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# Implications of the fluorescence for the conformational analysis of polymeric profisetinidins and procyanidins

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## Summary

The common monomeric units in the polymeric procyanidins are catechin and epicatechin, which have a hydroxyl group at C(5). This hydroxyl group is absent in the profisetinidins. The fluorescence properties have been characterized for the profisetinidin monomer and dimers, and compared with previous results for the procyanidins. There is a measurable heterogeneity in the fluorescence of fisetinidol, in contrast to the simpler fluorescence of the procyanidin monomers. This heterogeneity is attributed to differences in the photophysical properties of the aromatic A- and B-rings in fisetinidol. These differences are larger in fisetinidol than in catechin or epicatechin. The heterogeneity prevents determination by fluorescence of the conformations at the interflavan bond in the profisetinidins by the data analysis employed successfully with the procyanidins.

## Introduction

The procyanidins and profisetinidins are classes of condensed tannins, which are polyphenols that are widespread in the plant kingdom (1). Both the biological functions and the commercial applications of condensed tannins depend on their ability to form conformationally dependent complexes with other biological and synthetic polymers. Therefore understanding the conformational properties of condensed tannins is important.

The profisetinidin monomer is fisetidinol (Figure 1). The procyanidin monomers, catechin and epicatechin, have another hydroxyl group at C(5). In the polymers, the monomers are joined by interflavan bonds that are usually from C(4) to C(8), but sometimes from C(4) to C(6). Hindered rotation about the interflavan bond yields two rotational isomers with populations that are generally nonequivalent. The conformation of the chain is extended and helical if one rotational isomer completely dominates the other, but it is a more compact random coil if both rotational isomers are populated to an appreciable extent (2–4).

In typical solvents at ambient temperature, the interconversion of the two rotational isomers in underivatized procyanidin dimers is fast on the NMR time scale, but it is slow on the fluorescence time scale (5). The heterogeneity of the time-resolved fluorescence provides an experimental route to the populations of the rotational isomers at

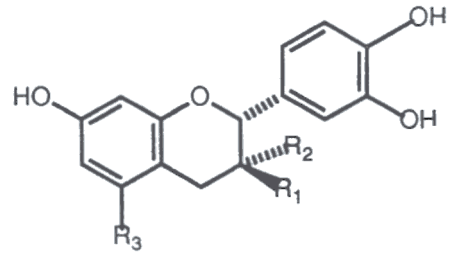


Figure 1. Fisetinidol ($R^1$ = OH, $R^2$ = $R^3$ = H), catechin ($R^1$ = OH, $R^2$ = H, $R^3$ = OH), and epicatechin ($R^1$ = H, $R^2$ = $R^3$ = OH).

the interflavan bond in the dimeric procyanidins in these solvents (5,6), and hence to information about the conformations of the polymers. This analysis takes advantage of the single-exponential decay of the fluorescence in the monomeric procyanidins and conformationally constrained dimeric procyanidins, in contrast with the heterogeneous decay of the fluorescence in unconstrained dimeric procyanidins (5).

The steady-state and time-resolved fluorescence of fisetinidol and four of its dimers are reported here. This data demonstrates that the equivalent analysis of the conformations at the interflavan bond is not feasible with the profisetinidins, due to a heterogeneity in the decay of the fluorescence of the profisetinidin monomer, fisetinidol.

## Materials

The fisetinidol monomer was isolated from a natural source while the four dimers were acquired via a biomimetic synthesis described below. Extensive TLC in different solvent systems (chromatographically pure) and 300 MHz $^1$H NMR indicated a purity of the compounds in excess of 95%.

Two grams of mollisacacidin (isolated from the heartwood of Acacia mearnsii) (7) was added in portions over 1 hr to a stirred solution of epicatechin, which was obtained from FLUKA Chemicals Switzerland, in 0.1 M HCl (150 ml). The mixture was stirred at room temperature for 24 hr and extracted with ethyl aceteate (5 × 150 ml). The freeze-dried ethyl acetate-solubles (6.0 g) were subjected to column chromatography on Sephadex LH-20 in ethanol (120 × 4.0 cm column, 15 ml/tube). Fraction 1 (tubes 35–90, 1.7 g) consisted of epicatechin, and fractions 2 (tubes 120–174, 1.1 g) and 3 (tubes 195–200, 1.5 g) consisted of fisetinidol-(4$\beta$ → 8)-epicatechin and fisetinidol-(4$\alpha$ → 8)-epicatechin, respectively, by comparison of the $^1$H NMR data of their heptamethyl ether diacetates with those of authentic samples (8). In a similar synthesis where catechin was substituted for epicatechin, the chromatography (100 × 2.5 cm column, 10 ml/tube) of the ethyl acetate-solubles (5.5 g) yielded catechin in fraction 1 (tubes 12–31, 2.5 g), fisetinidol-(4$\beta$ → 8)-catechin in fraction 2 (tubes 32–45, 0.85 g), and fisetinidol-(4$\alpha$ → 8)-catechin (tubes 50–80, 1.36 g).

