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Stable solid-supported leucoanthocyanidin variants for flavanoid biosynthesis elucidation

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ABSTRACT

Remarkable progress toward the complete elucidation of the biosynthesis of flavanoids has been accomplished during the last decade, but the final steps presumably involving the transformation of leucoanthocyanidins, which are highly unstable when free in aqueous solution, into both anthocyanidins and proanthocyanidins still remain to be fully understood. Herein is described the synthesis of stabilized solid-supported leucoanthocyanidin variants that should serve as valuable tools for in vitro studies aimed at investigating the metabolism of these seemingly fleeting common precursors of two main classes of flavanoids.

The biosynthesis of flavo/flavanoids is probably one of the most extensively studied plant metabolic pathways.[1] Most of the genes encoding the enzymes that are responsible for the catalysis of each individual step have been isolated from different plant sources.[2] However, the last steps leading to the formation of flavylium-based anthocyani(di)ns and flavan-3-ol-derived proanthocyanidins remain unclear and are still under debate due in part to the lack of in vitro evidence.[3] The putative biosynthesis pathway leading to these two important classes of flavanoids[4] is depicted in Scheme 1. Leucoanthocyanidins [i.e., (2*R*,3*S*,4*S*)-flavan-2,3-*trans*-3,4-*cis*-diols] are presumed to be the common precursors of both anthocyani(di)ns and proanthocyanidins. Results from in vitro studies have shown that Leucoanthocyanidin DiOXygenase (LDOX, also referred to as ANS for ANthocyanidin Synthase), a 2-oxoglutarate-dependent enzyme, is involved in the conversion of colorless leucoanthocyanidins into colored anthocyanidins 3-*O*-glucosides.[5] These anthocyanins can then undergo methylation of their ring-B hydroxyl groups.

However, the mechanistic details of the conversion of leucoanthocyanidins into anthocyanidins have not yet been fully elucidated due to the lack of structural information on the intermediate(s) entailed in this biotransformation.[6] Even more speculative are the final stages of the biosynthesis of proanthocyanidins.[7] These so-called condensed tannins would result from the polymerization of flavan-3-ols and/or flavan-3,4-diols (Scheme 1). At least two additional enzymes would be involved in the process leading to flavan-3-ols from leucoanthocyanidins. Thus, (2*R*,3*R*)-*cis*-flavan-3-ols such as (−)-epicatechin indirectly derive from leucoanthocyanidins through the action of an ANthocyanidin Reductase (ANR) on anthocyanidins,[3a] whereas a LeucoAnthocyanidin Reductase (LAR) directly converts leucoanthocyanidins into (2*R*,3*S*)-*trans*-flavan-3-ols such as (+)-catechin.[8]

Several questions about the (bio)polymerization leading to proanthocyanidins still remain unanswered such as those concerning the exact nature of the starter and extension units, as well as whether or not a dedicated enzyme (i.e., a 'proanthocyanidin synthase') is involved in the process.[7,9] The keystone position of leucoanthocyanidins in this biosynthetic scheme obviously makes them substrates of choice for in vitro experiments aimed at addressing all of the above issues remaining obscure in these final steps of the biogenesis of anthocyani(di)ns and proanthocyanidins. Unfortunately, such experiments are far from being easy to conduct because of the notable instability of these flavan-3,4-*cis*-diol compounds, which are prone to polymerize even under slightly acidic conditions.[1a,10] In aqueous solutions, highly reactive (protonated) *para*- and/or *ortho*-quinone methides resulting from a facile departure of the benzylic hydroxyl group at position 4 are probably responsible for the rapid formation of polymeric materials in an uncontrolled manner. This is also probably the main reason why 3,4-*cis* leucoanthocyanidins that have been previously generated by chemical synthesis were characterized under their protected methyl ether forms.[11] The very few enzymatic studies that have been carried out to date with these compounds in fact report the use of unprotected (2*R*,3*S*,4*R*)-diastereomers of

