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# New affinity-based probes for capturing flavonoid-binding proteins†

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**Flavonoid-bearing probes have been designed and synthesized to explore their ability to selectively capture target proteins or biosynthetic enzymes under oxidative activation. A proof-of-concept study using biotinylated (epi)catechin-bearing affinity-based probes herein demonstrates the ability of these probes to capture the LDOX flavonoid enzyme using sodium periodate as the oxidant.**

Flavonoids are among bioactive plant polyphenols that appear in high abundance in plant-derived foodstuffs and beverages whose regular consumption is claimed to be beneficial for the protection of human health.[1,2] Besides their well-known antioxidant properties,[1–3] plant polyphenols can also exert their protective actions against age-related illnesses and disorders (*e.g.*, cardiovascular and neurodegenerative diseases, cancer, diabetes) by directly binding to target proteins.[1,4] However, most protein-binding plant polyphenols are multi-target compounds capable of multiple biological effects,[5] and it remains experimentally difficult to identify the full range of proteins with which a given plant polyphenol interacts and which target proteins are predominantly responsible for a given biological effect. Moreover, enzymatic intervention in the biosynthetic metabolism of most plant polyphenols is still either uncertain or remains to be disclosed, such as in the case of flavanol oligomerization.[6]

Our search of appropriate tools for addressing these key issues of polyphenol–protein interactions was inspired by those relying on the use of molecular probes in "Activity-Based Protein Profiling" (ABPP), "Capture Compound Mass Spectrometry" (CCMS) and related methodologies,[7,8] and we thus decided to design polyphenol-bearing probes capable of covalently labeling and pulling down interacting proteins for their identification by mass spectrometry analysis. Examples of such proteomic probes equipped with plant polyphenols (or analogues thereof) are rare,[9] and the strategies used to covalently link the probes to the proteins are not universally applicable to the broad structural diversity of plant polyphenols.[1] Since most plant polyphenols feature catechol- and/or pyrogallol-type motifs, we opted to take advantage of the inherent sensitivity of these di- and/or trihydroxyphenyl units toward oxidation to electrophilic *ortho*-quinones as a simple and general means to induce *in situ* covalent linkages between polyphenol-bearing affinity-based probes and nucleophilic residues within the binding site of the proteins. This type of *in situ* oxidative activation of phenols for covalent bond formation with proteins has been used with success for cross-linking DOPA-containing peptide–protein complexes[10] and for trapping sulfhydryl proteins *in cellulo* using a biotinylated 3,4-dihydroxyphenyl acetic acid-bearing probe.[11] Here we report the synthesis of flavonoid-bearing probes **1a** and **1b** for which the catecholic flavan-3-ols (+)-catechin and (−)-epicatechin were selected both as affinity and oxidation-dependent reactive functions, and the evaluation of their performance towards achieving selective protein recognition and covalent capture by using the flavonoid leucoanthocyanidin dioxygenase (LDOX, also referred to as ANS for anthocyanidin synthase) and sodium periodate ($NaIO_4$) as an activating oxidant[10] (Scheme 1).

The LDOX, a 2-oxoglutarate-dependent enzyme, is involved in the last steps of the flavonoid biosynthesis and catalyses the conversion of colorless leucoanthocyanidins (flavan-3,4-diols) into colored anthocyanidins.[12] Recent *in vitro* experiments have shown that LDOX can also recognize (+)-catechin and (−)-epicatechin and selectively catalyse the conversion of (+)-catechin into a bisflavan-3-one dimer.[13] Therefore, the LDOX constitutes an appropriate model enzyme for this proof-of-concept study, since it should be competent for binding to both probes **1a** and **1b**.

The construction of these probes relied on amide bond formation between adequately protected or modified building blocks (see ESI† for details). First, a linker composed of a hydrophilic polyethylene glycol chain was coupled to biotin. The resulting block

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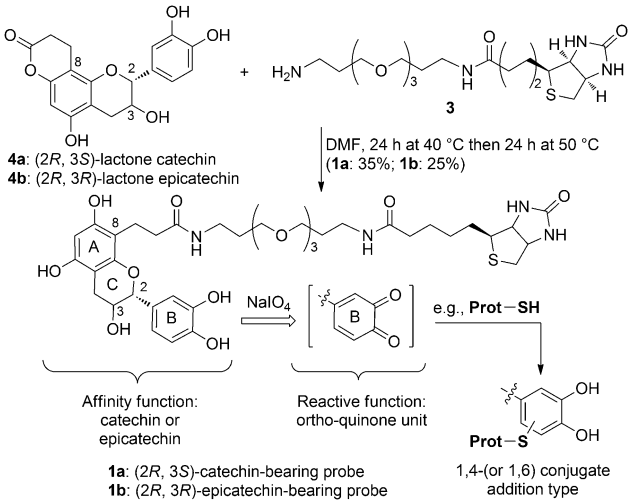


Scheme 1 Synthesis of flavonoid-bearing probes **1a** and **1b** and their oxidative activation into electrophilic *ortho*-quinones for covalent linkage to proteins.

