PERGAMON

Phytochemistry 51 (1999) 867–872

PHYTOCHEMISTRY

Binding affinities of gallotannin analogs with bovine serum albumin: ramifications for polyphenol-protein molecular recognition

K.S. Feldman*, A. Sambandam, S.T. Lemon, R.B. Nicewonger, G.S. Long, D.F. Battaglia, S.M. Ensel, M.A. Laci

Department of Chemistry, The Pennsylvania State University, University Park, PA 16802, USA

Received 15 September 1998; received in revised form 24 December 1998; accepted 24 December 1998

Abstract

A series of gallotannin analogs were prepared by chemical synthesis, and their affinity for the test-case protein bovine serum albumin was measured by equilibrium dialysis. The structure/activity data obtained suggest that the naturally occurring gallotannins, in fact, do not represent the optimal protein recognition agents amongst polyphenolated templates. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Gallotannin; Equilibrium dialysis; Protein binding

1. Introduction

The widespread occurrence and immense structural diversity among the tannin family of secondary plant metabolites has prompted much conjecture on the role of these polyphenolics in the plant's ecology (Bate-Smith, 1973; Feeny, 1976; Zucker, 1983; Beart et al., 1985). Both in vivo feeding studies and in vitro complexation experiments have highlighted the detrimental effects of condensed tannins (i.e. oligomeric hydroxylated flavanoids) on the availability and digestibility of feedstocks rich in these species (Hagerman & Butler, 1981; Butler et al., 1984; Asquith & Butler, 1986; Mole & Waterman, 1997, Hagerman & Robbins, 1992; Bernays et al., 1989). These observations have been taken in support of a hypothesis (Feeny, 1976) which describes the condensed tannins as antifeedant constituents of the plant that have benefited from positive selective pressure (Zucker, 1983). Protein–tannin interactions lie at the heart of this proposition, and the affinity of the condensed tannins for salivary and digestive proteins, inter alia, has been documented (Hagerman & Butler, 1981; Butler et al., 1984; Asquith and Butler, 1986; Hagerman and Robbins, 1992). Evidence which speaks to similar antifeedant properties for the hydrolyzable tannins (gallotannins and ellagitannins) is rather more scarce (Mole & Waterman, 1987; Bernays et al., 1989; Hagerman & Robbins, 1992), but arguments for their participation in the plant's defense follow those advanced for condensed tannins. Furthermore, the well-defined molecular structures of the hydrolyzable tannins, in contrast to the ill-characterized oligomeric nature of the condensed tannins, have enabled molecular-level analysis of protein recognition to proceed apace.

The structural basis for association between hydrolyzable tannins and select proteins has been probed in ever increasing detail. Initial assays of protein precipitating capacity by a wide range of structurally diverse gallotannins and ellagitannins (and some simple, well characterized condensed tannins) using hemoglobin as a model protein afforded an early glimpse of the value of increased galloylation as well as the penalty imposed by galloyl coupling on protein affinity

* Corresponding author.

PII: S0031-9422(99)00144-2

(Okuda, et al., 1985). Contemporaneous studies on β-glucosidase inhibition by various hydrolyzable tannins with and without competitive ligands present corroborated the protein precipitation results and suggested that the protein recognition properties of the hydrolyzable tannins were not tied to the particular assay employed (Haslam, 1974; Ozawa et al., 1987). These qualitative observations were placed on a more quantitative basis by equilibrium dialysis-based calculations of the free energy of transfer (ΔG_{tr}) of hydrolyzable tannin from buffer into a solution of protein (bovine serum albumin, BSA) in buffer at a designated ligand concentration of 4.5×10^{-5} M (McManus et al., 1985). This quantity is related to the free energy of binding, and at least for the test case protein BSA, the maximum affinity among the simple gallotannins attended β-D-pentagalloylglucose (β-D-PGG). Interestingly, putative downstream β-D-PGG-derived metabolites such as octagalloylglucose or hexahydroxydiphenoyl (HHDP)-containing ellagitannins offered no significant advantage in BSA complexation. More recent ^{1}H NMR studies have revealed the intimate molecular-level details of the contacts between β-D-PGG and short proline-rich peptides as models for tannin–protein interactions (Murray et al., 1994). A clear preference for association between the aryl ring of the gallotannin and the pyrrolidine ring of the proline residues was discerned, an observation consistent with earlier in vitro studies that showed a striking affinity for proline-rich proteins by condensed tannins (Hagerman & Butler, 1981). These observations have fueled speculation that hydrophobic interactions dominate tannin–protein recognition. While the relevance of BSA (or peptide fragment) binding to the larger issue of feeding deterrence can be brought into question, the observation that gallotannins, especially β-D-PGG, have significantly greater affinity for BSA than the PGG-derived ellagitannins may bear on the antiherbivory hypothesis. The fact that the plant constituents requiring more biosynthesis (ellagitannins) are demonstrably worse at protein binding has been used in support of the proposition that the assignment of hydrolyzable tannins as feeding deterrents may be premature (Beart et al., 1985).