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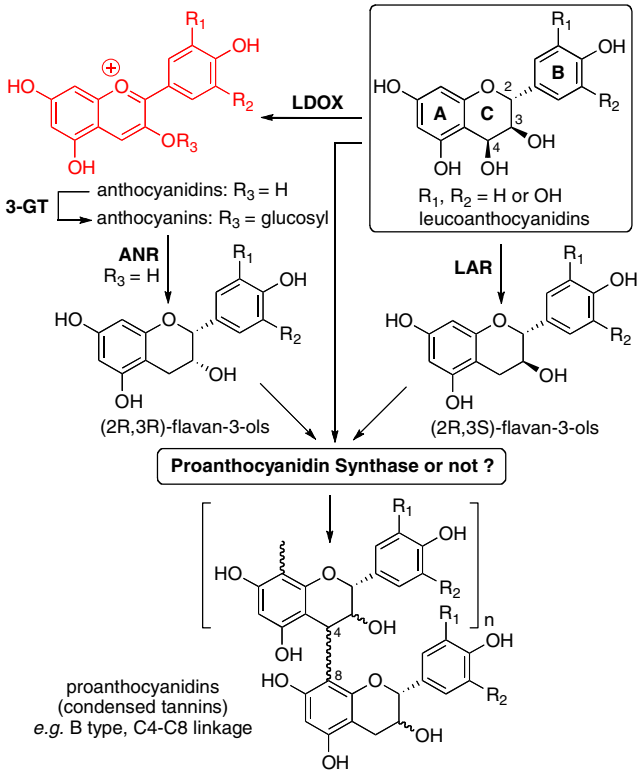


Scheme 1. Putative last steps of the biosynthesis of anthocyani(di)ns and proanthocyanidins.

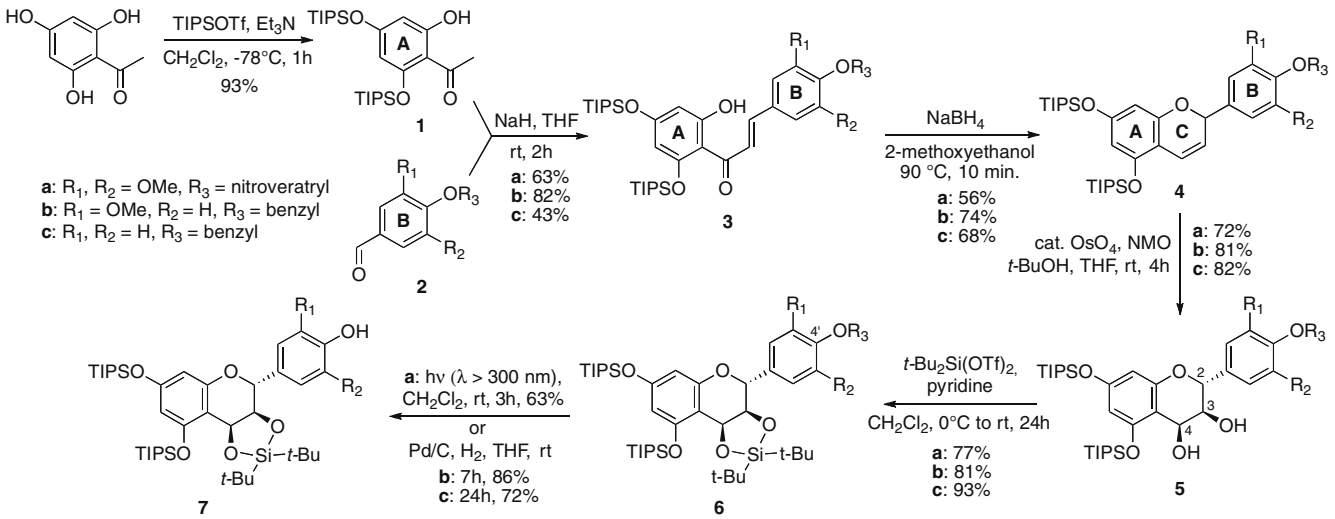
leucoanthocyanidins that are claimed to be easily converted into the desired natural isomers under the conditions used for the enzymatic assays.[5] However, the discrete chemical nature of the materials thus involved in these assays remains questionable.[10b]