## Measurements and Calculations

Ultraviolet absorption measurements were performed at ambient temperature with a Hewlet-Packard 8451A Diode Array Spectrophotometer. Steady-state fluorescence measurements were performed on an SLM 8000C Spectrofluorometer with a dual grating

Table 1. Fluorescence quantum yields in 1,4-dioxane (25°C, $\lambda_{exc} = 280$ nm)

| Profisetinidin[a] | $Q$ | Procyanidin[a] (14,15) | $Q$ |
|---|---|---|---|
| Fisetinidol | $0.14 \pm 0.01$ | Catechin | $0.224 \pm 0.012$ |
| | | Epicatechin | $0.228 \pm 0.017$ |
| Fis-(4$\alpha \rightarrow$ 8)-cat | $0.14 \pm 0.01$ | Cat-(4$\alpha \rightarrow$ 8)-cat | $0.092 \pm 0.007$ |
| Fis-(4$\alpha \rightarrow$ 8)-epi | $0.13 \pm 0.01$ | Cat-(4$\alpha \rightarrow$ 8)-epi | $0.080 \pm 0.004$ |
| Fis-(4$\beta \rightarrow$ 8)-cat | $0.09 \pm 0.01$ | Epi-(4$\beta \rightarrow$ 8)-cat | $0.140 \pm 0.011$ |
| Fis-(4$\beta \rightarrow$ 8)-epi | $0.11 \pm 0.01$ | Epi-(4$\beta \rightarrow$ 8)-epi | $0.149 \pm 0.009$ |

[a] Fis = fisetinidol, cat = catechin, epi = epicatechin.

excitation monochromator, a single grating emission monochromator, and an ozone free 450W xenon arc lamp. With the exception of the depolarization measurements, the polarizers were set for "magic angle" conditions (9). Quantum yields for fluorescence, $Q$, were determined from the integrated areas for the emission spectrum of the sample and quinine sulfate (10). The steady-state fluorescence depolarization measurements were performed at −20°C in propylene glycol. The fluorescence anisotropy, $r$, was calculated from the measured values of $I_{vv}/I_{hv}$ and $I_{vh}/I_{hh}$ as (9)

$$\frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} = \frac{R_{corr} - 1}{R_{corr} + 2} \tag{1}$$

where $G = I_{hv}/I_{hh}$, $R_{corr} = I_{vv}/I_{vh}G$, $I$ denotes the intensity of the emission, and the subscripts denote the positions of the polarizers for excitation and emission, respectively (h for horizontal, v for vertical).

The time-correlated single photon counting technique operating in the inverted configuration was used to determine the fluorescence lifetimes, $\tau$ (11,12). Excitation at 295 nm was achieved using the frequency doubled light from a Model 702 Coherent dye laser, synchronously pumped by the 532 nm light from a mode-locked, frequency doubled Nd:YAG laser (Coherent Antares Model 76-s). Emission was detected at $\lambda > 380$ nm by insertion of a Schott GG375 filter between the sample and detector (Hamamatsu R298 photomultiplier tube). The FWHM of the instrument response was about 750 ps. Residual solvent luminescence was subtracted by employing a "blank" of 1,4-dioxane. Standard methods were employed for analysis of data (11,12). Quality of each fit of the time-dependent intensity, $I(t)$, was judged using $\chi^2$ and weighted residual parameters.

$$I(t) = \alpha \exp(-t/\tau) \tag{2}$$

$$I(t) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) \tag{3}$$

In addition, the autocorrelation of the residuals and the Durbin-Watson parameter (12) were employed for cases where judging the goodness of fit from $\chi^2$ and the weighted residual parameter was ambiguous.

