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A Supramolecular Fluorescence Probe for Caffeine

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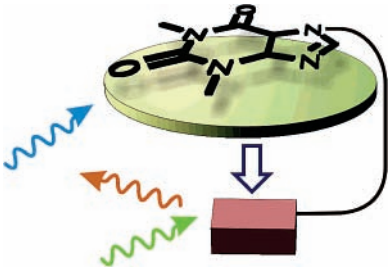
ABSTRACT



Supramolecular sensing is rather limited to charged analytes. This report describes a practicable assay for caffeine, which employs a competitive "indicator-displacement" approach and allows detection by fluorescence. A simple analytical protocol is described that allows quantitation of caffeine in beverages.

Caffeine (**2**) is among the mostly consumed alkaloidal compounds.[1] Besides the traditional sources of caffeine—coffee, black tea, and green tea—nowadays caffeinated beverages with increased levels of the alkaloid are gathering larger shares among soft drinks.[2] While caffeine is regarded to be harmless for adults in general, there are severe concerns about unfavorable influences of caffeine on young children and pregnant women, including the risk of fetal death.[3] The stimulating effect of caffeine is not only exploited in nutrient technology but also in cosmetics and pharmaceuticals, which accounts for the economic importance of this particular additive.[4] The easiest access to the alkaloid is by extraction from natural sources, which renders the caffeine content a key property for quality assurance.

For caffeine quantitation, chromatographic methods (HPLC, LC, CE) are still regarded as state-of-the-art. The desire for shorter analysis times drives the development in these fields and initiated other spectroscopic approaches[5] and the application of molecularly imprinted polymers (MIPs) in combination with quartz microbalances.[6] Such sensors are reusable and exhibit large applicable concentration ranges, but they still cope with response times of about 10 min, probably due to the bulk diffusion effects. Supramolecular chemists have devised several tailor-made chemoreceptors that allow a designed interaction in solution.[7] The triphenylene ketal based compounds contributed by our group exhibit the highest binding constants for caffeine.[8]

We recently described a novel assay for caffeine involving fluorescence spectroscopy.[8h] The interaction of caffeine and

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the receptor **1** (Figure 1) yields a bathochromic shift as well

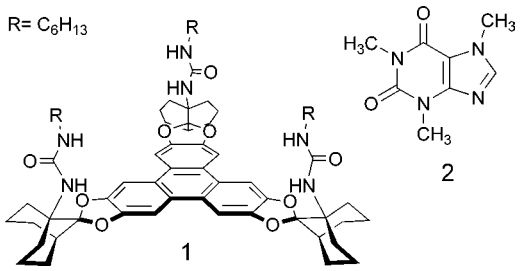


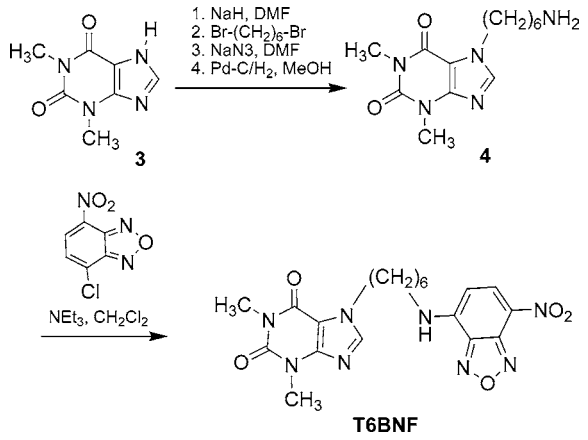
Figure 1. Receptor and caffeine.

as an increase of quantum yield by 30% in the complex (Figure 3; **I**/**II**).