We thus decided to engage in efforts aimed at the elaboration of leucoanthocyanidin variants of enhanced stability yet still amenable to enzymatic studies in vitro. Three flavan-3,4-*cis*-diol derivatives (**7a–c**) convertible into some of the most abundant anthocyanins found in berries [i.e., malvidin 3-*O*-glucoside ($R_1 = R_2 = OMe$), peonidin 3-*O*-glucoside ($R_1 = OMe$, $R_2 = H$), and pelargonidin 3-*O*-glucoside ($R_1 = R_2 = H$)][12] were synthesized and grafted onto a biocompatible solid support (vide infra). The premise behind this approach was that thus immobilized at an appropriate mutual distance on an insoluble cross-linked polymer, the selected leucoanthocyanidin variants should find themselves in conditions approaching those of high (infinite) dilution, hence minimizing the possibilities of intermolecular reactions.[13]

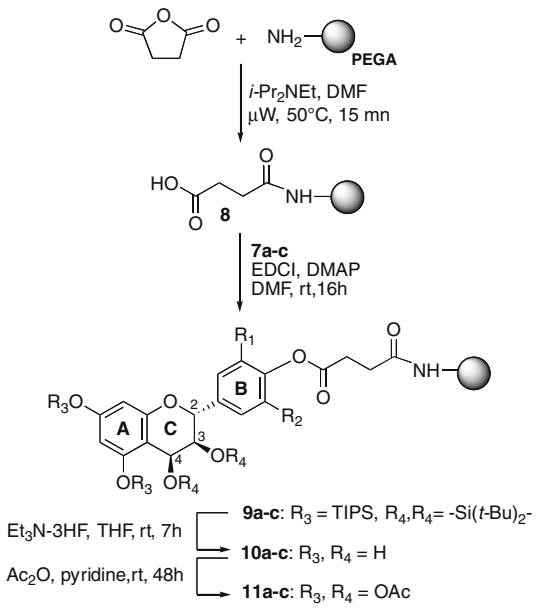
A synthesis of solid-supported leucoanthocyanidins relying on an early grafting step using the polymer support as a protecting group could have constituted a more elegant strategy, but the difficulty of structurally characterizing polymer-bound intermediates by means of spectroscopical methods led us to favor a more practical approach based on the synthesis of the desired leucoanthocyanidin molecules prior to their loading onto the polymer support. The synthesis of the racemic protected leucoanthocyanidins **7a–c** was thus first accomplished (Scheme 2). In each of the three cases, the phenolic hydroxyl function at the 4′-position of ring-B had to be orthogonally protected in anticipation of its exploitation for the attachment to the polymer support. In addition, the protecting groups for all of the other hydroxyl functions had to be chosen while keeping in mind the necessity of removing them under mild conditions without perturbing the fragile leucoanthocyanidin molecules still attached to the polymer support. To meet these requirements, silyl groups were selected to protect the hydroxyl functions at positions 3, 4, 5, and 7, whereas a photolabile 6-nitroveratryl or, alternatively, a classic benzyl group was used for the orthogonal protection of the phenolic 4′-hydroxyl function.

