Hydrazinoanthrylboronic Acids as Exciton-Coupled Circular Dichroism (ECCD) Probes for Multivalent Catechols, Particularly Epigallocatechin Gallate

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ABSTRACT

We report the use of exciton-coupled circular dichroism (ECCD) spectroscopy in multianalyte sensing systems in complex matrices. To prepare ECCD sensors, anionic anthrylhydrazides are reacted with formylphenylboronic acids to give hydrazinoanthrylboronic acids that in turn are reacted with multivalent catechols in aqueous solution. The ECCD signal between the anthracene chromophores in the resulting boronate ester products depends strongly on the structure of the boronic acid sensor and the polyphenol analyte. This dependence of the ECCD signal on analyte structure is interesting for sensing applications. Best ECCD response is found for epigallocatechin gallate (EGCG), a key polyphenol in green tea. Weakly bell-shaped pH profiles, sensitivity to ionic strength and decreasing ECCD with decreasing solvent polarity imply that the CD active product is stabilized by hydrophobic interactions between the anthracene chromophores and by formation of the conjugate bases of the boronic esters. Analyte screening reveals selectivity for divalent catechols, with effective concentrations down to $EC_{50} = 5 \mu M$ for EGCG. Monovalent or achiral catechols such as (+)-catechin, protocatechuate or homoprotocatechuate are not detected. However, the latter two become detectable when attached to a chiral, divalent 1,2-cyclohexylamine scaffold. Application of this simple, user-friendly ECCD system to polyphenol sensing in various green tea extracts delivers easily accessible and reproducible values in the expected range. *Chirality* 21:826–835, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: circular dichroism; exciton coupling; sensors; chromophores; boronic acids; polyphenols

INTRODUCTION

Our recent results on polyphenol sensing with synthetic pores were very surprising.1,2 Without going into technical details, the only convincing explanation of huge deviations from calibration curves implied that covalent capture of multivalent catechols such as epigallocatechin gallate (EGCG) 1, the most important multivalent polyphenol in green tea,3–7 by anthrylboronic acid 2 to give product 3 occurs with effective concentrations around 10 $\mu M$ (Scheme 1).1 This interpretation was difficult to accept because the suggested magnitude of molecular recognition is about 20-times better than with catechin ($EC_{50} = 204 \mu M$) under identical conditions,1 and clearly better than the dissociation constants of 2-anthrylboronic acid and catechol (330 $\mu M$),8 phenylboronic acid and catechol (303 $\mu M$, pH 8.5, 2 mM, pH 7.0),9 D-fructose (6.2 mM, pH 7.0),9 p-glucose (500 mM, pH 7.0),9 and so on,10–23 in water.9 This puzzling observation was further supported by a hypersensitive response of synthetic pore sensors to the formation of product 3.1 However, complexity and quality of these data was incompatible with publication, and the observation remained thus unexplained. In this report, we show that the superb molecular recognition of EGCG 1 by anthrylboronic acid 2 in water can be readily and unambiguously confirmed by exciton-coupled circular dichroism (ECCD). Moreover, we elaborate on the advantage of the preparation of sensing systems in situ using hydrazine methods,24–29 and demonstrate applicability of ECCD probes to polyphenol sensing in complex matrices using CD spectroscopy.