For sensitivity improvement, the application of a competitive guest was envisioned. The pioneering contributions by Anslyn et al. promoted the development of such competitive indicator displacement assays.[9] Since most of these assays rely on ionic or covalent interactions as main binding force and signaling pathway, the assays are commonly applicable even in aqueous media. However, like many other potential analytes, caffeine binding is restricted to hydrogen bonding as the key interaction. This may account for the small number of fluorescent chemosensors for such compounds.[10] Furthermore, it limits the number of appropriate solvents: We restrict our investigations to CH_2Cl_2, since it delivers good solubility for both receptor and caffeine.[11]

The synthesis of the labeled guest is very straightforward. By simple alkylation, the spacer is attached at N-7 of theophylline (**3**) (Scheme 1). The ω-amino moiety is introduced

Scheme 1. Synthesis of the Labeled Guest **T6BNF**



by reaction of the alkyl bromide with azide and subsequent catalytic hydrogenation. The most suitable fluorophore is obtained by attachment of the commercially readily available 7-chloro-4-nitrobenzofurazane.[12]

In presence of toluene, this compound yields dark red crystals that were analyzed by X-ray analysis (Figure 2). In

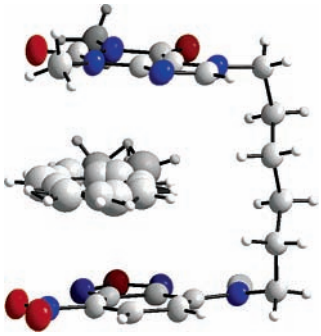


Figure 2. Molecular structure of **T6BNF** with intercalated toluene by X-ray analysis.

this structure, both heterocyclic moieties are arranged in a coplanar fashion in a distance of approximately 7 Å. This gap is filled with a toluene molecule, but when bound to the

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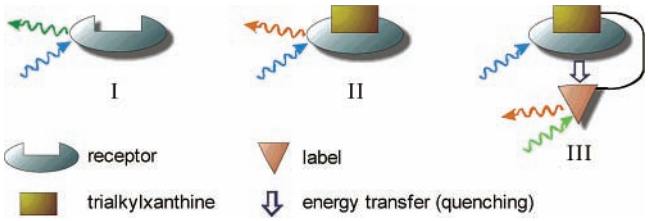

Figure 3. Effects on fluorescence upon binding of receptor **1** (**I**) to caffeine (**II**) and a competitive guest with a quenching label (**III**). Note that in **III** no emission from the receptor is observed. Colors are used to visualize different wavelengths.

receptor, a 2-fold π-stacking with the triphenylene unit could slightly increase the affinity and the spectral interactions.[13]

The beneficial spectroscopic properties of **T6BNF** allow an independent monitoring of the individual fluorescence signals of guest and host, respectively (see the Supporting Information). Excitation of the receptor is optimal at 352 nm since absorption of the labeled guest reaches a minimum at this wavelength.

The fluorescence spectrum of **T6BNF** reveals a broad band around 520 nm which experiences a red-shift upon addition of the receptor.[14] Addition of caffeine to this mixture results in a blue shift, while a control experiment in the absence of receptor does not. A pronounced effect is observed for the fluorescence of the receptor: Addition of only 8 equiv of **T6BNF** reduces the fluorescence by 80% (Figure 4). This

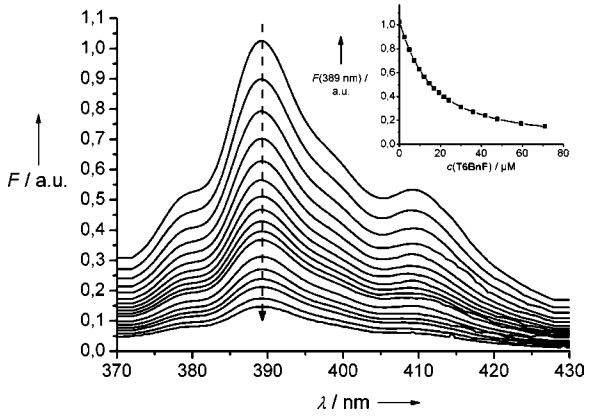

Figure 4. Decrease of fluorescence intensity upon addition of labeled **T6BNF** to receptor **1**; c(**1**) = 10 μM. Dilution effects were neglected since the total volume varied by <4% over the course of the experiment. Inset: Fluorescence intensity as a function of c(**T6BNF**).

