Surface Plasmon Resonance

Real-Time Analysis of Polyphenol–Protein Interactions by Surface Plasmon Resonance Using Surface-Bound Polyphenols

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Abstract: A selection of bioactive polyphenols of different structural classes, such as the ellagitannins vescalagin and vescalin, the flavanoids catechin, epicatechin, epigallocatechin gallate (EGCG), and procyanidin B2, and the stilbenoids resveratrol and piceatannol, were chemically modified to bear a biotin unit for enabling their immobilization on streptavidin-coated sensor chips. These sensor chips were used to evaluate in real time by surface plasmon resonance (SPR) the interactions of three different surface-bound polyphenolic ligands per sensor chip with various protein analytes, including human DNA topoisomerase IIα, flavonoid leucoanthocyanidin dioxygenase, B-cell lymphoma 2 apoptosis regulator protein, and bovine serum albumin. The types and levels of SPR responses unveiled major differences in the association, or lack thereof, and dissociation between a given protein analyte and different polyphenolic ligands. Thus, this multi-analysis SPR technique is a valuable methodology to rapidly screen and qualitatively compare various polyphenol–protein interactions.

Introduction

Plant polyphenolic compounds, which are abundant in fruits and vegetables, and present in many plant-derived foodstuffs and beverages, are usually acclaimed for their antioxidant activity, and yet they can also play biologically relevant roles by interacting with cellular proteins.[1] Today, plant polyphenolic extracts are essentially used in the production of food supplements, parapharmaceuticals, and cosmetics, but the natural products they contain have not yet been fully exploited by the pharmaceutical industry. Among the reasons for this mere relegation of plant polyphenols to natural antioxidant agents are mainly concerns about their possible toxicity, poor bioavailability, and lack of specificity in interacting with proteins. If certain polyphenols indeed only act as nonspecific protein precipitating agents (i.e., tanning action),[1g] which are usually characterized by relatively low binding affinities (i.e., dissociation constants K_D in the micromolar to millimolar range), other polyphenols can exhibit much higher and protein-specific affinities.[1,2] This is, among numerous examples, the case for the tight binding of the soy isoflavone genistein to the estrogen receptor alpha,[2j] for the inhibition of an ATP synthase by the stilbenoid resveratrol,[2k] for the submicro- to nanomolar binding of the tea flavanoid epigallocatechin gallate (EGCG) to 1) the T cell-expressed ZAP-70 tyrosine kinase,[2i] 2) the metastasis-associated 67 kDa laminin receptor,[2o] and 3) the pro-angiogenic vascular endothelial growth factor,[2c] as well as for the inhibition of the DNA topoisomerase IIα[2d,n,r] or for the perturbation of actin cytoskeletal dynamics by the oak C-glucosidic ellagitannins vescalagin and vescalin.[2a,e]

Investigations on polyphenol–protein interactions are commonly performed in aqueous solutions by using various analytical techniques, such as those based on NMR spectroscopy,[3a–f] including saturation transfer difference spectroscopy,[3e,f] isothermal titration calorimetry (ITC),[3f–m] mass spectrometry (MS),[3d,n–s] FTIR spectroscopy,[3k,t] UV/Vis absorption and fluorescence spectroscopy,[3k,4a–h,o] circular dichroism (CD),[3d,k,4c,d,f,g,i,j] small-angle X-ray scattering (SAXS),[4j–l] dynamic light scattering (DLS),[4a,d,i–k] and flow nephelometry.[4d,m,n] These techniques enable one to determine the binding sites and strengths, as

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well as the general thermodynamic parameters of the interactions, and to decipher their possible modus operandi and the nature of the resulting complexes. However, specific bindings of polyphenols, especially those of higher molecular mass (e.g., flavanolic proanthocyanidins, pyrogallolic gallotannins, and ellagitannins) to proteins are unfortunately often difficult to discriminate from nonspecific bindings because certain proteins can become rapidly heavily coated with these highly hydroxylated aromatic compounds (tanning action) during the course of most analyses. Considering this problematic issue, we envisioned taking advantage of surface plasmon resonance (SPR), which enables the detection of molecular interactions in real time with high sensitivity using very small quantities of molecular partners in microfluidic devices.[5] This refractometric optical measurement technique, which is based on the modulation of the resonance of an incoming light wave with an excited surface plasmon wave from a metallic layer (e.g., gold) on a glass sensor surface, the refractive index of which is affected by the accumulation of mass through adsorption of molecules from a circulating aqueous solution,[5] has found many applications in the analysis of bio(macro)molecular interactions.[5a,6] One of the interacting partners, referred to as the ligand, is immobilized on the sensor surface, and the other partner in solution, referred to as the analyte, is eluted over the surface. In classical SPR experiments involving proteins and small molecules, the simplest method is to make the protein the immobilized ligand, since commercial sensor chips are conveniently provided with already functionalized surfaces that facilitate the covalent immobilization of proteins.[7] However, using polyphenolic molecules as analytes, which are therefore used in excess in the microfluidic mobile phase, would render the discrimination between specific and nonspecific interactions with proteins difficult, due to the aforementioned tendency of some polyphenols to invariably stick to most proteins through multiple binding interaction.

This is the reason why the SPR method we developed is instead based on the immobilization of the polyphenol molecule on the surface of the sensor chip.[2e,8] In such a reverse SPR experiment, the protein becomes the analyte and can be supplied over the sensor chip surface bearing the immobilized polyphenolic ligand in a continuous-flow mode at low microfluidic concentrations, which could enable the observation of specific higher-affinity interactions to be emphasized and that of nonspecific lower-affinity interactions to be limited. Our first few implementations of this reverse SPR technique met these expectations by enabling us to unveil the high affinity of the ellagitannin vescalin for human DNA topoisomerase IIα[8a] and the preferential interaction of its congener vescalagin with filamentous actin over that with globular actin.[2e] Herein, we report the results of our work aimed at generalizing the implementation of this reverse SPR technique for studying polyphenol–protein interactions using several polyphenols of four different structural types (i.e., ellagitannins, flavanols, proanthocyanidins, and stilbenoids; Figure 1) and several proteins with different structural criteria.