The syntheses started with the partial silylation of commercially available 2,4,6-trihydroxyacetophenone using triisopropylsilyl triflate (TIPSOTf) and triethylamine (Et_3N) in CH_2Cl_2 at low temperature to furnish the phenolic ketone **1** in high yield. The phenolic hydroxyl group of commercial syringaldehyde was protected via a Williamson reaction using 4,5-dimethoxy-2-nitrobenzyl bromide to furnish **2a** in 97% yield (see Supplementary data). The other two ring-B precursors, that is, 4-benzyloxy-3-methoxybenzaldehyde (**2b**) and 4-benzyloxybenzaldehyde (**2c**), are both commercially available. The phenolic ketone **1** was then submitted to a Claisen–Schmidt aldol condensation reaction with each of the three aldehydes **2a–c** to deliver the expected chalcone products **3a–c** in moderate to good yields.[11c,14] The chalcones **3a–c** were then cyclized to give rise directly to the flavenes **4a–c** according to the Clark-Lewis method using sodium borohydride in 2-methoxyethanol (Scheme 2).[11c,15,17a]

The following cis-dihydroxylation of the flavenes **4a–c** was performed using a catalytic amount of osmium tetraoxide in the presence of *N*-methylmorpholine N-oxide (NMO)[16] to furnish the expected racemic 2,3-*trans*-3,4-*cis*-leucoanthocyanidins **5a–c** in



Scheme 2. Synthesis of silylated leucomalvidin (**7a**), leucopeonidin (**7b**), and leucopelargonidin (**7c**).



Scheme 3. Grafting of leucoanthocyanidins onto a functionalized PEGA resin. (**a**) R_1, R_2 = OMe, (**b**): R_1 = OMe, R_2 = H; (**c**): R_1, R_2 = H.

good yields.[17] The 2,3-trans-3,4-cis configuration was confirmed by ^{1}H NMR analysis, which indicated for each compound a $J_{2,3}$ coupling constant of about 10 Hz and a $J_{3,4}$ of 3 to 4 Hz. Next, the *cis*-3,4-diol units of **5a–c** were protected with a di-*tert*-butylsilyl group (Scheme 2). Previous attempts to independently protect these vicinal hydroxyl groups with triisopropylsilyl group failed, as only one silyl group could be introduced using TIPSOTf in the presence of Et_3N. The ultimate step consisted in the deprotection of the 4′-hydroxyl group. Thus, the flavan-3,4-diol **6a** was subjected to irradiation using a high-pressure mercury lamp ($\lambda > 300$ nm) to furnish the silyl-protected leucomalvidin **7a** in 11% overall yield. The corresponding leucopeonidin **7b** and leucopelargonidin **7c** were obtained after hydrogenolysis of **6b** and **6c**, respectively, in 32% and 15% yields over the six steps of their synthesis.

The instability of free leucoanthocyanidins was here verified by monitoring via ^{1}H NMR analysis the *quasi* immediate and steady degradation of a sample of leucopeonidin **7b** in $CDCl_3$ undergoing desilylation with a solution of TBAF in THF at room temperature (see Supplementary data).

With adequately protected leucoanthocyanidins **7a–c** in hand, we turned our attention to the selection of a resin on which to graft them with the following criteria in mind. Such a resin should be not only appropriately functionalized to enable the attachment of **7a–c** via their free phenolic hydroxyl group, but also made of a polymeric material capable of swelling extensively in aqueous media for allowing access of proteins (enzymes) into the resin beads during biochemical assays. Moreover, the loading onto the resin beads should not be too high to avoid the risk of intermolecular reactions between leucoanthocyanidins once deprotected. The polyethyleneglycol-polyacrylamide (PEGA) resin with a potential maximum loading of 0.4 mmol/g meets these criteria.[18]

Succinic anhydride was used to install a functionalized linker compatible with the attachment of **7a–c** (Scheme 3). This requisite adaptation of the PEGA resin was conveniently achieved in the