Table 2. Fluorescence lifetimes in 1,4-dioxane ($\lambda_{exc}$ = 295 nm)

| Dimer[a] | $\tau_1$ (ns) | $\tau_2$ (ns) | $\alpha_1/(\alpha_1+\alpha_2)$ | $\chi^2$ |
|---|---|---|---|---|
| Fis-(4$\alpha$ → 8)-cat | 2.09 ± 0.12 | 1.09 ± 0.13 | 0.32 | 1.52–1.60 |
| Fis-(4$\alpha$ → 8)-epi | 2.45 ± 0.57 | 1.21 ± 0.16 | 0.14 | 1.45–1.53 |
| Fis-(4$\beta$ → 8)-cat | 2.70 ± 0.26 | 1.01 ± 0.10 | 0.12 | 1.48–1.60 |
| Fis-(4$\beta$ → 8)-epi | 3.28 ± 0.57 | 1.11 ± 0.06 | 0.08 | 1.51–1.52 |

Fis = fisetinidol, cat = catechin, epi = epicatechin.

The energies of the molecular orbitals were calculated using MOPAC 6.0. The covalent structures were first constructed and optimized using Sybyl 6.03. Then the energies of HOMO, HOMO-1, and LUMO were calculated with the AM1 Hamiltonian. All internal degrees of freedom were marked for optimization. The calculation used increased convergence criteria with keywords GNORM-0.01 SCFSRT=1.D-9.

## Results and Discussion

For the profisetinidin monomer and dimers in 1,4-dioxane, the only feature in each of the absorption spectra above 250 nm is a single broad structureless band with a maximum absorbance at 284 nm. This broad absorption band arises from contributions from the phenolic rings (A- and B-ring, Figure 1). The fluorescence emission spectra exhibit broad structureless bands with maxima at 320–325 nm, which is similar to the results for the procyanidins, where the maxima are at 314–324 nm (13,14). The $Q$ obtained upon excitation at 280 nm are listed in Table 1. Prior results for several procyanidins are included for comparison. While $Q$ for fisetinidol is significantly lower than $Q$ for catechin and epicatechin, the results for the profisetinidins fall within the range observed previously for the procyanidins (14,15). The fluorescence lifetimes for the dimers, Table 2, are also in the range observed previously for $\tau_1$ and $\tau_2$ of the dimeric procyanidins (5,6).

While there are substantial similarities in the steady-state fluorescence of the profisetinidins and procyanidins, as documented above, their monomers have fluorescence that differ in several important aspects. The $Q$ for fisetinidol exhibit a small systematic dependence on the wavelength for excitation, as shown in Table 3. This wavelength dependence of $Q$ was not observed with catechin and epicatechin. The excitation and emission spectra also depend slightly on the emission and excitation wavelength, respectively. An example of this dependence is presented in Figure 2. The anisotropy of the fluorescence of fisetinidol in propylene glycol at −20°C also exhibits a dependence on excitation wavelength, as shown in Figure 3.

Another difference is revealed in the fluorescence lifetime measurements. Prior work has shown that both of the procyanidin monomers had decay curves for the fluorescence intensity that were best described by a single exponential, Eq. (2), with lifetimes of 2 ns (6). With fisetinidol, however, fitting the decay curve to a single exponential resulted in a large $\chi^2$ of 1.64 and non-random distribution of the residuals. The best fit, assuming a single exponential decay, required $\tau$ = 2.25 ns. A better fit was ob-

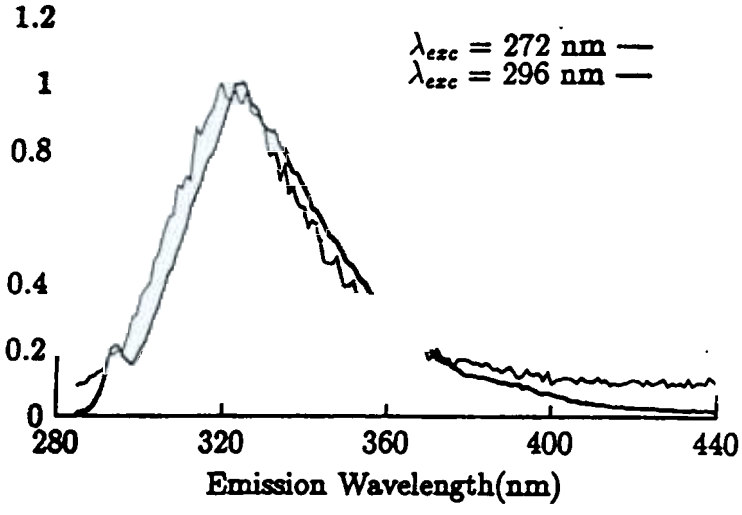


Figure 2. Normalized fluorescence emission spectra for fisetinidol upon excitation at 272 or 296 nm in 1,4-dioxane at 25°C.