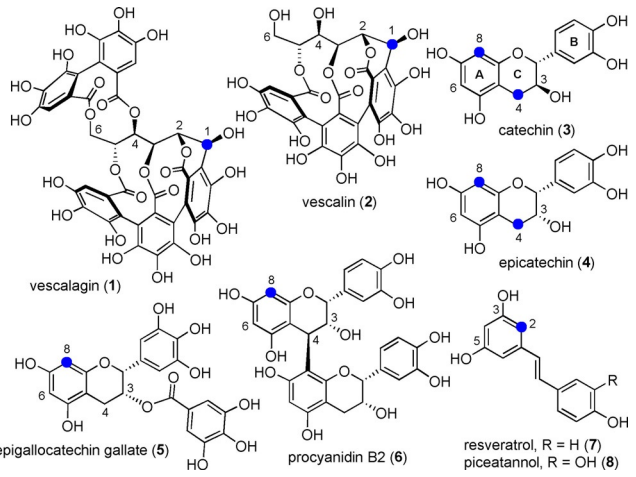


Figure 1. Selected plant polyphenols of different representative structural classes. Blue circles indicate the carbon sites of attachment of biotin-terminated units to these polyphenolic molecules.

Results and Discussion

Synthesis of SPR-ready biotinylated polyphenolic probes

The selected plant polyphenols belong to different structural classes and are representatives of the differences in chemical composition, size, morphology, and conformational freedom encountered for this large family of natural products.[1g] The polyhydroxylated terarylic C-glucosidic ellagitannins vescalagin (**1**) and vescalin (**2**) are globular, propellerlike and rather large and rigid compounds. The di- and triphenolic flavanols catechin (**3**), epicatechin (**4**), and epigallocatechin gallate (EGCG, **5**) are small yet rather flexible stereoisomeric molecules, and their oligomeric proanthocyanidin variants, here represented by the dimeric procyanidin B2 (**6**), are large elongated, threadlike molecules. The diphenolic stilbenes resveratrol (**7**) and its catecholic variant piceatannol (**8**) are small, flat, and quite rigid compounds.

Studying the interactions of these polyphenols with proteins by our reverse SPR method necessitates relying on chemical synthesis for modifying their structure with a view to their immobilization on the sensor-chip surface. We chose to use streptavidin-coated sensor chips for immobilizing biotinylated derivatives of our selected polyphenols by taking advantage of the strong noncovalent interaction between streptavidin and biotin ($K_D \approx 10^{-15}$ M), as shown in Figure 2.[5a] First, a linker had to be introduced on these polyphenols at specific sites, which were chosen to limit as much as possible any interference with the binding to proteins. Therefore, the phenolic hydroxyl groups were left intact because the binding to proteins is usually sealed through hydrogen bonding with these groups.[1g] Instead, only reactive carbon centers served to install the linker (Figure 1), which was equipped with either a terminal sulfhydryl group or a terminal carboxyl group for subsequent coupling with either the biotinylated maleimide derivative **9** or the biotinylated polyethylene glycol (PEG) derivative **10** (Figure 2).

This mode of immobilization based on the biotin–streptavidin interaction proved to be advantageous over the disulfide-

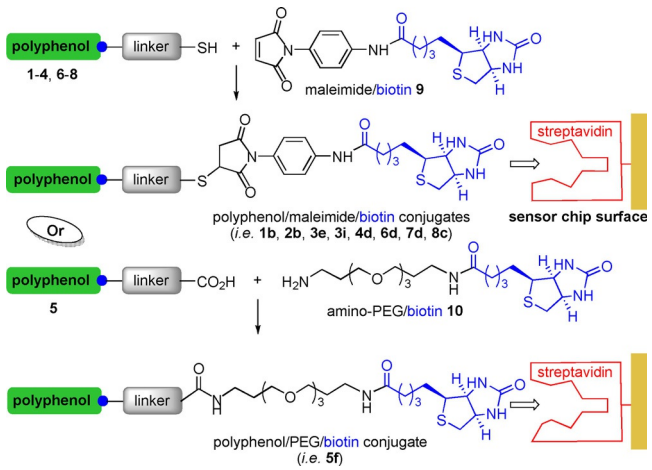
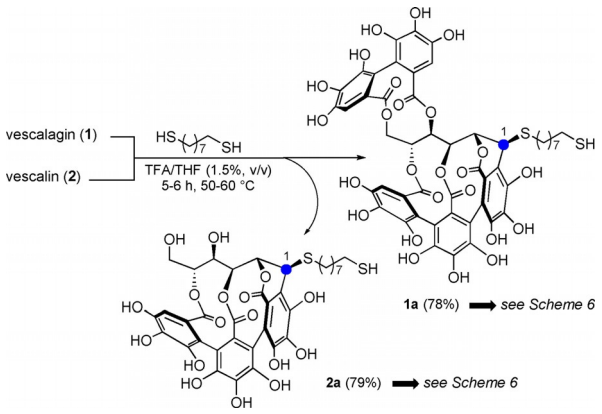


Figure 2. Schematic illustrations of the design and synthesis of polyphenol–biotin conjugates and their binding to streptavidin-coated sensor chips.



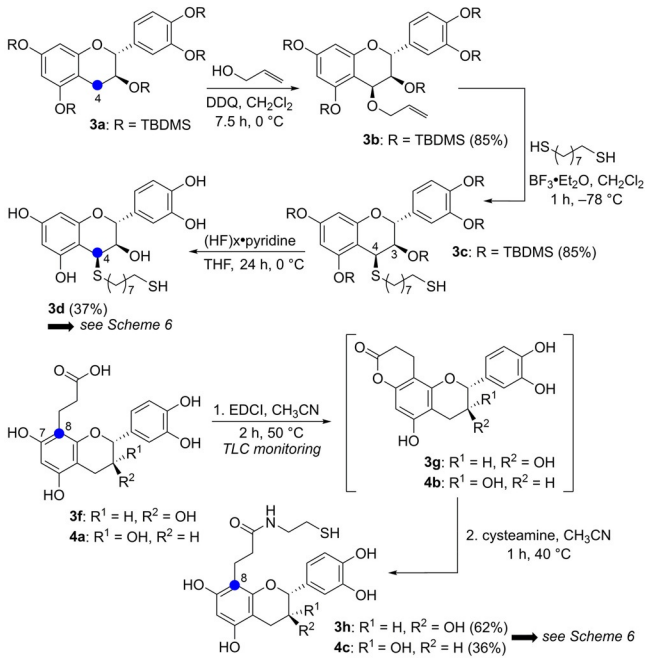
Scheme 1. Synthesis of the C1-deoxy vescalagin- and vescalin-C1-thioether sulfhydryl derivatives **1a** and **2a**.[2e, 8a]