All of these studies relied on naturally occurring tannins out of necessity. However, extension of the pool of gallotannin probe molecules to include unnatural analogs available through chemical synthesis offers the opportunity to explore some otherwise inaccessible structure/binding questions. The quantifiable equilibrium dialysis assay with BSA developed by Haslam and Lilley (McManus et al., 1985) will be used to facilitate comparison with earlier data. The binding results described herein offer some insight into the significance of stereochemistry, galloyl loading and hydroxylation patterns on the overall affinity of gallotannins for the test case protein BSA.

2. Results and discussion

Synthesized samples of the naturally occurring tannins β-D-pentagalloylglucose (**1**) and tellimagrandin I (2) and as well as the gallotannin analogs **3**–**6** were examined in this study (Fig. 1). The first two species provide a tie-in with the earlier work of Haslam and Lilley on BSA affinity for plant-derived hydrolyzable tannins (McManus et al., 1985). β-D-PGG serves as a reference point from which the effect of structural variation for the analogs **3**–**6** on BSA binding can be gauged. The influence of absolute stereochemistry was probed with the enantiomer of the natural product, β-L-PGG (**3**), while the C(1) diastereomer α-D-PGG (**4**) provided insight into the importance of relative stereochemistry. Unfortunately, further mapping of BSA affinity as a function of epimer position within the β-D-PGG core was thwarted by the limited solubility of the β-pentagalloylgalactose and β-pentagalloylmannose analogs. An interest in assessing the relative contributions of hydrophobic vs H-bonding interactions in

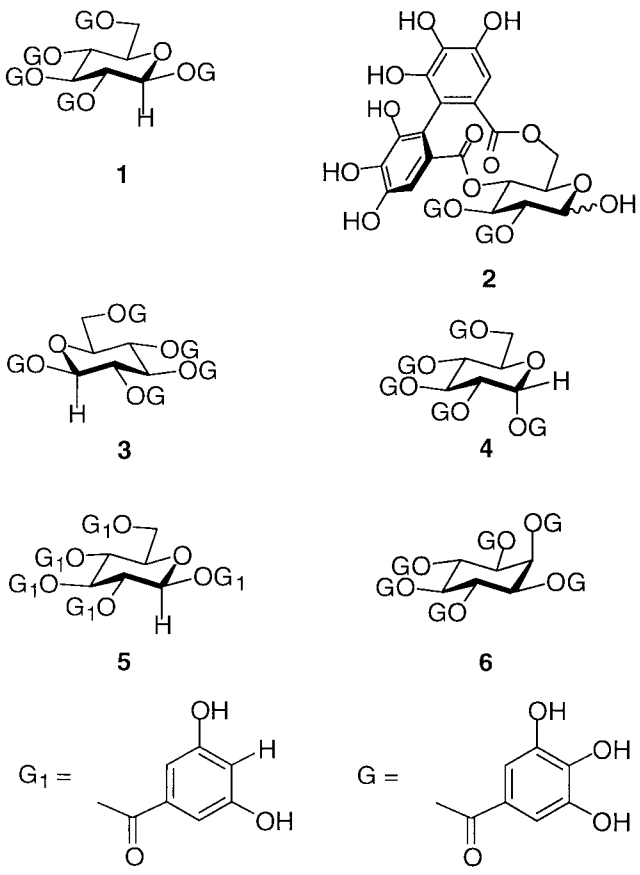

Fig. 1. Polyphenolic ligands examined in this study.