ECCD is routinely used in organic chemistry and related fields to determine absolute configuration and enantiomeric purity of molecules such as natural products or synthetic drugs.30–34 Moreover, ECCD is broadly appreciated as invaluable, highly sensitive and selective method to study conformational changes, self-assembly and ligand binding of proteins, DNA, RNA and complex synthetic...
In contrast to this frequent use in structural studies, ECCD is rarely used to study functions. This is surprising because advantages known from structural studies, such as high sensitivity without interference from achiral components of complex systems or the ratiometric detection of the bisignate ECCD Cotton effect to exclude artifacts, apply for functional studies in complex systems as well. In our ongoing program on CD methods development at the "bio-nano" interface, we have introduced ECCD probes to detect osmotic pressure or the activity of ion channels and pores. Many elegant biosensors and chemosensors that have appeared over the past decades, however, operate usually with colorimetric, fluorometric, or electrochemical detection. Interestingly, early chemosensors as well as the first fluorescent sensing system that operate on analyte-sensitive photoinduced electron transfer (PET) from proximal tertiary amines are based on anthrylboronic acids. Several divalent PET sensors with anthracene fluorophores carrying two boronic acids followed. Further extension toward covalent bischromophoric systems with two boronic acids was of interest to combine PET and excimer emission for ratiometric detection. In contrast, divalent anthrylboronic acid systems as ECCD sensors are unexplored. Here, we introduce anthrylboronic acid as ECCD probe for the sensing of the content of multivalent catechols as key component of polyphenols in complex matrices such as green tea.

**RESULTS AND DISCUSSION**

Anthrylboronic acid was prepared in situ from anthrylhydrazide and formylphenylboronic acid (Scheme 1). The precise concentration of the obtained product was naturally unknown and necessarily lower than expected for quantitative conversion. Decreasing ECCD activity at extralong reaction times suggested decreasing concentration of sensor under these conditions. This observation implied that short and well controlled reaction times are preferable to minimize interference from the competing polymerization of anthrylhydrazide on the multiequilibrium system. This dynamic nature of sensor naturally limits batch-to-batch reproducibility but is absolutely unproblematic as long as the same material is used.

Solutions of the achiral anthrylboronic acid in neutral water were CD silent as expected. However, the addition of EGCG caused the appearance of a first negative CD Cotton effect at 345 nm followed by a positive CD Cotton effect at 310 nm (Fig. 1A). Intensity, location, and bisignate nature were all in qualitative support of exciton coupling between two dialkoxyanthracene chromophores in complex matrices such as green tea.
close proximity as origin of these CD signals. The occurrence of ECCD implied that two pyrogallols in EGCG 1 react with two anthrylboronic acids 2 to give product 3 (Scheme 1). Interestingly, no anthracene excimer emission was observed under these conditions, indicating that ECCD chemosensors can be more sensitive than fluorescence probes.

At constant concentrations of anthracene 2, the ECCD signal of product 3 increased with increasing concentration of EGCG 2 (Fig. 1A). Compared to the dramatic changes in the CD spectrum during the formation of product 3, the changes in the absorption spectrum were very small and clearly insufficient for any sensing application (Fig. 1B). Contributions from the EGCG absorption did not interfere with the first ECCD signal at 345 nm. However, the absorption around the second ECCD signal at 310 nm increased steadily with increasing EGCG concentration owing to the intrinsic absorption of EGCG. Only the first ECCD signal was therefore used for data analysis. Hill analysis of the dose response curve gave an effective concentration EC$_{50}$ = 6.2 ± 0.3 µM at pH 7.5 (see Fig. 2). This result was in perfect agreement with the surprising findings with synthetic pore sensors$^{1}$ concerning the outstanding molecular recognition of EGCG 1 by anthrylboronic acid 2.

The CD activity of sensor 2 saturated at high EGCG concentration with only very minor decreases at excess analyte (see Fig. 2). Independent of pH, this trend demonstrated that the accumulation of ECCD silent 1:1 products 6–9 is not preferred also under these favorable conditions (Scheme 2). This finding suggested that the stability of the ECCD active 2:1 product 3 exceeds that of any possible 1:1 product by far. The preference of 2:1 over 1:1 products originates necessarily from interactions between the anthryl groups. In water, hydrophobic interactions should dominate. Further stabilization by flanking hydrogen bonds between the adjacent acylhydrazones and partially protonated carboxylates appears likely.

To better understand the origin of the stability of 2:1 products of sensor 2, the dependence of the ECCD signal of product 3 on pH, ionic strength, solvent polarity and buffer was determined first. Between pH 6.0 and pH 7.5, strongly increasing CD activity was found for increasing pH (Figs. 3–4). No significant CD activity was observed below pH 6. Mildly decreasing CD activity above pH 7.5 produced a slightly bell-shaped pH profile (Fig. 4, $\lambda$). The EC$_{50}$'s naturally followed the same trend below pH 7.5 and showed a small but significant decrease above pH 7.5 (Fig. 4, $\circ$).