bond exchange-based mode previously used for the immobilization of vescalin (**2**),[8a] as it is performed in only one step from the biotinylated polyphenolic conjugates and allows for better stability over time in comparison to the fragile disulfide covalent bond. Eight of these nine different sensor-surface-bound polyphenolic systems were then utilized to examine their interactions with proteins of varying structures, sizes and functions: 1) the α isoform of human DNA topoisomerase II (Top2α, 175 kDa), a nuclear enzyme targeted in anticancer chemotherapies and inhibited by ellagitannins,[2d,n,r,9] 2) bovine serum albumin (BSA, 66 kDa), a loosely structured globular protein serving as a key transporter (e.g., for fatty acids) in the circulatory system and the principal protein model used in numerous studies on polyphenol–protein interactions,[2t, 3i, 10] 3) myoglobin (Mb, 17 kDa), here used as a model for small and tight globular metalloproteins, 4) streptavidin (54 kDa), the biotin-binding tetrameric protein, 5) type I collagen (ca. 300 kDa), a triple-helical fibrillar structural protein, 6) leucoanthocyanidin dioxygenase (LDOX, 43 kDa), an enzyme involved in the biosynthesis of flavonoids,[11] and 7) the B-cell lymphoma 2 (Bcl-2) apoptosis regulator protein (27 kDa), an anti-apoptotic protein known to be downregulated by resveratrol and thought to be inhibited through direct interactions with other polyphenols.[12]

For vescalagin (**1**) and its simpler congener vescalin (**2**), the syntheses of their corresponding SPR-ready probes started with the incorporation of a sulfhydryl group mounted onto an appropriate linker. Octane-1,8-dithiol was chosen for this purpose, and its installation was performed in only one step without any prior protection of **1** or **2** by taking advantage of the remarkable chemo- and stereoselective reactivity expressed at their C1 center.[2n, 13] As previously reported,[2e, 8a] the desired thioether sulfhydryl C1-deoxy derivatives **1a** and **2a** were both obtained by acid-catalyzed nucleophilic substitution reactions in good yields (Scheme 1).

The analogous derivatization of catechin (**3**) and epicatechin (**4**) was envisioned by installing a sulfhydryl-terminated linker at their C4 or C8 centers (Figure 1). The C4 benzylic position of catechin (**3**) can be easily functionalized by oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in the presence of a wide range of oxygen-based nucleophiles.[14] However, since sulfhydryl-based nucleophiles are sensitive to oxidation to disulfides with DDQ, a direct oxidative C4 thioetherification could not be envisaged. To circumvent this problem, octane-1,8-dithiol was introduced through nucleophilic substitution of an allyl ether derivative of **3**, which was obtained by oxidative C4 etherification.[14d] Thus, the phenolic and secondary hydroxyl groups of catechin (**3**) were first protected by silylation with *tert*-butyl(chloro)dimethylsilane (TBDMSCl) to furnish **3a**, as previously described.[15] The resulting persilylated catechin derivative was then treated with DDQ in the presence of allyl alcohol to furnish allyl ether **3b** in 85% yield as a single stereoisomer, in agreement with previous related works (Scheme 2).[14b,d,e] The nucleophilic substitution reaction of **3b** with octane-1,8-dithiol was promoted by the use of the Lewis acid $BF_3{\cdot}Et_2O$[14b,d] at low temperature in CH_2Cl_2 to cleanly afford the thioether **3c**. The stereochemistry at its C3 and C4 centers was confirmed to be *cis* by ^1H NMR analysis, again in agreement with previous related works.[14b,d] The desilylation of **3c** was not as trivial as expected. In fact, our choice of using silyl protecting groups instead of benzyl groups, which are classically used for flavonoid derivatization,[14,16] was based on the risk of poisoning the metal catalyst with the sulfur-containing groups of **3c** during hydrogenolytic cleavage of the benzyl ether groups. However, several attempts to cleave the TBDMS ether groups by using tetra-*n*-butylammonium fluoride (TBAF), 3 HF·Et_3N, or KF in the presence of 18-crown-6 were all fruitless, giving only partially desilylated products, even after extended reaction times. Fortunately, the removal of all TBDMS groups could be achieved with $(HF)_x$·pyridine in THF, which furnished the desired catechin-C4-thioether sulfhydryl derivative **3d** in a moderate but sufficient yield of 37% (Scheme 2).

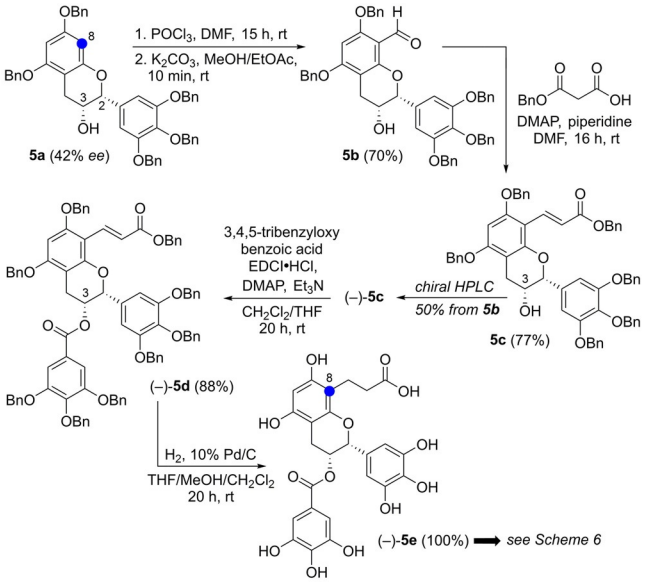
For the derivatization of catechin (**3**) and epicatechin (**4**) through the A ring, the greater inherent nucleophilic character of their C8 centers[17] was exploited to install a propanoic acid tether, by following a five-step reaction sequence that we previously described.[18] The resulting carboxylic acids **3f** and **4a**[18] were initially intended to be converted to thioester deriv-



Scheme 2. Synthesis of the catechin-C4-thioether sulfhydryl derivative **3 d** and of the catechin- and epicatechin-C8-propanamido sulfhydryl derivatives **3 h** and **4 c**.

atives by using octane-1,8-dithiol under standard Steglich-type esterification conditions in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI). However, a δ-lactonization involving the carboxyl group and the phenolic hydroxyl group at the C7 center of **3 f** (see **3 g** in Scheme 2) prevailed over the expected thioesterification. An attempt to proceed with a lactone-opening transesterification using octane-1,8-dithiol was also inoperative. Performing this reaction under basic conditions to enhance the nucleophilicity of octane-1,8-dithiol as thiolate anion was not envisaged because of the risk of generating phenolate anions, which are prone to cause autoxidative degradation of such polyphenolic substances. Therefore, we decided to use another aliphatic-type sulfhydryl linker unit equipped with a primary amino group, and we opted for cysteamine. The carboxylic acids **3 f** and **4 a** were then each allowed to react with the EDCI coupling agent in CH_3CN at 50 °C until complete formation of the corresponding lactones **3 g** and **4 b**, as monitored by TLC. Cysteamine was added directly to the reaction mixtures and rapidly promoted opening of these lactonic flavanoids to give rise to the formation of the desired catechin- and epicatechin-C8-propanamido sulfhydryl derivatives **3 h** and **4 c**, which were isolated in 62 and 36% yield, respectively (Scheme 2).