tannin/BSA complexation prompted the choice of the C(4) hydroxyl deletion analog **5**. Removal of the most acidic phenolic moiety (Ackerman et al., 1970) while maintaining the overall hydrophobic character of the aroyl rings may impact on both ligand–H_2O interactions (H-bond dominated) and ligand–BSA interactions (hydrophobic and H-bond?) with **5** compared with the fully hydroxylated β-D-PGG. Finally, the value of increasing the galloyl load was tested with the hexagalloylated *myo*-inositol derivative **6**. Early reports indicated that only a modest increase in BSA affinity resulted from addition of three depsidically linked galloyl rings to β-D-PGG (e.g. octagalloylglucose) (McManus et al., 1985). However, analog **6** increases galloylation directly on the glucopyranose periphery and may extend the denticity of the ligand in a more productive manner.

β-D-PGG (**1**) and α-D-PGG (**4**) were prepared by the method of Khanbabaee (Khanbabaee & Lötzerich, 1997), while β-L-PGG (**3**) was available by adapting this same chemistry to L-glucose. The synthesis of tellimagrandin I (**2**) has been described (Feldman et al., 1994). The 3,5-dihydroxybenzoyl compound **5** and the *myo*-inositol analog **6** were synthesized by acylation of either D-glucose or *myo*-inositol with an excess of an appropriate per-*O*-benzylated benzoyl derivative in the presence of base, followed by hydrogenolytic removal of the benzyl ethers. All polyphenolic compounds were stored under Ar at or below 0°C to minimize adventitious oxidation, and solutions were made just prior to the dialysis runs. All of the dialysis experiments were conducted in rotating Teflon cells maintained at 25.0 ± 0.2°C using a 27 μM Spectrapore cellulose acetate membrane (6000–8000 MW cutoff). The dialyses were run in pH = 2.25 ± 0.05 buffer to suppress polyphenol oxidation and facilitate comparison to earlier work. No evidence for polyphenol–BSA precipitation was detected in the concentration regime and at the pH value studied. Time runs indicated that equilibration was complete by 23 ± 1 h, and so that time point was adopted throughout. Binding isotherms for all of the compounds examined are shown in Fig. 2. The data treatment developed by Haslam and Lilley (McManus et al., 1985) was employed to calculate the ΔG_{tr} values recorded in Table 1.

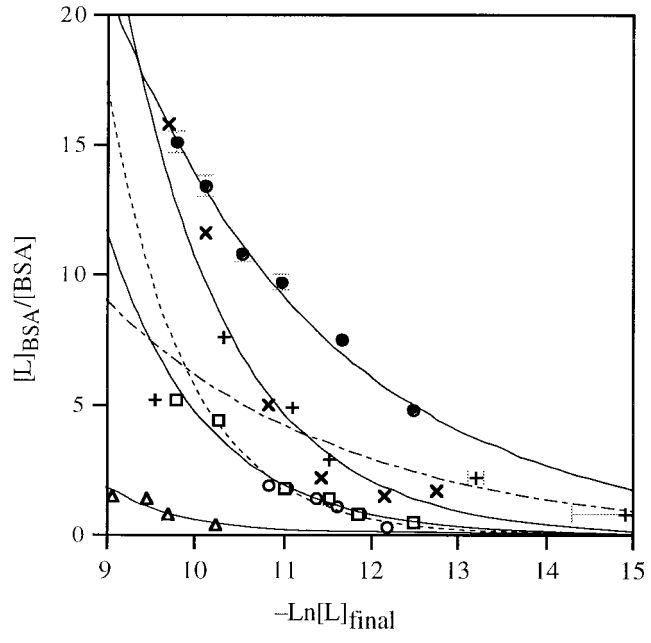

Fig. 2. Binding isotherms for **1**–**6**. **1** (□), $r^2 = 0.98$; **2**(△), $r^2 = 0.93$; **3** (○), $r^2 = 0.87$; **4** (+), $r^2 = 0.89$; **5** (×), $r^2 = 0.92$; **6** (●), $r^2 = 0.99$.