The pH profiles of product 3 were consistent with the acid/base chemistry of boronic acids and esters (Scheme 2). In general, boronic acids such as 2 are less acidic than cyclic boronic esters such as 10. The distortion of the bond angles in the trigonal boron atom in cyclic boronic esters accounts for this difference. For example, phenylboronic acid has pK$_a$ of $\sim$9.0, whereas boronic esters formed with $\alpha$-fructose under the same conditions have pK$_a$ of $\sim$5.2.$^{12}$ Boronic acid 2 thus exists in neutral water without significant traces from the conjugate base 11, although an ortho-fluoride increases acidity and thus reactivity. This activated trigonal boron electrophile reacts readily with catechol 1 to give boronic ester 10. In neutral water, this boronic ester 10 is acidic enough to react with water and then release protons to give the conjugate base 3. The tetrahedral boron anions in 3 are nucleophiles rather than electrophiles and thus stable toward ester hydrolysis. As a result of this thermodynamic cycle, product 3 is comparably stable in neutral water and produces a strong ECCD signal.

Under more acidic conditions, the stable base 3 transforms in its conjugate acid 10 with trigonal boron electrophiles. Under more basic conditions, the reactive boronic acid 2 transforms in its conjugate base 11 with tetrahedral boron anions. Detailed analyses of the situation with sugars suggest that the overall equilibrium constant from 11 to 3 operating under more basic conditions should be larger than the one from 2 to 10 operating under more acidic conditions.$^{12}$ In any case, the high asymmetry of bell-shaped pH profile confirms that the stabilization of boronate ester 10 as conjugate base 3 is the most important process to understand the activity of boronic acid 2 as polyphenol sensor.

The CD activity of product 3 decreased with decreasing solvent polarity (see Fig. 5). Increasing MeOH content in the aqueous solution caused rapid decrease of the maximal magnitude of the first CD Cotton effect around 345 nm. In dose response curves for EGCG 1 at constant concentrations of anthrylboronic acid 2, the EC$_{50}$’s increased with decreasing $\Delta$E$_{MAX}$. With 30% MeOH, Hill analysis revealed a substantial destabilization to EC$_{50}$ = 40.0 ± 2.0 µM (Fig. 5, $\square$). The coinciding decrease of both CD activity ($\Delta$E$_{MAX}$) and product stability (EC$_{50}$) was as in pH profiles (see Fig. 4). Sensitivity toward solvent polarity confirmed that hydrophobic interactions between the two anthracene chromophores, contribute significantly to the stability of the CD active product 3. This trend is contrary to the usual behavior of boronic esters.$^{8–23}$

The EC$_{50}$’s of product 3 decreased slightly with increasing ionic strength (not shown). This very weak trend confirms that neither ion pairing nor charge repulsion are...
involved and may further support that the contributions of hydrophobic interactions to the stability of product 3 are important. The CD activity of product 3 was roughly independent of the nature of the buffer. Dose response curves obtained with Tris and HEPES under otherwise identical conditions were nearly superimposable (not shown).

The ECCD signal between the anthracene chromophores in product 3 depends strongly on both the structure of the anthrylboronic acid and the polyphenol substrates. In situ access to ECCD sensors 2 from anthrylhydrazide 4 and formylphenylboronic acid 5 is naturally ideal to elaborate on structural aspects of the sensing system (Scheme 1). EGCG binding with isomer 12 was similar to that with isomer 5, whereas the Δ6_MAX was very weak (Table 1, Entries 1 and 2, Fig. 6). Presumably originating from the formation of a less CD active product, the precise origin of this poor CD sensitivity is unknown. The ECCD responsiveness toward EGCG 1 of the defluorinated hydrazinoanthrylboronic acid obtained from anthrylhydrazide 4 and para-formylphenylboronic acid 13 was similar to that of the original sensor 2 (Table 1, Entries 1 and 3). This finding suggested that structure and stability of product 3 do not strongly depend on the acidity of the involved boronic acids.