The modification of epigallocatechin gallate (**5**) was next considered by adopting the same strategy as for the C8 derivatization of (epi)catechins **3** and **4**. Starting from commercially available (−)-**5** was not viewed as a convenient option, because this natural flavanol enantiomer is rather expensive and not fully adapted to our derivatization protocol. Therefore, a complete chemical synthesis of the required derivative of **5** was carried out by adapting some of the methods developed for the total synthesis of **5**[19] to meet our objective. Inspired by Chan and Li's enantioselective synthesis of (−)-**5**,[19a] we converted 3,4,5-tribenzyloxycinnamyl alcohol and 3,5-dibenzyloxyphenol to the pentabenzylated epigallocatechin **5 a**, which was obtained as a mixture of enantiomers (the major isomer was the pentabenzylated (−)-(2*R*,3*R*)-*cis*-epigallocatechin, *ee* = 42%) in seven steps and 31% overall yield from the starting cinnamyl alcohol.[19a,20] This poorly enantioenriched **5 a** was formylated at its C8 center by a Vilsmeier–Haack reaction (Scheme 3), followed by a short methanolysis to cleave the formiate group concomitantly installed on the secondary hydroxyl group at C3.

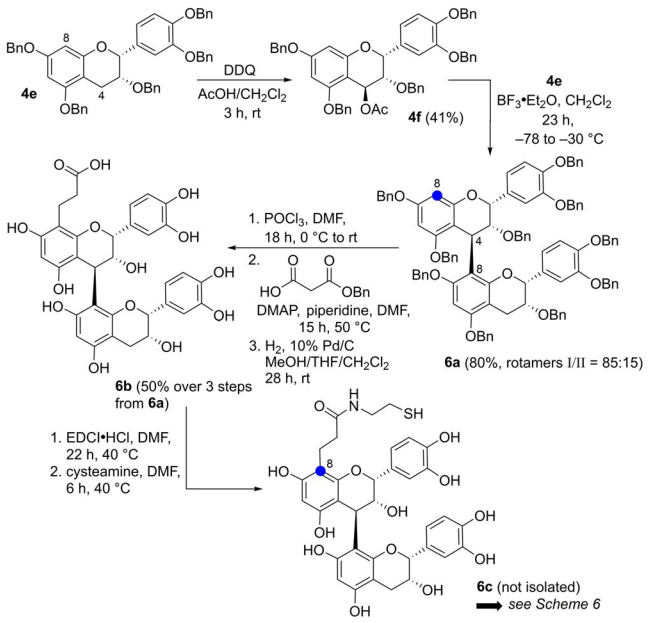


Scheme 3. Synthesis of the epigallocatechin gallate-C8-propanoic acid derivative **5 e**.

The resulting aldehyde **5 b** was thus obtained in 70% yield, and was then converted to the α,β-unsaturated ester **5 c** in 77% yield thanks to a Doebner–Knoevenagel reaction with monobenzyl malonate in the presence of 4-(dimethylamino)-pyridine (DMAP) and piperidine catalysts in DMF.[21] At this stage we separated the major enantiomer from our working mixture, and this purification was performed by semipreparative chiral HPLC to furnish enantiopure (2*R*,3*R*)-*cis*-epigallocatechin derivative (−)-**5 c** in 50% yield from the mixture **5 b** (see the Supporting Information for details). This compound was then galloylated at its C3 position in high yield with 3,4,5-tribenzyloxybenzoic acid under Steglich-type conditions by using EDCI hydrochloride as coupling agent,[19d] and the resulting gallate **5 d** was finally subjected to classical Pd-catalyzed hydrogenation conditions to promote the reduction of its olefinic bond, as well as the removal of its nine benzyl groups, to quantitatively afford the desired C8-propanoic acid epigallocatechin gallate derivative (−)-**5 e** (Scheme 3). The same lactonization as for **3 f** and **4 a** (Scheme 2) was followed by the addition of cysteamine to generate the expected analogous sulfhydryl derivative, but complications arose during the purifica-

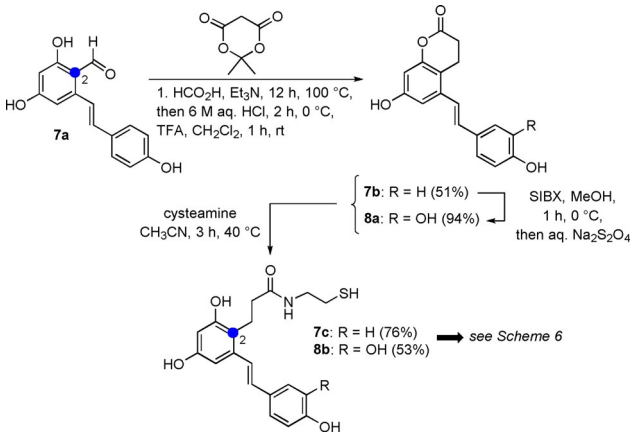
tion of this coupling product. Therefore, we opted for another solution that involved the amidation of **5e** with the amino-PEG/biotin derivative **10** (see Figure 2 and Scheme 6).