Table 1
ΔG_{tr} (free energies of transfer calculated at [polyphenol] = 4.5×10^{-5} M, kcal/mol) for polyphenolic ligands from pH 2.25 buffer into pH 2.25 buffer containing bovine serum albumin

Polyphenol ligand	ΔG_{tr} (kcal/mol), 25°C, pH 2.25
1 β-D-PGG	−3.2
2 Tellimagrandin I	−0.34
3 β-L-PGG	−3.0
4 α-D-PGG	−9.7
5 β-D-penta(3,5-dihydroxybenzoyl) glucopyranose	−7.9
6 Hexagalloyl *myo*-inositol	−20.2

Initial trials with β-D-PGG (**1**) and tellimagrandin I (**2**) were used to compare the current experimental procedures with those of Haslam and Lilley. The ΔG_{tr} values derived for these species (−3.2 kcal/mol for **1**, −0.34 kcal/mol for **2**) are approximately half the values reported by Haslam and Lilley (−6.4 kcal/mol for **1**, −0.66 kcal/mol for **2**). This discrepancy could plausibly arise from the different membrane thickness used. The Sheffield group employed a 20 μM thick membrane compared to the 27 μM membrane used in these experiments. Since the membrane itself competes for ligand and the quantity of membrane-bound tannin must be factored into the ΔG_{tr} computations, it is understandable how the thicker membrane leads to an apparent decrease in the calculated ΔG_{tr}. Multiple, independent measures of polyphenol-membrane binding at the concentration ranges of interest displayed a remarkable consistency in the amount of ligand bound. Thus, once this "polyphenol sink" is taken into account in the ΔG_{tr} calculations, the relative affinities of different polyphenols for BSA should be revealed. For example, the ratio of ΔG_{tr}'s between **1** and **2** remained approximately constant under the two systems, lending confidence to the use of the thicker membrane dialysis technique for detecting relative differences in BSA affinity among the structurally diverse ligands.

The free energy of transfer for the unnatural enantiomer β-L-PGG (**3**) is indistinguishable from that of

the natural stereoisomer β-D-PGG (**1**). The chiral "receptor" BSA has similar affinity for both enantiomers of β-PGG, reinforcing the notion that a precise "lock-and-key" fit between tannin and protein is not required for complexation. These observations provides no support for earlier speculation that precise, stereospecific interactions at well-defined receptor cites underlie gallotannin/protein interactions (Zucker, 1983).

The *O*(1)-diastereomer of β-D-PGG, α-D-PGG (**4**), appears to have a measurably greater affinity for BSA than does the natural stereoisomer. This observation may have its origins in the hydrophobic association model discussed earlier. Thus, if availability of hydrophobic surfaces scales with protein affinity among the hydrolyzable tannins, then the axial disposition of the *O*(1) galloyl ring should better expose the *O*(1) aroyl surface as well as "open up" the *O*(2) galloyl ring for hydrophobic interactions compared to the more compact, all-equatorial pentagalloyl species **1**. This rationale is supported by relative affinity data in a hemoglobin precipitation assay (Okuda et al., 1985). Thus, the *O*(1)-α-galloyl-2,3- and 4,6-HHDP-containing ellagitannin potentillin is 1.25 times as effective as its *O*(1)-β-galloyl diastereomer casuarictin at effecting hemoglobin precipitation. Interestingly, while naturally occurring *O*(1)-α-ellagitannins such as potentillin have been identified, no *O*(1)-α-gallotannin has been reported to date.