The change from para-formylphenylboronic acid 13 to meta-formylphenylboronic acid 14 caused a minor

**Scheme 2.** The thermodynamic cycle resulting from the coupled equilibria of the formation of boronate ester 3 and acid/base chemistry of boronic acid 2 and boronic ester 10. In general, cyclic esters (10) are stronger acids than boronic acids (2), because of the distorted boron atom, whereas the trigonal boronic acids (2) and esters (10) are better electrophiles than their tetrahedral conjugate bases (3, 11). Proximity effects on the acidity of proximal boronic and carboxylic acids and esters are not shown for clarity. 2:1 Products (3, 10) are preferred over 1:1 products (6–9) because of additional interactions between the aromatic chromophores.
decrease in EC_{50}'s together with a more substantial decrease in De_{MAX} (Table 1, Entries 3 and 4). This suggested that the product obtained from meta-formylphenylboronic acid 14 is at least as stable as product 3, whereas the distance and/or angle between the coupled anthracene chromophores changes to a less ECCD active arrangement. With the ortho-formylphenylboronic acid 15, no CD activity was found (Table 1, Entry 5). This suggested that either the anthrylboronate esters do not form or the resulting products are CD silent.

Results with synthetic pores sensors suggest that covalent capture of (+)-catechin by the resulting hydrazinoanthrylboronic acid 16 (EC_{50} = 208 μM) is as good as with the sensor 2 (EC_{50} = 204 μM), presumably because of the coordinative N-B bond. However, the binding of a second anthracene 16 required for ECCD response might still be unfavorable, possibly because of steric reasons. The addition of hydrazinoanthrylboronic acid 16 to the CD active product 3 did not cause CD silencing. This competition experiment confirmed that ortho-isomers 16 cannot form CD silent products that are as stable as the CD active product 3 in water, and thus confirmed the preference for multivalent catechols with stabilizing contributions between the hydrophobic anthracenes.

The dependence of the ECCD response on the nature of the catechol was explored next using the standard hydrazinoanthrylboronic acid sensor 2 (Scheme 1). Covalent capture of (+)-catechin 17 (see Fig. 7) did not give a CD response at concentrations up to 1.25 mM. Considering the EC_{50} = 204 μM obtained from synthetic pores under

| Table 1. CD response of anthrylhydrazide 4 after reaction with formylphenylboronic acids 5 and 12–15 in the presence of catechols 1 and 17–25. |
|---|---|---|---|
| Catechol | Aldehyde | ΔDe_{MAX} (M^{-1} cm^{-1}) | EC_{50} (μM) |
| 1 | 5 | 4.1 | 6.2 ± 0.3 |
| 2 | 12 | 1.0 | 4.1 ± 0.2 |
| 3 | 13 | 4.9 | 6.5 ± 0.3 |
| 4 | 14 | 2.2 | 4.9 ± 0.5 |
| 5 | 15 | 4.1 | 4.9 ± 0.5 |
| 6 | 17 | 5 | 8.8 ± 0.4 |
| 7 | 18 | 5 | 8.8 ± 0.4 |
| 8 | 19 | 5 | 8.8 ± 0.4 |
| 9 | 20 | 5 | 8.8 ± 0.4 |
| 10 | 21 | 5 | 8.8 ± 0.4 |
| 11 | 22 | 5 | 8.8 ± 0.4 |
| 12 | 23 | 5 | 8.8 ± 0.4 |
| 13 | 24 | 5 | 8.8 ± 0.4 |
| 14 | 25 | 5 | 8.8 ± 0.4 |