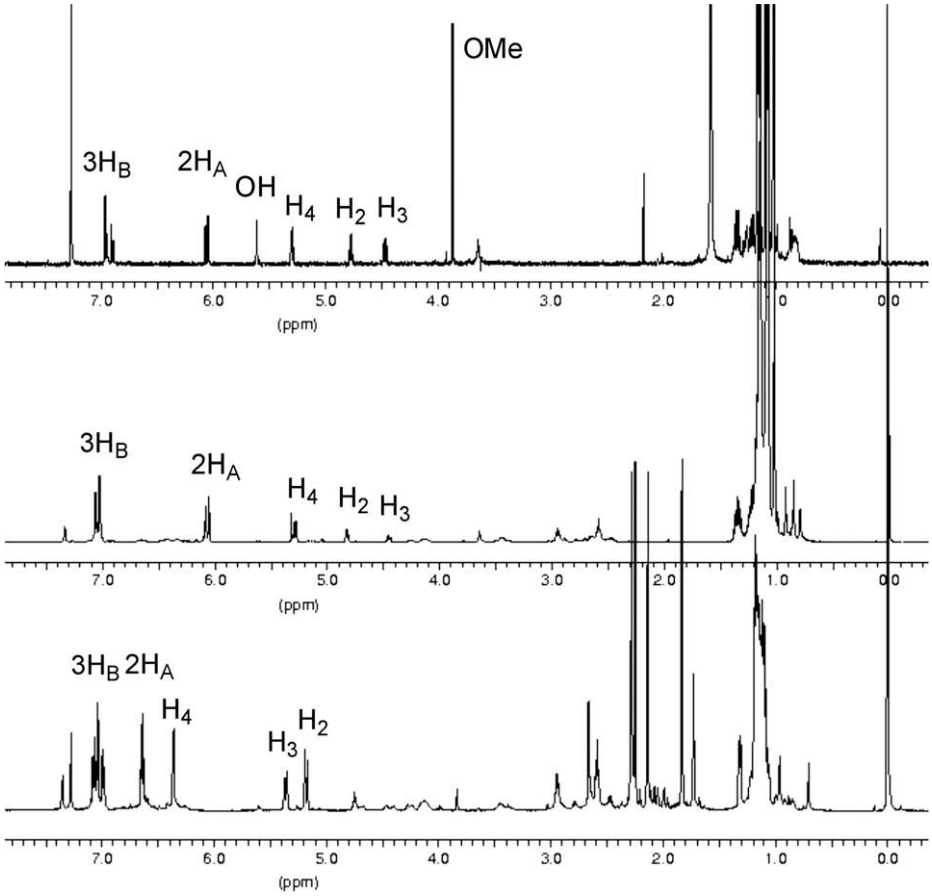


Figure 1. (Top) ^{1}H NMR spectrum of silylated leucopeonidin **7b** in $CDCl_3$; (middle) HR-MAS ^{1}H NMR spectrum of PEGA-supported silylated leucopeonidin **9b** in $CDCl_3$; and (bottom) HR-MAS ^{1}H NMR spectrum of PEGA-supported acetylated leucopeonidin **11b** in $CDCl_3$.

presence of Hunig's base in DMF.[19] Activation by microwaves permitted to drastically reduce the reaction time from about 5 days to only 15 min at 50 °C (see Supplementary data). Then, esterification of the resulting carboxylic acid **8** to the free phenolic function of **7a–c** was accomplished in a standard fashion using EDCI/DMAP as the condensation reagents (Scheme 3). To evaluate the extent of the loading onto the resin, an aliquot of the PEGA-supported silylated leucoanthocyanidin **9b** was treated with sodium methoxide in a CH_2Cl_2/MeOH (2:1) mixture. The quantity of **7b** thus released and estimated by HPLC analysis indicated a loading onto the resin of about 50% (i.e., 0.2 mmol/g).