BSA exhibits a significantly greater affinity for the C(4) hydroxyl deletion analog (**5**) compared to the fully hydroxylated reference point β-D-PGG (**1**). This result might be interpretable in light of the "hydrophobic binding" hypothesis as well. If hydrophobic association between a galloyl ring and a hydrocarbon-rich epitope on BSA dominates complexation, then it would be difficult to advance a compelling argument for why either **1** or **5** would have a significant advantage in BSA binding. On the other hand, several lines of indirect evidence support the contention that **5** may be much less well solvated in aqueous solution. For example, an octanol/water partition coefficient (P_{ow}) of 5.0 was measured for 3,4,5-trihydroxybenzoic acid while 3,5-dihydroxybenzoic acid exhibited a larger value (7.2) (Howard & Meylan, 1997). In addition, the ΔG_{tr} for resorcinol is 50% larger than that for pyrogallol (vs BSA, pH = 2.2, calculated at [phenolic] = 1.5 mM) (McManus et al., 1985). From this perspective, the larger ΔG_{tr} for this compound might result from ground-state elevation of **5** relative to **1**, even as the $(\mathbf{5})_n$·BSA and $(\mathbf{1})_n$·BSA complexes are of similar energy.

The final compound examined, the hexagalloyl *myo*-inositol derivative (**6**), provided the largest ΔG_{tr} value of any of the species examined either in this study or reported earlier by Haslam and Lilley. Clearly, packing a sixth galloyl unit around the six-membered ring core provides a great advantage in BSA complexation, although the axial disposition of one of the galloyl rings clouds interpretation.

In summary, this study demonstrates that it is possible, even trivial, to prepare gallotannin analogs that display higher affinities for BSA than all of the naturally occurring species examined to date. If these results can be extrapolated to other proteins of interest, an important question which bears on the gallotannin's role in the plant's ecology can be brought into focus: If gallotannins are defensive compounds, and if defense equates with protein binding ability, then just what are the optimum protein binding agents among this family of natural products?

3. Exprimental

Nuclear magnetic resonance spectra (^{1}H NMR, ^{13}C NMR) are reported in δ units vs TMS as an internal standard. Low-resolution fast atom bombardment mass spectra (FABMS) were obtained in either a 2-nitrophenyl octyl ether matrix or in a nitrobenzyl alcohol matrix, while +MALDI spectra were obtained using a 2,5-dihydroxybenzoic acid matrix. Liquid (flash) column chromatography (Still et al., 1978) was carried out using 32–63 μm silica gel and the indicated solvent. THF was purified by distillation from sodium/benzophenone under nitrogen. CH_2Cl_2 was distilled from CaH_2 under nitrogen. Moisture sensitive reactions were carried out in pre-dried glassware under an inert atmosphere of Ar. Polyphenols **1**, **2**, and **4** were prepared by literature methods (Feldman et al., 1994; Khanbabaee & Lötzerich, 1997), while **3** was prepared in a manner analogous to the synthesis of **1**. Dialyses were performed using a Spectrum 20-cell dialyser immersed in a constant temperature water bath.

β-D-Pentakis(3,5-dibenzyloxybenzoyl)glucopyranose. Triethylamine (2.77 ml, 21.5 mmol) was added to a stirring solution of dimethylaminopyridine (240 mg, 1.95 mmol), D-glucose (348 mg, 1.95 mmol), and 3,5-dibenzyloxybenzoyl chloride (3.44 g, 9.8 mmol) in 200 ml of CH_2Cl_2 and the mixture was brought to reflux and held there for 20 h. At that time, the solution was cooled to room temperature, poured into 200 ml of 1 M H_3PO_4, and the aqueous layer was removed and extracted with 50 ml of CH_2Cl_2. The combined organic phases were washed with brine, dried over Na_2SO_4, concentrated, and the residue was purified by flash column chromatography on SiO_2 with EtOAc–hexane (2:5) as eluent to furnish 1.70 g (50%) of the title compound as a light yellow foam. ν^{CDCl_3} cm^{-1}: 1737 (C=O); ^{1}H NMR (200 MHz, acetone-d_6): δ 4.50 (1H, *dd*, *J* = 13.1, 5.4 Hz, H-6), 4.77 (2H, *m*, H-5 and H-6′), 4.99 (4H, *s*, OCH_2Ph), 5.01 (4H, *s*,