a Determined from CD dose response curves for catechols at constant hydrazide concentration in 10 mM HEPES, 100 mM NaCl, pH 7.5 (compare Scheme 1 for reactions and Figure 1 for spectra).
b For structures, see Figure 7.
2 For structures, see Figure 6.
3 Maximal Δc of the first CD Cotton effect at 340–350 nm (324 nm for 14) at saturation in dose response curves, calculated based on the initial concentration of anthracene 4. The reported values represent intrinsic underestimates considering both incomplete conversion of 4 into sensor 2 and analogs as well as subquantitative formation and 2:1 stoichiometry of the CD active products.
4 Effective concentration to observe 50% of ΔDe_{MAX} of the first CD Cotton effect in dose response curves.
5 CD silent, no Δc observed.
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similar conditions, this insensitivity suggested that products obtained from (+)-catechin 17 with boronic acid 2 are CD silent. With (+)-catechin 17 containing only one catechol group, this result implied that the binding of more than one anthracene is needed to obtain CD active products, that is, the occurrence of ECCD. Identical CD silence was found for monovalent catechols 18–20.

The dose response curves for pure EGCG 1 and a 1:1 mixture of EGCG 1 and gallate 18 were superimposable. This insensitivity of EGCG products toward interference from gallate corroborated the selectivity of anthrylboronic acid 2 for multivalent catechols and thus highlighted the importance of hydrophobic interactions in product 3.

To detect single, achiral catechols such as protocatechuate 19 or homoprotocatechuate 20, they were transformed into divalent, chiral dimers. The classical ECCD scaffold for this purpose is 1,2-diaminocyclohexane. Reaction of each enantiomer with homoprotocatechuate 20 gave the enantiomeric diamides 21 and 22. Addition of the (1S,2S)-enantiomer 21 to a solution of ECCD sensor 2 caused the appearance of a positive ECCD doublet (Fig. 8 and Table 1, Entry 10) consistent with the positive sense of twist expected between the two anthracene chromophores when attached to the (1S,2S)-diaminocyclohexane scaffold. A negative ECCD doublet of identical magnitude found for (1R,1R)-enantiomer 22 confirmed the validity of this interpretation (Fig. 8 and Table 1, Entry 11). The absolute magnitude of the first CD Cotton effect was about 6-times weaker for the homoprotocatechuate dimers 21 and 22 compared with EGCG 1 (Table 1, Entries 1 and 11). The EC50 = 11.4 μM was identical for both enantiomers and about two times that of EGCG 1. Compared with the parent product 3, the products obtained from enantiomers 21 and 22 had thus similar stability but reduced CD activity.

The protocatechuate dimer 23 was obtained by reaction of veratric acid with (1R,2R)-1,2-diaminocyclohexane and subsequent deprotection of the methylethers with BBr3. Addition of 23 to 2 resulted in a negative CD doublet centered around 333 nm followed by a second doublet around 301 nm consistent with the negative helical twist of the N–C–C–N dihedral angle. The EC50 = 8.8 ± 0.4 μM fell within the range of the other multivalent catechols, and the absolute maximal molar circular dichroism was twice as large as for the more flexible homolog 22 (Table 1, Entries 11 and 12).

The compatibility of boronate ester formation from diols rather than catechols with ECCD sensor 2 was explored with quercetin-3-β-D-glucoside 24. However, no CD signal was observed for glucoside 24 up to saturation around 625 μM in presence of 200 μM anthrylboronic acid 2 in a CD cell with 1 mm pathlength (Table 1, Entry 13). This
result suggested that a single catechol plus hydrophobic interactions between two anthracenes 2 fails to overcompensate the weak binding of glucose to boronic acids. Reaction of sensor 2 with procyanidin B2 (25), which is the main constituent of cacao polyphenols, was found to be five times less efficient than with EGCG, whereas the obtained molar circular dichroism was of comparable magnitude with opposite sign (Table 1, Entries 1 and 14).