The derivatization of procyanidin-B2 (**6**) was also chosen to take place at its available A-ring C8 center. Again, a complete chemical synthesis of the required derivative of **6** was carried out (Scheme 4). The procyanidin core was elaborated by following the procedures described by Suzuki, Ohmori, and co-workers.[14b,16c] The pentabenzylated epicatechin **4e**[18] was first acetoxylated at its benzylic C4 center by oxidation with DDQ in a mixture of acetic acid and CH_2Cl_2 to give the β-acetoxy derivative **4f** in 41% yield as the sole diastereomer.[14b] The Lewis acid activation of **4f** with BF_3·OEt_2 in the presence of a threefold excess of **4e**[16c] led, after 23 h at low temperature, to the formation of the expected flavonoid dimer **6a** as the sole stereodimer in 80% yield. Full NMR analysis confirmed both the C4-C8 regioselectivity and the β orientation of the interflavan bond, in agreement with the literature data.[14a,16d–f] Moreover, the examination of the NMR spectra of **6a** in [D_6]acetone and complementary NOESY data indicated that this dimer is a mixture of two rotamers I and II in a ratio of approximately 85:15 (see the Supporting Information).[14a,16d–f] With this protected procyanidin B2 **6a** in hand, we installed a propanoic acid tether at its available C8 center by following a sequence of reactions similar to that used for the derivatization of epigallocatechin gallate (**5**), that is, Vilsmeier–Haack formylation, Doebner–Knoevenagel olefination, and hydrogenation; see Scheme 4 and the Supporting Information for experimental details. The resulting modified procyanidin B2 **6b** was then lactonized under Steglich-type conditions in DMF and then allowed to react with cysteamine (Scheme 4). In this case, no purification of the expected sulfhydryl derivative **6c**, which was found to be particularly sensitive to oxidation to the disulfide, was attempted, and it was instead directly engaged in a conjugate nucleophilic addition reaction with the maleimide/biotin derivative **9** (see Figure 2 and Scheme 6).



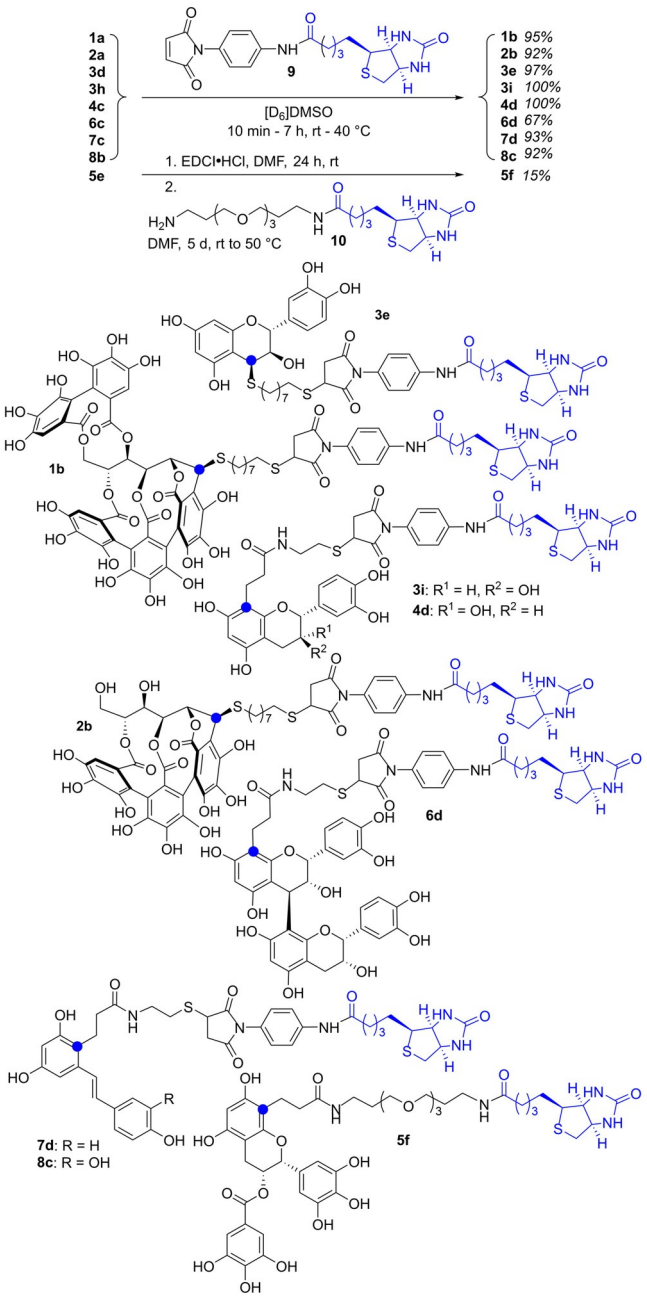
Scheme 4. Synthesis of the procyanidin B2-C8-propanamido sulfhydryl derivative **6c**.

Finally, resveratrol (**7**) and its natural catecholic analogue, piceatannol (**8**), were also equipped with a propanamido sulfhydryl moiety at their aromatic C2 centers (Scheme 5). Without any protection of its phenolic hydroxyl groups, **7** was regioselectively formylated by a Vilsmeier–Haack reaction in high yield, as previously reported.[22] The resulting aldehyde **7a** was this time treated with Meldrum's acid in the presence of triethylammonium formiate to furnish, after acidic treatment, the desired lactone **7b** in 51% yield.[23] This lactone was subjected to our SIBX-mediated phenolic *ortho*-hydroxylation conditions to generate the corresponding piceatannol derivative **8a** in high yield.[24] Lactones **7b** and **8a** were then each allowed to react with cysteamine to deliver the expected resveratrol- and piceatannol-C2-propanamido sulfhydryl derivatives **7c** and **8b** in reasonable yields (Scheme 5).



Scheme 5. Synthesis of resveratrol- and piceatannol-C2-propanamido sulfhydryl derivatives **7c** and **8b**.

All of the sulfhydryl derivatives **1a**, **2a**, **3d**, **3h**, **4c**, **6c**, **7c**, and **8b** were each rapidly coupled by Michael addition to the maleimide/biotin unit **9**[2e,25] to give the required SPR-ready biotinylated polyphenolic probes in good to high yields (see Figure 2 and Scheme 6). The low solubility of the polyphenolic sulfhydryl derivatives in standard organic solvents led us to perform these coupling reactions in [D_6]DMSO, which conveniently enabled us to monitor the progress of the reactions by ^{1}H NMR analysis. The resulting products were precipitated from the [D_6]DMSO solution by addition of mixtures of Et_2O and $CHCl_3$, and then, if deemed necessary, further purified by semipreparative HPLC, to afford the biotinylated polyphenolic probes **1b**, **2b**, **3e**, **3i**, **4d**, **6d**, **7d**, and **8c** in yields ranging from 67 to 100% (Scheme 6 and see the Supporting Information for details). As mentioned above, the biotinylated EGCG probe was instead finally built by lactonizing the carboxylic acid derivative **5e** (see Scheme 3), directly followed by opening of this lactone by using the amino-PEG/biotin derivative **10** (see Figure 2 and Scheme 6). The low reactivity of the lactone



Scheme 6. Final step(s) of the elaboration of nine SPR-ready biotinylated polyphenolic probes.

and tedious purification of the amide product **5 f** enabled us to generate this biotinylated EGCG probe in only 15% yield (Scheme 6), but in largely sufficient quantities to carry out several SPR experiments.

