

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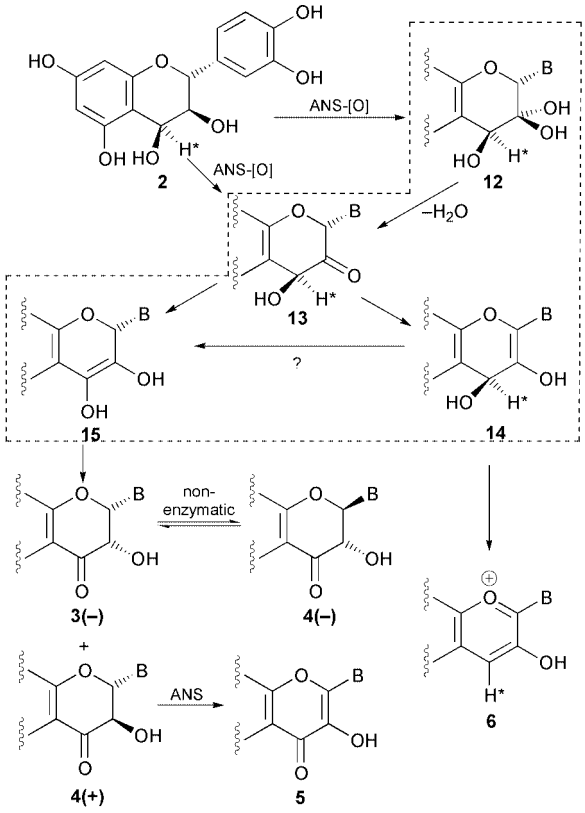
 DOI: 10.1039/b105576n

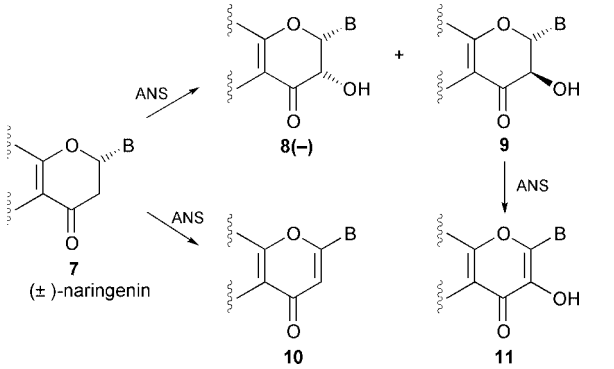


Scheme 3 *In vitro* ANS catalysed oxidation of leucocyanidin.**

To obtain NMR evidence for stereochemical assignments, it was necessary to purify the products from the crude incubation mixtures. Preparative HPLC was attempted, but the acidic conditions required to prevent co-elution and precipitation of flavonoids resulted in isomerisation and degradation of the *cis*-DHQ **3**.[11] Instead reverse phase C-18E solid phase extraction columns (eluting with water–MeOH) were used to isolate the dihydroflavonols from the crude incubation mixture. The optical purity of *O*-methylated DHQ's has been assessed by ^{1}H NMR using lanthanide chiral shift reagents.[11,12] This resulted in enantiomer dependent splitting of the C-5 hydroxy proton resonance (δ11.5 for DHQ). Eu(hfc)$_3$ was found to be of particular utility over the analogous Pr(tfc)$_3$ and Eu(tfc)$_3$ derivatives, as it was more soluble in CD_3CN and provided improved peak separation.¶

The NMR spectra of the DHQ produced in the leucocyanidin **2** assay revealed a *cis*-**3**:*trans*-**4** DHQ ratio of *ca.* 2:1, significantly less than observed by analytical HPLC, indicating *cis*- to *trans*-isomerisation had occurred during work-up. Subsequent addition of shift reagent revealed *cis*-DHQ **3** was present in high enantiomeric excess (>90%) assigned as the (−) form, assuming conservation of C-2 chirality. The high enantiomeric excess of the *cis*-isomer implies it is an enzymatic product. In contrast, *trans*-DHQ **4** was observed as a mixture of enantiomers. Formation of the (−)-*trans*-DHQ **4** enantiomer is probably due to isomerisation of the enzymatically produced (−)-*cis*-DHQ **3** (Scheme 3). The mechanism of *cis–trans* DHQ interconversion implies that the (+)-*trans*-DHQ **4** formed is also a direct enzymatic product.‖ The (+)-*trans*-DHQ **4** formed is depleted by its ANS catalysed desaturation to quercetin.[3]

Previous *in vivo* feeding studies using 4-[^{3}H]-leucocyanidin **2** demonstrated the tritium label was conserved in biosynthetic anthocyanin **1** and higher derivatives, indicating cyanidin formation does not proceed *via* C-4 oxidation.[13] To investigate the site of initial oxidation, ANS was incubated with the flavanone (±)-naringenin **7**, an unnatural substrate (Scheme 4).



Scheme 4 Reaction of ANS with naringenin (only major isomers shown).

The major product was dihydrokaempferol (DHK), with a *cis*-**8** to *trans*-**9** ratio of *ca.* 4:1 by ^{1}H NMR analysis after work-up. Chiral shift NMR analyses indicated the presence of *cis*-DHK in high enantiomeric excess (>70%) again with the (−)-enantiomer predominant. Apigenin **10** and trace amounts of kaempferol **11** were also observed. The former presumably results from a competing desaturation mechanism while the latter from ANS catalysed desaturation of enzymatically produced *trans*-DHK **9**.

Assuming the site of initial oxidation is the same for cyanidin and dihydroflavonol formation, the evidence presented here implies initial substrate reaction occurs at C-3, possibly *via* 'α face'-hydroxylation to give **12**, although direct formation of a ketone **13**** is also possible (Scheme 3). Subsequently, enolisation to form a flav-2-en-3,4-diol **14** leads to cyanidin **6** whilst formation of a flav-3-en-3,4-diol **15** can lead either to (−)-*cis*-DHQ **3** or (+)-*trans*-DHQ **4** in a second bifurcating process. If the ANS mechanism proceeds as proposed, it suggests that FLS and FNS I catalysis may also occur *via* hydroxylation at C-3 followed by dehydration, instead of the vicinal desaturation processes previously proposed.[5] Factors influencing the outcome of the complex *in vitro* ANS reaction and the relevance of the bifurcation steps to the *in vivo* situation are under investigation.

We thank the BBSRC, EPSRC, MRC and EU for financial support of the work and Dr R. T. Aplin for MS analyses.

Notes and references

‡ Also known as Leucoanthocyanidin (di)oxygenase.
§ Saito *et al.* have also reported the *in vitro* conversion of **2** to **6** with HPLC monitoring at 520 nm (non-optimal for detection of dihydroflavonols).[4]
¶ (hfc)$_3$-(+):tris(3-heptafluoropropylhydroxymethylene)-(+)-camphorate; (tfc)$_3$-(+):tris(3-trifluoromethylhydroxymethylene)-(+)-camphorate.
‖ Absolute chirality was assigned by doping a racemic mixture of *trans*-DHQ with pure (+) *trans*-DHQ **4** by NMR.
** Ketone intermediate should be drawn as **13** and not as in ref. 3.

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