**3** was then directly fixed to the lactonic catechin **4a** or epicatechin **4b**[13b] by a simple nucleophilic attack of the free amine function of **3** onto the lactonic carbonyl group of **4a** or **4b** (Scheme 1). This mode of attachment of flavanols through their ring-A carbon-8 center was privileged over any attachment through their more-facile-to-transform phenolic hydroxyl functions in order to limit interference in the LDOX binding, which is sealed through hydrogen bonds with these functions.[13b,14]

Moreover, in order to compare the effectiveness of the oxidation-activated capture mode of probes **1a/b** with that of the classically used photo-activated capture mode, we also synthesized an analogous catechin-bearing probe **2** additionally equipped with a photo-activable aryl azide reactive function (Fig. 1, see ESI† for details).

With these flavanol-bearing probes in hand, we first verified the capacity of probes **1a/b** to bind to and covalently label the LDOX enzyme under $NaIO_4$-mediated oxidative activation. Briefly, probes **1a** and **1b** (9.1 μM) were incubated with some partially purified LDOX (5.3 μM) in 0.2 M phosphate buffer (pH 7) at 23 °C for 30 min and then treated (or not) with an excess (5 mM) of aqueous $NaIO_4$ in order to generate the requisite flavanol-derived *ortho*-quinone for covalent linkage with the protein. The reaction mixtures were quenched 20 min after the addition of $NaIO_4$ using a phosphate buffer containing dithiothreitol (DTT, 50 mM). For comparison, probe **2** was also incubated with the LDOX enzyme under the same conditions, but

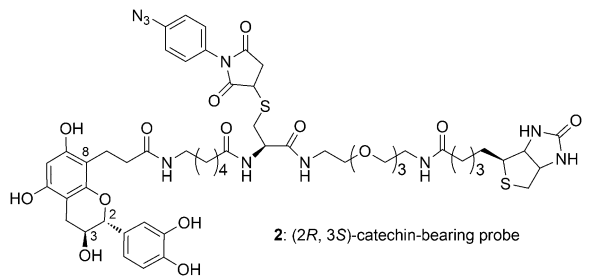


Fig. 1 Photo-activable catechin-bearing probe **2**.

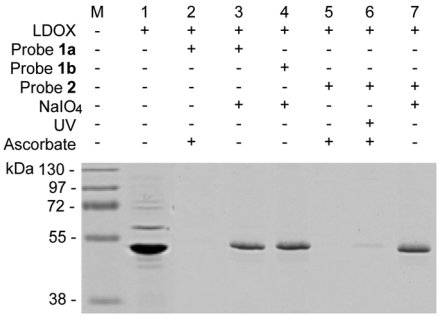


Fig. 2 Coomassie blue-stained SDS-PAGE comparative analysis of oxidation- and photoirradiation-activated capture of the LDOX enzyme by probes **1a/b** and **2**; see ESI† for details.

instead irradiated (or not) under UV light at 312 nm for 20 min. In each case, free proteins were washed away after sorting out the desired biotinylated probe-labeled proteins with streptavidin-coated magnetic beads. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Fig. 2) was realized including the two negative controls run in the absence of $NaIO_4$ (lane 2) or UV irradiation (lane 5). In both cases, sodium ascorbate (0.5 mM) was added to prevent autoxidation of the probes in the presence of any residual molecular oxygen in the buffer solution. These negative controls confirmed the absence of any covalent linkage between the LDOX enzyme and probes **1a** and **2** without appropriate activation, even when longer incubation times were applied in the absence of ascorbate (not shown, see ESI† for details).

Gratifyingly, probes **1a** and **1b** successfully bound to and covalently labeled the LDOX enzyme under oxidative activation as expected (lanes 3 and 4) showing a protein band at a molecular mass close to 40 kDa (*i.e.*, LDOX, lane 1). The capture efficiency for these probes was evaluated to be around 13%. Longer incubation times did not provide any improvement of this capture process (see ESI†). This somewhat modest yield is however high enough to envisage identification of a protein by subsequent mass spectrometry analysis. Probe **2** was much less efficient in capturing the LDOX enzyme under UV light irradiation, as evidenced by the significant decrease in band intensity (lane 6, Fig. 2). The results obtained with the structurally simpler probes **1a/b** thus attest to the superiority of the oxidation-activated capture mode (densitometric analysis indicated a 30-fold excess of LDOX capture with probes **1a/b** as compared to that with probe **2**). Furthermore, a strong band is again observed when probe **2** is oxidatively activated using $NaIO_4$ (lane 7). This result also indicates that the lack of efficiency of probe **2** under irradiation is not due to any structural interference of the recognition of the probe by the protein.