Compared to other divalent catechols present in green tea, exceptional responsiveness of sensor 2 to EGCG was of interest for sensing applications. With synthetic pores, polyphenol sensing was complicated because calibration was possible for (+)-catechin but not for the more relevant EGCG. Calibration of the dose response curves obtained for polyphenol, a commercial polyphenol extract, for (+)-catechin gave polyphenol levels that were 5.8-times higher than expected (Table 2, Entry 1). For polyphenol sensing in green tea, the values found with calibration for (+)-catechin were with 286 mg/g similarly excessive (Table 2, Entry 2). However, correction by a factor of 5.8 for the overestimate with polyphenol, that is calibration for polyphenol, gave with >49 mg g⁻¹ a polyphenol level that was reasonably near the expected value of 72 mg.

With the introduction of ECCD sensing, the origin of drastic overestimates obtained with synthetic pores calibrated for (+)-catechin is now understood. Contrary to synthetic pores, ECCD probe 2 is insensitive to (+)-catechin but responds to EGCG, the most relevant polyphenol in green tea. The compatibility of ECCD probe 2 with polyphenol sensing in complex matrices was explored first with polyphenol. Titration of hydrazinoanthrylboronic acid 2 with polyphenol gave an ECCD response as for EGCG (Figs. 1–4). Calibration of the dose response curve for EGCG gave a polyphenol content of 50% (Table 1, Entry 1). This value was near the ≥60% provided by the supplier.

The ECCD response of sensor 2 to green tea extracts was similarly convincing. Calibration for EGCG without any further correction gave 89 mg g⁻¹ polyphenol for Geneva bags (Table 2, Entry 2). This value was reasonable, 24% higher than expected but clearly lower than the mass overestimates obtained from synthetic pores calibrated for (+)-catechin. Correction of these values for the measured polyphenol levels caused a slight increase from 89 mg g⁻¹ to 107 mg g⁻¹ with ECCD sensor, whereas the values from synthetic pores dropped dramatically to 49 mg g⁻¹.

The polyphenol levels found in Shincha leaves with EGCG-calibrated ECCD sensors 2 were with 104 mg g⁻¹ not much higher than in Geneva bags (Table 2, Entries 2 and 3). This finding differed from synthetic pores, where polyphenol levels in Shincha leaves more than doubled those in Geneva bags. However, considering many possible contributions such as sample age, sample preparation, and so on, we felt that this difference was probably not very significant. Considering the responsiveness of ECCD and pore sensors to different processes and complexity of polyphenol mixtures in green tea extracts, we rather felt that the results from two complementary sensing systems should be considered as surprisingly consistent. For example, higher polyphenol levels found with both methods in Shincha leaves compared to Geneva tea bags were in agreement with expectations from suppliers and literature.

### CONCLUSIONS

The evaluation of the responsiveness of hydrazinoanthrylboronic acids 2 as ECCD sensor reveals poor binding and CD silence for monovalent catechols and catechol-diol hybrids. Excellent binding without much selectivity is found for divalent catechols. However, the magnitude of the ECCD response to the binding of EGCG is outstanding among divalent catechols present in green tea. This excellent selectivity for EGCG is applicable to ECCD sensing of polyphenols in green tea extracts using CD spectroscopy.

Experimental evidence for ECCD chemosensors as well as the applicability of CD spectroscopy to sensing in complex matrices as such is an interesting breakthrough. More reservation seems appropriate, at least at this point, concerning the perspectives to generalize the successful approach from an isolate highlight toward a more general approach to sensing. An understanding of the observed selectivity for EGCG on the structural molecular level will be unavoidable for any eventual progress in this direction. In general, highly selective signal transducers are undesirable in a broader context because they are incompatible with multianalyte sensing. Design strategies for reduced selectivity (but increased sensitivity) of ECCD transducers will be needed to move on, in concert with variable signal generators such as enzymes, antibodies or aptamers, toward multianalyte ECCD sensing systems. Beyond these concerns with regard to sensing applications in a broader context, multianalyte ECCD sensing systems should be considered as surprisingly consistent.
context, there is much potential in exploring ECCD chromophors with regard to red-shifted absorption, higher ECCD amplitudes, supramolecular finetuning, and covalent capture chemistry beyond catechols and boronic acids.