OCH_2Ph), 5.05 (4H, *s*, OCH_2Ph), 5.08 (4H, *s*, OCH_2Ph), 5.14 (4H, *s*, OCH_2Ph), 5.9 (2H, *m*, H-3 and H-4), 6.28 (1H, *t*, $J=9.5$ Hz, H-2), 6.50 (1H, *d*, $J=8.0$ Hz, H-1), 6.8 (5H, *m*, Ar–H), 7.3 (60H, *m*, Ar–H); ^{13}C NMR (90 MHz, acetone-d_6): δ 70.8, 70.9, 71.01, 71.03, 71.3, 72.8, 73.8, 74.28, 74.33, 108.2, 109.1, 109.3, 109.4, 109.55, 109.61, 128.7, 128.8, 128.91, 128.97, 129.48, 129.51, 131.6, 132.0, 132.1, 132.8, 137.8, 137.9, 138.1, 160.97, 161.06, 161.08, 161.11, 161.16, 165.0, 165.95, 165.98, 166.2, 166.28; +MALDI *m/z* (rel. int) 1783 $[M+Na]^+$ (29). Found: C, 75.4; H, 5.4. $C_{111}H_{92}O_{21}$ requires: C, 75.7; H, 5.3.

β-D-Pentakis(3,5-dihydroxybenzoyl)glucopyranose (**5**). β-D-Pentakis(3,5-dibenzyloxybenzoyl)glucopyranose (200 mg, 0.11 mmol) and 5%Pd/C (75 mg) in 6 ml of THF were purged with Ar and then charged with H_2 and stirred under a balloon of H_2 for 5 h. TLC analysis indicated the consumption of starting material at that time, and so the solution was purged with Ar, filtered through Celite, and concentrated in vacuo to yield 107 mg of **5** (~100%) as a white solid. ν^{CHCl_3} cm^{-1}: 3416 (OH), 1714 (CO); ^1H NMR (200 MHz, acetone-d_6): δ 4.54 (2H, *m*, H-6), 4.64 (1H, *m*, H-5), 5.67 (1H, *t*, $J=8.3$ Hz, H-2), 5.73 (1H, *t*, $J=9.9$ Hz, H-3 or H-4), 6.10 (1H, *t*, $J=9.7$ Hz, H-3 or H-4), 6.5 (6H, *m*, Ar-H, H-1), 6.88 (2H, *d*, $J=2.2$ Hz, Ar-H), 6.92 (2H, *d*, $J=2.2$ Hz, Ar–H), 6.99 (2H, *d*, $J=2.2$ Hz, Ar–H), 7.05 (2H, *d*, $J=2.2$ Hz, Ar–H), 8.6 (10H, *s*, OH); ^{13}C NMR (90 MHz, acetone-d_6): δ 62.4, 68.9, 71.4, 73.0, 73.2, 92.8, 107.7, 107.8, 107.9, 108.0. 108.1, 108.2, 108.35, 108.37, 108.47, 108.5, 130.8, 131.28, 131.34, 132.1, 158.78, 158.84, 158.90, 159.1, 164.4, 165.11, 165.13, 165.4, 165.8; FABMS *m/z* (rel. int) 860 $[M]^+$ (1).

Hexakis(3,4,5-tribenzyloxybenzoyl)myo-inositol. A solution of *myo*-inositol (329 mg, 1.83 mmol), 3,4,5-tribenzyloxybenzoic acid (4.70 g, 10.7 mmol), dimethylaminopyridine (700 mg, 5.57 mmol), dimethylaminopyridine hydrochloride (874 mg, 5.57 mmol) and dicyclohexylcarbodiimide (2.77 g, 13.5 mmol) in 55 ml of CH_2Cl_2 was brought to reflux and held there for 70 h. The solution was then cooled to room temperature and HOAc (0.25 ml) in CH_3OH (1.3 ml) was added. The mixture was filtered through SiO_2, diluted with 50 ml of Et_2O, washed with water and then brine, and dried over Na_2SO_4. The residue was purified by flash column chromatography on SiO_2 with EtOAc–hexane (2:5) as eluent to afford 3.20 g (65%) of the title compound as an off-white foam. ν^{CHCl_3} cm^{-1}: 1725 (C=O); ^1H NMR (200 MHz, $CDCl_3$,): δ 4.9 (36H, *m*, OCH_2Ph), 5.86 (2H, *dd*, $J=10.2$, 2.8, Hz, H-2), 6.12 (1H, *t*, $J=10.2$ Hz, H-4), 6.35 (1H, *t*, $J=2.7$ Hz, H-1), 6.48 (2H, *t*, $J=10$ Hz, H-3), 7.3 (102H, m, Ar-H); ^{13}C NMR (90 MHz, $CDCl_3$): δ 70.3, 70.4, 70.9, 71.06, 71.14, 71.3, 71.7, 75.14, 75.20, 75.22, 75.25, 109.2, 109.3, 123.7, 123.87, 123.91, 124.1, 127.64, 127.66, 127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.35, 128.37, 128.5, 128.6, 128.7, 136.4, 136.49, 136.54, 137.45, 137.49, 137.51, 137.6, 143.1, 143.2, 143.5, 152.6, 152.7, 152.9, 165.1, 165.4, 165.7, 165.8; FABMS *m/z* (rel. int) 2713 $[M+H]^+$ (25). Found: C, 76.5; H, 5.4. $C_{174}H_{144}O_{30}$ requires: C, 77.0; H, 5.4.