effect is reversed upon addition of caffeine. Furthermore, a bathochromic shift by 5 nm is observed. The latter seems to occur generally when a xanthine compound binds the receptor. The absence of this shift upon addition of **T6BNF** and the signal reduction indicate the presence of a quenching mechanism: As soon as the receptor binds to **T6BNF**, its fluorescence is quenched—most likely by a FRET mechanism (Figure 3, **III**). Caffeine competes with the labeled guest for the receptor and yields the fluorescent complex **II**, which causes both signal augmentation and bathochromic shift in the spectrum.

A linear Stern−Volmer plot further underlines the quenching hypothesis. The size of the Stern−Volmer constant (K_{SV}) of 87000 M^{-1} indicates a static quenching mechanism, since these values cannot be caused by diffusion at the given concentrations.[15] Thus, K_{SV} represents the binding constant. Compared to caffeine, the number is slightly elevated (NMR: 36500 M^{-1}), which might be due to the additional attractive interactions with the benzofurazyl moiety underlined by NMR spectroscopic data.

The xanthinyl unit is a prerequisite for this static quenching. This is demonstrated by mixing the receptor with **4BNF**, which only consists of a butyl chain and the fluorescent marker. The decrease in fluorescence is now significantly smaller and follows linearly the concentration of **4BNF**.[17] Therefore, this effect is attributed to absorption by the slightly colored benzofurazyl system. Since the UV spectra of **4BNF** and **T6BNF** are very similar in the relevant range (>330 nm) the contribution of absorption to the total decline can be estimated to make up 3% and 16% after mixing the receptor with 1 and 2 equiv of **T6BNF**, respectively.

The beneficial competitiveness of the assay is demonstrated by comparing the capacity of the systems (Figure 5).

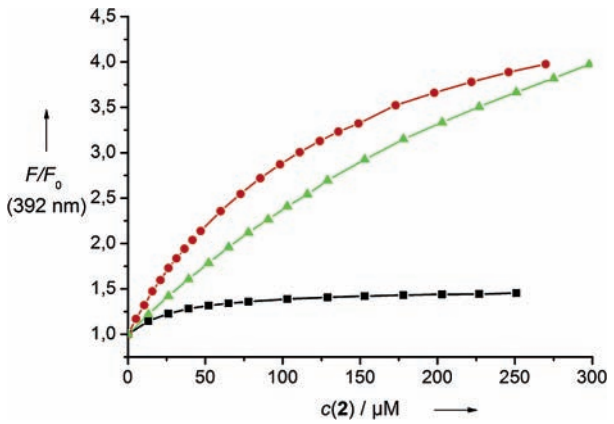

Figure 5. Signal increase is more pronounced in the competitive protocols (c(**T6BNF**) = 110 μm (▲)/50 μm (●)) than in absence of the quencher. (■). c(**1**) = 10 μm, c(**2**) ≈ 5 μm. Furthermore, the capacity is significantly higher.

Whereas titration of the pure receptor with caffeine rapidly reaches saturation, the competitive approach still provides distinct signal changes over a broad range of concentrations.

(12) Caffeine solutions in CH_2Cl_2 are obtained during economically important decaffeination processes, and we will demonstrate later how to easily exchange water with CH_2Cl_2 in "real life" samples. CH_2Cl_2 is used in direct and "indirect" decaffeination methods. Other solvents for extraction are ethyl acetate, water, and $scCO_2$. See also: http://www.ico.org.

(13) We also tested the dansyl and fluoresceinyl moiety. Synthesis of (9-anthranyl)methylidenes proved to be very challenging. The benzofurazyl modified compounds stand out in terms of solubility and superior spectroscopic properties and are consequently the only probes discussed herein.