MATERIALS AND METHODS

General

Analytes, buffers, salts, reagents, solvents, and polyphenon were from Fluka, Aldrich, and Sigma. Tea leaves (Shincha from Shizuku) and tea bags were obtained from supermarkets in Japan and Switzerland, respectively. Flash column chromatography was carried out using Silica Gel 60 (Fluka, 0.04–0.063 mm, 230–400 mesh). Anthrylboronic acid hydrazide 4 was synthesized following previously reported procedures.2

Synthesis

Chiral homoprotocatechuate dimer 21 was synthesized by dissolving 20 (148 mg, 0.88 mmol) and (1S,2S)-1,2-diaminocyclohexane (50 mg, 0.44 mmol) in anhydrous DMF (2.5 ml). After cooling to 0°C, PyBOP (458 mg, 0.88 mmol) and triethylamine (267 mg, 2.64 mmol) were added. The solution was stirred for 16 h, while it was allowed to warm up to room temperature. The solvent was removed in vacuo and the pale yellow oil was purified by flash column chromatography (CH2Cl2/MeOH 95:5) as a colorless solid.

Preparation of Hydrazinoanthrylboronic Acids

Hydrazide 4 (5 μmol) and formylphenylboronic acids 5 and 12–15 (5 μmol) in DMSO (500 μl) were incubated for 2 h at 50°C in a Grant-bio (Cambridge, UK) PHMT thermostaker. The freshly prepared solution was directly used for spectroscopic measurements without further workup.

Titrations

UV-Vis and CD spectra were recorded in quartz glass cuvettes (1 cm path length, 2 ml total volume if not stated otherwise) using a JASCO V-650 spectrophotometer and a JASCO J-815 spectropolarimeter, both equipped with a circulating water bath to maintain constant temperature (25°C).

Cooperative binding of hydrazinoanthrylboronic acids toward multivalent catechols and pyrogallols was analyzed by nonlinear least squares fitting of the titration data (polyphenol concentration c plotted versus the molar circular dichroism Δc) with the Hill equation 1 to obtain the effective concentration EC50

\[ \Delta c = \Delta c_{\text{MAX}} + (\Delta c_0 - \Delta c_{\text{MAX}})/\left\{1 + (c/EC_{50})^n\right\}, \]

where Δc0 is Δc in absence of polyphenols, Δc_{MAX} is Δc with excess polyphenols, and n the Hill coefficient.4

Polyphenol Sensing

Ten milligrams of dry tea leaves were incubated with 1 ml of preheated hot water at 100°C for 10 min in a Grant-bio (Cambridge, UK) PHMT thermostaker. After centrifugation, the supernatant was collected and stored at -20°C or directly used for titrations. A modified version of the Hill equation was used for data analysis (eq. 2)
\[ \Delta e = \Delta e_{\text{MAX}} + \left( \frac{\Delta e_0 - \Delta e_{\text{MAX}}}{1 + \left( V/V_0 \right)^n} \right), \]

in which \( V \) is the volume of tea added and \( V_{50} \) the volume tea needed to afford 50% of maximal molar circular dichroism. Care was taken that increases in total cuvette volume by addition of titrant were negligible in all cases (<2.5%). The polyphenol concentration \( \beta_{PP} \) (in mg ml\(^{-1}\)) in green tea was calculated from the EC\(_{50}\) of epigallocatechin gallate EC\(_{50,\text{EGCG}}\), the molar mass of EGCG \( M_{\text{EGCG}}\), the cuvette volume \( V_{\text{cuvette}} \), and the \( V_{50}\) of the tea preparation by using eq. 3.

\[ \beta_{PP} = \left( \frac{\text{EC}_{50,\text{EGCG}} \cdot V_{\text{cuvette}} \cdot M_{\text{EGCG}}}{V_{50}} \right) \]

Considering the amount of tea used for sample preparation (10 mg) one obtains the polyphenol content of the tea in mg g\(^{-1}\) dry weight.

**LITERATURE CITED**


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