We further confirmed the covalent labeling of the LDOX enzyme by both types of probes **1a/b** and **2** by taking advantage of their biotin unit in Western blot experiments using a mouse antibody against biotin (see ESI,† Fig. S5). Moreover, a MALDI-TOF mass spectrometric analysis of the reaction mixture of capture of the LDOX enzyme by probe **1a** revealed that only a monoadduct was formed (see ESI,† Fig. S6).

The catechin-bearing probe **1a** was next utilized in competitive binding assays to evaluate the level of selectivity of its interaction with the LDOX enzyme. To this aim, we chose the globular bovine

 

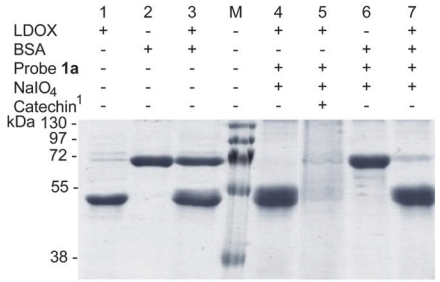


Fig. 3 Coomassie blue-stained SDS-PAGE analysis of competitive binding assays of probe **1a** and catechin with the LDOX and BSA proteins; [1]100 fold-excess; see the ESI† for details.

serum albumin protein (BSA), which is known to form a 1 : 1 complex with catechin.[15] Following the same experimental protocol, probe **1a** was thus co-incubated with LDOX, BSA or a 1 : 1 mixture of LDOX and BSA, and then oxidatively activated using $NaIO_4$. Analysis by SDS-PAGE (Fig. 3) shows that BSA is captured by probe **1a** in the absence of LDOX (lanes 2 and 6). However, when a 1 : 1 mixture of LDOX and BSA (lane 3) is treated with probe **1a** (lane 7), LDOX is quasi exclusively captured at a level seemingly identical to that observed in the control experiment run in the absence of competing BSA (see lane 7 as compared to lane 4).

The higher affinity of the catechin-bearing probe **1a** for LDOX as compared to that for BSA is quite remarkable if one considers the relatively low Km value of 175 μM of LDOX with its catechin ligand.[13a]

The specificity of binding of probe **1a** to the LDOX enzyme was also unambiguously confirmed by using a 100-fold molar excess of catechin, in which case **1a** was prevented from binding to the LDOX's active site thereby occupied by catechin. This is evidenced by the disappearance of the corresponding probe-labeled LDOX band (Fig. 3, see lane 5 as compared to lane 4). These results confirmed that the flavanol-bearing probe **1a** (and **1b**, data not provided) can be used to capture the LDOX enzyme. Thus, we next evaluated the capacity of probe **1a** to capture the LDOX enzyme present as a minor component in a complex protein mixture. To this aim, we used a bacterial lysate of *E. coli* supplemented with 0.5%/w of LDOX in the presence of only *ca.* 2 equiv. of **1a** relative to the added LDOX. Under these conditions of low abundance of the LDOX target, we could not visualize the result of its oxidative capture by SDS-PAGE or Western blotting. However, a shotgun proteomic analysis of the same mixture of proteins permitted us to unambiguously identify LDOX among only less than 50 bacterial proteins (see ESI,† Table S2) out of about 500 proteins otherwise detected by the same analysis in the absence of **1a**.

In summary, we have successfully designed and prepared simple affinity-based probes to investigate flavanoid-protein specific interactions. We have demonstrated that the (epi)catechin-bearing probes **1a** and **1b** can be used to efficiently capture the LDOX enzyme under oxidative activation by simply exploiting the inherent chemical reactivity of the polyphenolic entity, and provided evidence that these probes form a covalent adduct with the LDOX enzyme, most likely involving nucleophilic residues at the enzyme's active site. More importantly, the catechin-bearing probe **1a** can discriminate interactions with different proteins, as shown herein using LDOX and BSA, and can still capture LDOX in low abundance in a complex protein mixture.

This proof-of-concept study thus constitutes a solid groundwork for future utilization of such catechin-bearing probes (or flavonoid analogues thereof) in chemical proteomic work aimed either at identifying proteins involved in flavonoid biosynthetic metabolism or at profiling ranges of flavonoid target proteins pulled-down from plant cell extracts or human cell lysates.

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