The PEGA-supported silylated leucoanthocyanidins **9a–c** were characterized by high-resolution magic angle spinning (HR-MAS) NMR analysis. Comparison of their HR-MAS ^{1}H NMR spectra with the standard ^{1}H NMR spectra of the silylated leucoanthocyanidins 7a–**c** in solution indicated an excellent level of concordance between the diagnostic signals of these pairs of spectra (see Fig. 1 and Supplementary data). The absence of methoxy signal(s) in the spectra of **9a** and **9b** (see Fig. 1 for **9b**, middle spectrum) is a consequence of the presaturation applied at 3.6 ppm to suppress the overwhelming ethylene signals from the PEG chains of the resin.[20]

The desilylation of **9a–c** caused us some difficulties, as the use of TBAF in THF or cesium fluoride in DMF consistently led to cleavage of the ester linkage to the resin.[21] We thus had to rely on the use of Et_3N–3HF in THF at room temperature (Scheme 3), but the corresponding HR-MAS ^{1}H NMR spectra run in either $CDCl_3$ or a $CDCl_3$/DMSO (1:1) solvent mixture exhibited such a low resolution that confirmation of the efficiency of this desilylation step leading to **10a–c** could not be made by this means. Nevertheless, good quality spectra were obtained in $CDCl_3$ after peracetylation leading to the PEGA-supported leucoanthocyanidin derivatives **11a–c** (see Fig. 1 for **11b**, bottom spectrum). The coupled proton spin system of their cycle C (i.e., H_2, H_3, and H_4) could clearly be assigned by 2D COSY HR-MAS NMR experiments (see Fig. 2 for **11b**). These satisfactory HR-MAS NMR analyses of **11a–c** not only confirmed the success of the preceding desilylation step (Scheme 3), but also provided a sound indication that the resulting PEGA-supported leucoanthocyanidins **10a–c** are thus granted with a much higher stability than their free counterparts, since they survive successive exposures to desilylation, HR-MAS NMR analysis, and acetylation conditions (see Supplementary data).[22]

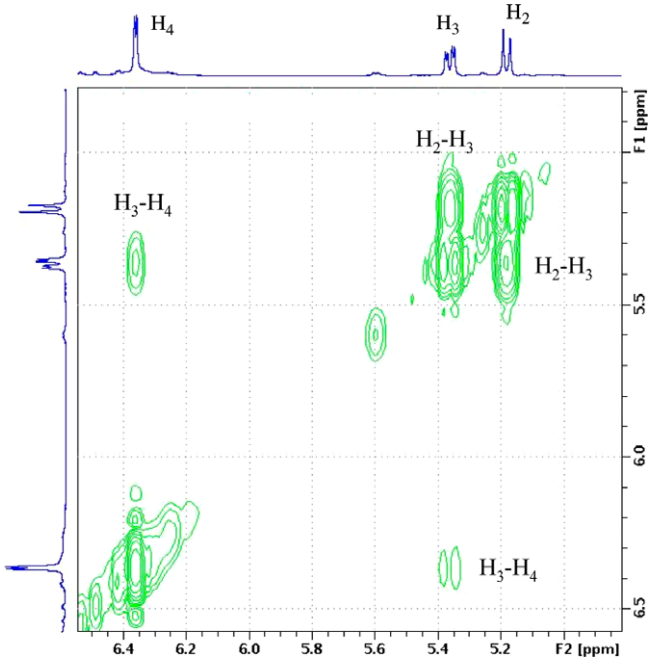


Figure 2. Diagnostic portion of ^{1}H–^{1}H HR-MAS COSY map of PEGA-supported acetylated leucopeonidin **11b** in $CDCl_3$.

In summary, the synthesis of three leucoanthocyanidin derivatives immobilized on a biocompatible PEGA resin via their 4′-hydroxyl group was achieved. These materials constitute stabilized variants of these otherwise fleeting flavan-3,4-diol species that should serve, in spite of their racemic nature, as valuable tools for in vitro enzymatic studies aimed at elucidating the final steps of the biosynthesis of both anthocyanidins and proanthocyanidins. Affinity and biochemical reactivity tests with LDOX are in progress and the results will be reported in due course.

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Supplementary data

Supplementary data (experimental procedures, characterization data, and NMR spectra for all major compounds) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.09.045.

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