Real-time SPR analysis of polyphenol-protein interactions

The classical manner of running SPR experiments by immobilizing proteins on sensor surfaces has been implemented in a few studies aimed at examining the interaction(s) of a given protein with a particular polyphenol or a selection thereof.[2o,26] In some rare cases, the specific high-affinity interaction of a given protein with a given polyphenol could be measured in such a way, for example, in the cases of the nanomolar binding of EGCG with the metastasis-associated laminin receptor,[2o] and of the submicromolar binding of EGCG to the signal transducer and activator of transcription-1 (STAT1) protein[26e] and to the protein phosphatase-1.[26f] In most cases, however, the discrimination between specific and nonspecific polyphenol–protein interactions was less evident,[26a–d,h,i] likely due to the aforementioned tendency of certain polyphenols to form precipitating complexes with some proteins (tanning action), and even more so when solutions of polyphenolic mixtures (e.g., tannin extracts) are used as analytes.[26i]

Far from pretending that our reverse SPR technique constitutes a panacea for the analysis of polyphenol–protein interactions, it offers an alternative that enables a rapid qualitative and comparative screening of the interactions of a selection of proteins with several adequately modified (and pure) polyphenols. Our biotinylated polyphenolic probes were immobilized on streptavidin-coated sensor surfaces (see Figure 2). Each sensor chip was divided into four separate flow cells, one of which was kept blank to serve as a control surface, whereas the three other flow cells were used to immobilize three different polyphenolic probes. These three probes per sensor chip were selected on the basis of the structural class of the polyphenol they bear and the type of proteins to be tested, so we decided to leave the piceatannol-bearing probe **8 c** aside from this experimental development. The following four sensor chips **A**–**D** were prepared: **A** (**1 b**, **2 b**, **3 e**), **B** (**3 e**, **3 i**, **4 d**), **C** (**1 b**, **3 i**, **7 d**), and **D** (**5 f**, **6 d**, **7 d**). Thus, this multi-analysis SPR setup enabled us to study in real time the behavior of a given protein towards three different polyphenols in a single experiment run, and to screen the interaction of other proteins with the same three polyphenols in subsequent runs. Equimolar quantities of each polyphenolic probe were immobilized over the three different flow cells of the same sensor chip. Briefly, 500 μM stock solutions of the biotinylated polyphenolic probes were prepared by dissolving them in DMSO, and then diluted to 25–50 nM concentrations with the SPR aqueous running buffer (50 mM sodium phosphate, pH 7, 150 mM NaCl, 0.005% of surfactant Tween-20). These ligand solutions were then injected at a flow rate of 5 $\mu L\,min^{-1}$ over the flow cell surface until the desired level of immobilization, which is expressed in resonance units (RU), was obtained (see the Supporting Information for details). The SPR protein binding assays were next performed (vide infra) by injecting the protein solutions in increasing order of concentration, without any regeneration of the sensor surfaces between injections (i.e., single-cycle kinetics).[27] This modus operandi offers the advantage of allowing the detection of interactions at different protein concentrations, without any risk of damaging the sensor surface by using a potentially ineffective regenerating agent. Thus, each of the protein solutions of increasing concentration was injected over a period of about 3 min (association phase), which was followed by a period of about 10 min to observe the dissociation of the polyphenol–protein complex. At the end of these runs, if no remaining complex was observed, the sensor chip surface was simply rinsed by three injections of running

buffer, and a solution of a different protein could be injected. However, in cases in which some remaining polyphenol–protein complex was still observed, two or three injection pulses of 20 μL of a 0.05% solution of sodium dodecyl sulfate (SDS) were carried out at a flow rate of 20 μL min^{-1} to regenerate the sensor surface (see the Supporting Information for details). SDS was the best surface regenerant we could find for these polyphenol–protein complexes, but we observed that somehow it altered the functionalized surfaces. Despite our efforts, it was very difficult, if not impossible, to obtain perfectly superimposed sensorgrams for repeated binding cycles (e.g., see Figure 3). However, the SPR responses followed the same trend and therefore do not alter the conclusions drawn from this work.

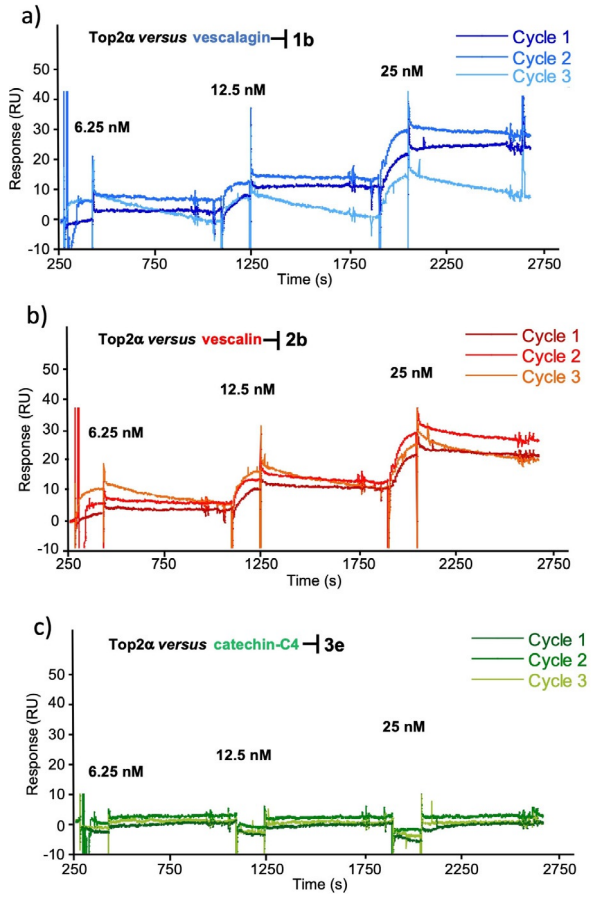


Figure 3. Chip-**A** sensorgrams recorded on injections of Top2α over a) the vescalagin-bearing probe **1b**, b) the vescalin-bearing probe **2b**, and c) the catechin-bearing probe **3e**.