Hexakis(3,4,5-trihydroxybenzoyl)myo-inositol (**6**). Hexakis(3,4,5-tribenzyloxybenzoyl)*myo*-inositol (1.66 g, 0.61 mmol) and 10% Pd/C (163 mg) in 25 ml of 95% EtOH was purged with Ar, charged with H_2, and stirred under a balloon of H_2. After 5 h the mixture was purged with Ar, filtered through Celite, and concentrated in vacuo to furnish 460 mg (69%) of a light-gray solid. ν^{KBr} cm^{-1}: 3441 (OH), 1719 (C=O); ^1H NMR (200 MHz, acetone-d_6): δ 5.84 (2H, *dd*, $J=9.6$, 2.7 Hz, H-2), 6.13 (1H, *t*, $J=10$ Hz, H-4), 6.15 (2H, *t*, $J=9.7$ Hz, H-3), 6.24 (1H, *t*, $J=2.7$ Hz, H-1), 6.95 (4H, *s*, Ar–H),), 6.98 (4H, *s*, Ar–H), 6.99 (2H, *s*, Ar–H), 7.23 (2H, *s*, Ar–H), 8.2 (18H, *br. s*, OH); ^{13}C NMR (90 MHz, acetone-d_6): δ 68.9, 69.0, 70.2, 70.9, 109.6, 109.7, 119.9, 120.17, 120.21, 138.59, 138.63, 138.8, 139.0, 145.3, 145.4, 145.7, 165.2, 165.3, 165.4, 165.5; FABMS *m/z* (rel. int) 1093 $[M+H]^+$ (40).

Equilibrium Dialysis of **1** vs BSA. The measurement of concentration data for **1** is representative of all of the equilibrium dialysis experiments (Table 2). One side of each of 18 Teflon dialysis cells equipped with a Spectrapore 27 μm cellulose acetate membrane presoaked sequentially in doubly distilled water, 90:10 ethanol/water and then pH 2.25 buffer was charged with 0.5 mL of **1** in a pH 2.25 buffer prepared by serial dilution from a 1.00 mM stock solution (23.5 mg

Table 2
Experimental data for the calculation of ΔG_{tr} for β-D-PGG (**1**). All concentration values are $\times 10^{-5}$ M

Sample	$[\beta\text{-D-PGG}]_{initial}$	$[\beta\text{-D-PGG}]_{final}$	$[\beta\text{-D-PGG}]_{mem.\ bound}$	$[\beta\text{-D-PGG}]_{BSA}$
A	58.7±0.4	5.64±0.04	33.4±0.2	14.1±0.4
B	46.7±0.3	3.51±0.03	27.7±0.2	12.0±0.4
C	24.2±0.2	1.63±0.02	16.0±0.1	4.9±0.3
D	17.0±0.2	1.00±0.02	11.0±0.07	4.0±0.2
E	12.3±0.2	0.71±0.02	8.53±0.06	2.3±0.2
F	7.5±0.2	0.38±0.02	5.32±0.04	1.4±0.2