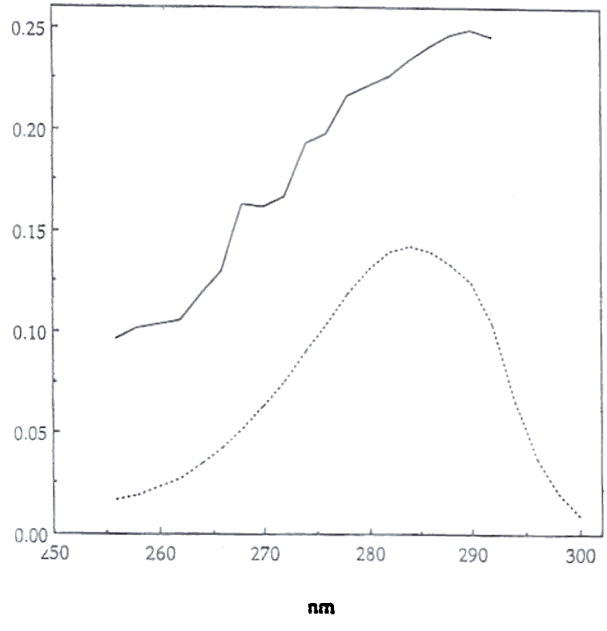


Figure 3. The fluorescence anisotropy (—, top curve) and absorbance bottom curve) for fisetinidol at −20°C in propylene glycol.

Table 3. The $Q$ ($\pm 0.01$) for fisetinidol in 1,4-dioxane (25°C, $\lambda_{exc}$ in nm)

| $\lambda_{exc}$ | 274 | 280 | 282 | 284 | 286 | 288 | 290 | 294 |
|---|---|---|---|---|---|---|---|---|
| $Q$ | 0.135 | 0.140 | 0.087 | 0.088 | 0.086 | 0.091 | 0.087 | 0.027 |

tained using the sum of two exponentials, which decreased $\chi^2$ to 1.02 and resulted in a more random distribution of the residuals. The two lifetimes components were 2.63 and 1.18 nm, with pre-exponential factors, $\alpha_i$, of 0.116 and 0.046, respectively. Two additional statistical tests (the Durbin-Watson parameter (12) and autocorrelation function) also suggest that the decay of the fluorescence intensity for fisetinidol should be described by the sum of two exponentials, Eq. (3).

Taken together, the wavelength dependence of $Q$, $r$, and the shape of the emission spectrum, and the two-exponential decay of the intensity of the fluorescence, suggest that two distinguishable electronic transitions contribute to the broad emission band for fisetinidol. These electronic transitions are assigned to the A- and B-rings. AM1 calculations for fisetinidol were used to estimate the energies for the HOMO → LUMO and for the HOMO-1 → LUMO transitions, which would be the transitions of the two chromophores in fisetinidol. The difference in these two transition energies was about 1.1%, which would correspond to a separation of the electronic bands of less than 10 nm.

Separate transitions from these two rings could not be resolved for the monomeric procyanidins, presumably because the A- and B-rings are photophysically less different in catechin and epicatechin than they are in fisetinidol.

## Conclusion

The heterogeneity of the decay of the fluorescence from the monomer of the condensed tannins is affected by hydroxylation at C(5). The simpler decay, which can be described by a single exponential, is obtained when the monomer bears a hydroxyl group at C(5). Removal of this hydroxyl group produces a heterogeneous decay of the fluoresence, which can be described as the sum of two exponentials. The A- and B-rings in the monomers are indistinguishable, from the viewpoint of fluorescence, when there is a hydroxyl group at C(5), but their contributions become resolvable when this hydroxyl group is removed.

In the absence of a conformational constraint that forces the occupation of a single rotational isomer at the interflavan bond, dimers of the procyanidins and profisetinidins exhibit heterogeneous decay of the fluorescence that is described by the sum of two exponentials. The heterogeneous decay in the dimers can be used to assign the populations of the two rotational isomers at the interflavan bond in the procyanidin dimers (5,6), but this interpretation cannot be extended to the profisetinidin dimers due to the heterogeneous decay of fisetinidol.

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