The first protein binding assays were performed with sensor chip **A** and solutions of human DNA topoisomerase 2α (Top2α) by using a commercial aqueous stock solution, which was diluted to the desired 6.25, 12.5, and 25 nM concentrations with the running buffer. The recorded sensorgrams are shown in Figure 3. The SPR responses increased in a dose-dependent manner when Top2α solutions were injected over immobilized vescalagin- and vescalin-bearing probes **1b** and **2b** (see Figure 3a and b). After each injection of the Top2α solutions, the sensorgrams showed that, at best, the level of SPR responses very slowly decreases during the dissociation phase of the SPR experiment, and hence indicate that Top2α remains associated with these two ellagitannins. These observations are in sharp contrast with those made in the case of the catechin-bearing probe **3e**, for which no interaction was detected with Top2α (see Figure 3c).

These first results were already qualitatively significant, as they demonstrate that this reverse SPR method is capable of discriminating the interactions, or lack thereof, between polyphenols of different structural classes and a given protein. The SPR responses observed for both Top2α–vescalin and Top2α–vescalagin interactions do not allow one to determine unambiguously which of these two ellagitannins is the best Top2α ligand. Nevertheless, these responses are in accordance with our previous results on the inhibition of Top2α-mediated decatenation of kinetoplast DNA by these ellagitannins[2d,n] and on the identification of vescalagin as a preferential catalytic inhibitor of the α-isoform of Top2 both in vitro and in cellulo.[2d] The relatively low level of SPR signals observed for the vescal(ag)in probe systems relative to a 1:1 model of interaction with Top2α suggests either that only a small amount of the protein could approach the immobilized polyphenols **1b** and **2b**, perhaps due to some steric impediment of the access of the protein analyte in the microfluidic mobile phase, and/or that the binding mode is complex. Therefore, the recorded sensorgrams do not fit a 1:1 interaction model, enjoining us from calculating K_D values for these ellagitannin–Top2α binding systems.

The significant differences in interaction behaviors between the vescal(ag)in probes **1b/2b** and the catechin probe **3e** towards Top2α could be simply attributed to the fact that the catechin unit of **3e** has a much lower number of hydroxyl groups and aromatic rings, which confer combined hydrophilic and hydrophobic characters to polyphenols in their interactions with proteins. This possibility would suggest that the interactions observed by SPR for the vescal(ag)in probes **1b/2b** would be of a nonspecific nature and should thus also be observed regardless of the tertiary structure or size of the protein involved in such interactions. To exclude such a possibility, sensor chip **A** was subjected to successive injections of solutions of proteins of different sizes and structures. No SPR response was observed after the injection of solutions of BSA, myoglobin, streptavidin, or type I collagen in the same concentration range (see the Supporting Information, Figure S1). These absences of SPR signals indicate that these proteins do not interact with the three polyphenols mounted on this first series of probes, and that the differences in behavior observed between these proteins, including Top2α, and the polyphenolic probes **1b**, **2b**, and **3e** are not simply due to their number of phenolic groups, but to some privileged polyphenol–protein binding relationships (or lack thereof).

At higher concentrations (i.e., 125, 250, 500 nM), type I collagen started to behave more like Top2α (see Figure 3a and b) in a dose-dependent manner with rather rapid associations and slow dissociations with/from the vescalagin- and vescalin-bearing probes **1b** and **2b** (Figure 4a). Again, no major differ-

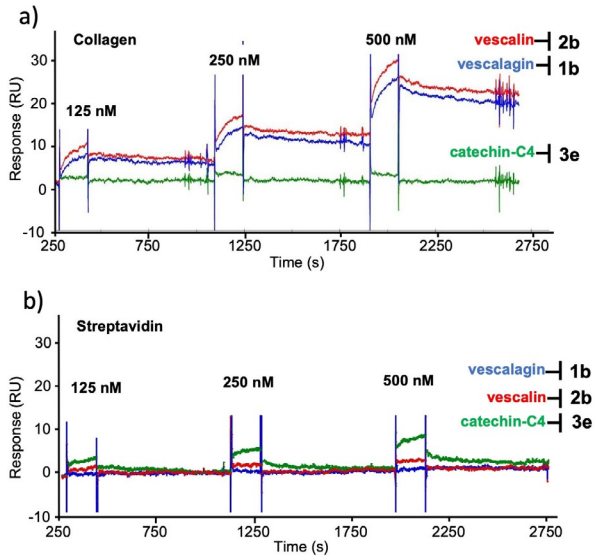


Figure 4. Chip-**A** sensorgrams recorded on injections of a) type I collagen and b) streptavidin at 125–500 nM concentration.

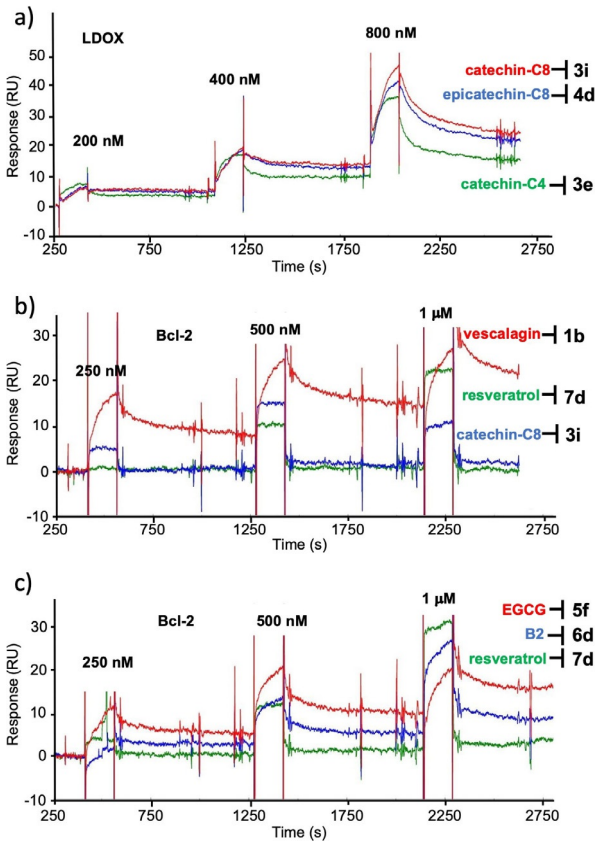


Figure 5. a) Chip-**B** sensorgrams recorded on injections of LDOX at 200–800 nM concentrations. b) Chip-**C** and c) chip-**D** sensorgrams recorded on injections of Bcl-2 at 250–1000 nM concentrations.

ence in the level of SPR responses with **1b** and **2b** was observed, and still no distinctive signal was observed with the catechin-bearing probe **3e**. BSA and myoglobin still did not show any evidence of binding to these polyphenolic probes (see the Supporting Information, Figure S2), but very weak SPR responses with fast dissociations could be detected for streptavidin interacting with the vescalin-bearing probe **2b** and with the catechin-bearing probe **3e** (Figure 4b).