An increasing fluorescence of **T6BNF·1** upon adding alcohols and DMF underlines the importance of hydrogen bonding for the stability of the complex. Thus, compounds with comparable hydrogen-bonding acceptors were the focus of selectivity experiments, among them barbituric acid (**4**) and a cyanuric acid derivative, benzimidazoles (**5**), and phenolic compounds (Figure 6). The latter were included,

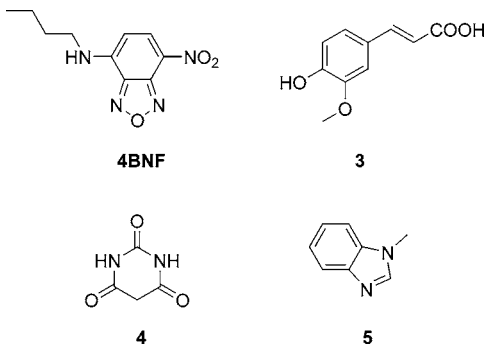

Figure 6. Selected, alternative guest molecules. For a full list of guests employed in the selectivity study, refer to the Supporting Information.

since they are a major constituent of coffee beverages.[1] Except for ferulic acid (**3**), which interferes by its own fluorescence, none of the examined compounds gave a pronounced interference.[18]

As proof of principle, we determined the caffeine content in caffeinated soft drinks and a coffee beverage. Although the selectivity of the system is promising, a brief sample preparation proved helpful when analyzing these samples. For the soft-drinks, matrix reduction and exchange of the solvent (water → CH_2Cl_2) was achieved simultaneously by a simple solid-phase extraction (SPE) using reversed-phase materials corresponding more to a filtration than to a chromatographic separation.[19] After adjustment of the volume, the eluate was directly used for caffeine determination and gave correct values in a range of 50−300 mg/L. The coffee sample was prepared by filtration over a polyamide phase. After extraction of the aqueous eluate with CH_2Cl_2, the organic phase was used for quantitation. The caffeine content was determined to be 585 mg/L which is in good accordance with the reference values determined by HPLC. Recovery rates in all double measurements were in the range of 91−114%, whereas most cases gave even better agreement.

By combination of receptor **1** with **T6BNF** as competitive guest, the first practicable chemosensor assay for caffeine was developed. If bound to the receptor, **T6BNF** quenches efficiently the fluorescence of the triphenylene fluorophore. Displacement of this labeled guest by caffeine leads to a signal increase by a factor up to four and creates a reliable and fast way for the determination of caffeine content in beverages. The assay can be applied over a broad range of concentrations. However, this system represents a powerful chemosensor for a difficult, neutral medicinally and technically highly relevant analyte.

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Supporting Information Available: Synthetic procedures and characterization data for **3**, **4** and **T6BNF**, NMR binding studies, fluorescence binding studies with **4BNF**, **T6BNF**, solvent effects, selectivity studies and detailed analytical protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Single crystals appropriate for X-ray analysis of receptor **1** with **T6BNF** could not be obtained. However, molecular modeling supports this hypothesis.

(15) Note that the receptor neither absorbs nor emits in this wavelength range.

(16) Titrations conducted at different temperatures with a different receptor (R = menthyl) revealed that increasing the temperature leads to lower K_{SV}. This is typical for static quenching processes.

(17) In the UV, **4BNF** differs from **T6BNF** solely by the lack of the short-wavelengths trialkylxanthine bands. Since the analysis is performed at significantly higher wavelengths, this should not cause interferences.

(18) Ferulic acid can be easily removed during sample preparation (vide infra).

(19) SPE is a common sample preparation technique which is easily parallelized and available as automated application. The application of the column takes less than 1 min (excluding equilibration and drying times). A fractionated collection of the eluate is not required. SPE is also commonly used for raising the sample concentration and could be applied for the transfer of this method to trace analyses.