of **1** in 25.0 mL of pH 2.25 buffer). Six concentrations were loaded in triplicate, and each concentration was measured precisely by UV spectrophotometry ($\varepsilon_{\beta\text{-D-PGG}}$ (216 nm) = 9.742×10^4/mol cm): 0.587 mM, 0.467 mM, 0.243 mM, 0.171 mM, 0.124 mM, and 0.0746 mM. The second compartments in 12 of the cells (two per concentration value) were charged with 0.5 mL of a pH 2.25 buffer solution containing 0.0272 mM BSA (from ca. 20 mg of BSA in 10.0 mL of 2.25 buffer, concentration measured precisely by spectrophotometry ($\varepsilon_{BSA} = 2.781 \times 10^6$/mol cm)). The second compartments in the remaining six cells (one per concentration value) were filled with pH 2.25 buffer to permit quantitation of irreversible membrane binding. The entire Spectrum dialyser was immersed in a 25.0 ± 0.5°C water bath, and the cells were rotated at 15 rpm for 24 h. At that time, measured aliquots (Pipetman) from the compartments which initially were charged with **1** were removed and assayed for the concentration of **1** using UV spectrophotometry. The amount of **1** irreversibly bound to the membrane at each concentration was calculated as the difference between $[\mathbf{1}]_{init}$ and $2 \times [\mathbf{1}]_{final}$ in the blank runs.

Acknowledgements

We thank the National Institutes of Health (GM35727) for support of this work.

References

Ackerman, G., Hesse, D., & Volland, P. (1970). Methyl gallate: $pK_a(1) = 7.85$, pK_a (2) = 10.0. *Zeitschrift für Anorganische und Allgemeine Chemie*, *377*, 92.

Asquith, T. N., & Butler, L. G. (1986). *Phytochemistry*, *25*, 1591.

Bate-Smith, E. C. (1973). *Phytochemistry*, *12*, 907.

Beart, J. E., Lilley, T. H., & Haslam, E. (1985). *Phytochemistry*, *24*, 33 and references cited therein.

Bernays, E. A., Driver, G. C., & Bilgener, M. (1989). In M. Begon, A. H. Fitter, E. D. Ford, & A. MacFadyen, *Advances in ecological research* (pp. 262–302). London: Academic Press and references cited therein.

Butler, L. G., Riedl, D. J., Lebryk, D. G., & Blytt, H. J. (1984). *Journal of the American Oil Chemists' Society*, *61*, 916.

Feeny P. (1976). Wallace J.W., Mansell R.L. (Ed.), *Recent advances in phytochemistry* (vol. 10, pp. 1–40). New York: Plenum Press.

Feldman, K. S., Ensel, S. M., & Minard, R. D. (1994). *Journal of the American Chemical Society*, *118*, 1742.

Hagerman, A. E., & Butler, L. G. (1981). *Journal of Biological Chemistry*, *256*, 4494.

Hagerman, A. E., & Robbins, C. T. (1992). *Canadian Journal of Zoology*, *71*, 628.

Haslam, E. (1974). *Biochemistry Journal*, *139*, 285.

Howard, P. H., & Meylan, W. M. (1997). *Handbook of physical properties of organic compounds*. Boca Raton: Lewis Publishers.

Khanbabaee, K., & Lötzerich, K. (1997). *Tetrahedron*, *53*, 10725.

McManus J.P., Davis K.G., Beart J.E., Gaffney S.H., Lilley T.H., Haslam E. (1985). *Journal of the Chemical Society, Perkin Transactions II* 1429.

Mole, S., & Waterman, P. G. (1987). In G. R. Waller, *Allelochemicals: Role in agriculture and forestry* (pp. 572–587). Washington, D.C., A.C.S: .

Murray, N. J., Williamson, M. P., Lilley, T. H., & Haslam, E. (1994). *European Journal of Biochemistry*, *219*, 923.

Okuda, T., Mori, K., & Hatano, T. (1985). *Chemical and Pharmaceutical Bulletin*, *33*, 1424.

Ozawa, T., Lilley, T. H., & Haslam, E. (1987). *Phytochemistry*, *26*, 2937.

Still, W. C., Kahn, M., & Mitra, A. (1978). *Journal of Organic Chemistry*, *43*, 2923.

Zucker, W. W. (1983). *American Naturalist*, *121*, 335.