Thus, at these higher concentrations, the elongated fibrillar type I collagen protein showed a clear preference for binding to the ellagitannins vescal(ag)ins (see Figure 4a), whereas the globular homotetrameric streptavidin protein could appear to slightly prefer to bind to the flavanol catechin (see Figure 4b), although such an interpretation of the very weak signals observed remains subject to caution. Notwithstanding these concerns, the observations made again demonstrate the value of this SPR technique to rapidly examine and qualitatively distinguish the interactions of different polyphenols with different proteins.

Sensor chip **B**, which is equipped with the C4-linked catechin probe **3e** and the C8-linked (epi)catechin probes **3i** and **4d**, was used to examine interactions with the LDOX flavonoid enzyme in solutions at concentrations of 200, 400, and 800 nM (Figure 5a). The resulting SPR responses increased in a dose-dependent manner, which clearly revealed binding of LDOX to these immobilized flavanols in agreement with the results of our previous work on affinity chromatography and affinity-based chemoproteomic capture of LDOX by these flavanols.[18,28]

All three interactions are again globally characterized by rather rapid associations and slow dissociations. These last results further confirm that this SPR technique can be used to unveil structure–protein binding relationships with our polyphenolic probes, since the interaction of the LDOX flavonoid enzyme with the catechin probe **3e** was expectedly detected,[18,28] whereas the same probe gave very weak or no SPR binding signals with Top2α, BSA, collagen, myoglobin, and streptavidin (see Figures 3 and 4, as well as the Supporting Information, Figures S1 and S2).

The sensor chips **C** and **D**, which are equipped with probes bearing polyphenols of different structural types (i.e., the ellagitannin vescalagin, the stilbenoid resveratrol, the flavanoids catechin and EGCG, and the dimeric procyanidin B2), were used to examine interactions with Bcl-2. The SPR responses obtained on injecting solutions of Bcl-2 at concentrations of 250, 500, and 1000 nM (see Figures 5b and c) revealed that Bcl-2 binds to the vescalagin moiety of probe **1b** in a manner similar to that of the interactions of **1b** with other proteins, which are characterized by rather rapid associations and slow dissociations. The small increase of the SPR signal between Bcl-2 concentrations of 500 and 1000 nM would indicate that this interaction reaches a level of saturation around 1000 nM (Figure 5b).

The SPR responses obtained with the resveratrol probe **7d** increased in a dose-dependent manner and are characterized by both very fast association and dissociation phases. Such a touch-and-go interaction between Bcl-2 and resveratrol was similarly observed on both sensor chips **C** and **D** (Figure 5b and c). The SPR monitoring of the interaction between Bcl-2 and the C8-linked catechin probe **3i** was more erratic when comparing signals obtained at 500 and 1000 nM, but again

this interaction is characterized by very fast association and dissociation phases (Figure 5b). Finally, as compared with resveratrol, the two larger and more highly hydroxylated polyphenols EGCG and procyanidin B2 exhibited a behavior more similar to that of vescalagin in their interaction with Bcl-2 with a rather fast association phase and a slow dissociation phase. This is even more the case for the EGCG probe **5f**, for which the interaction with Bcl-2 appears to reach saturation around 500 nM, in contrast to the case of the procyanidin B2 probe **6d**, for which the SPR response seems to be still dose-dependent between Bcl-2 concentrations of 500 and 1000 nM (Figure 5c). It is also noteworthy that the injections of BSA on these sensor chips **C** and **D** again revealed no significant binding with these five polyphenolic probes in this same range of concentrations for BSA (see the Supporting Information, Figure S3).

Conclusion

The series of biotinylated polyphenolic probes we have synthesized using polyphenols of different structural classes [i.e., the ellagitannins vescalagin (**1**) and vescalin (**2**), the flavanols catechin (**3**), epicatechin (**4**) and epigallocatechin gallate (**5**), the dimeric flavanoid procyanidin B2 (**6**) and the stilbenoids resveratrol (**7**) and piceatannol (**8**)] constitutes a useful toolbox of SPR ligands to examine qualitatively their interactions with various proteins in real time. Even though the recorded sensorgrams did not allow us to determine the dissociation equilibrium constants for these polyphenol–protein complexes, our multi-analysis reverse SPR setup enabled the rapid and convenient comparative analysis of the interaction behavior (i.e., type and level of binding) between a given protein analyte and different immobilized polyphenolic ligands, as well as between a given polyphenolic ligand and different protein analytes. Thus, privileged and dose-dependent interactions between the two ellagitannins vescalagin (**1**) and vescalin (**2**) and human DNA topoisomerase IIα (Top2α) were revealed by SPR, in accordance with previous results on their inhibitory effect on Top2α.[2d,n] At higher concentrations of proteins (>100 nM), type I collagen also binds to the same ellagitannins, but streptavidin, myoglobin, and BSA do not significantly interact either with these ellagitannins or with catechin (**3**). However, this flavanol **3** and its epimer epicatechin (**4**) expectedly gave SPR responses in a dose-dependent manner on interacting with the flavonoid enzyme LDOX. The SPR responses of the interactions between the small globular Bcl-2 apoptosis regulator protein and five different polyphenolic ligands [i.e., the ellagitannin vescalagin (**1**), the flavonoids catechin (**3**), epigallocatechin gallate (**5**), procyanidin B2 (**6**) and the stilbenoid resveratrol (**7**)] revealed different behaviors, such as touch-and-go interactions with the smaller polyphenols **3** and **7**, and interactions characterized by rather rapid associations and slower dissociations with the larger and more highly hydroxylated polyphenols **1**, **5**, and **6**. Of particular note is that BSA, the protein standard in studies of polyphenol–protein interactions, gave no significant SPR response with all of the polyphenolic ligands tested herein. These biotinylated polyphenolic entities and any future additions to this toolbox of immobilizable ligands will certainly find numerous and valuable applications, not only for screening polyphenol–protein interactions by SPR, but also for identifying cellular proteins targeted by bioactive polyphenols or plant proteins involved in their biosynthesis through the implementation of affinity chromatographic techniques or more modern affinity-based mass spectrometry-aided (chemo)proteomic protocols.[28]

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Conflict of interest

The authors declare no conflict of interest.

Keywords: analytical methods · immobilization · polyphenols · proteins · surface plasmon resonance

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