Colorimetric Sensors

A Color Sensor for Catecholamines**

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Dedicated to Professor Christian Reichardt on the occasion of his 70th birthday

Catecholamines are characterized by their phenylethylamine skeleton, which carry two phenolic *ortho*-hydroxy groups. The level of these and related compounds in blood and urine reflects the biodegradation efficiency of aromatic amino acids, especially tyrosine, and plays an important role in diagnostic purposes. Patients suffering from phaeochromocytoma display a critically elevated concentration of the catecholamine adrenaline in their blood which inevitably leads to heart failure and death.[1] Malfunction of the catecholamine dopamine-responsive neurons is a key feature for Parkinson's disease.[2] Usually, noradrenaline and adrenaline are determined in blood and urine samples through HPLC analyses with electrochemical or neurochemical detection.[3] In recent years, a large number of catecholamine-receptor structures have been designed for organic solvents, some as ditopic host molecules with recognition sites

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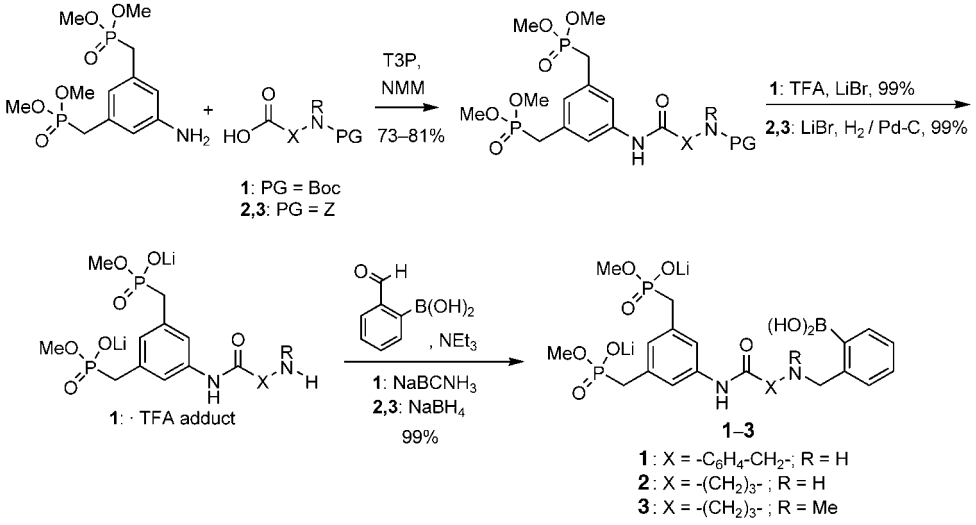
for both parts of the guest.[4] However, only very few of these receptors operate in pure aqueous solution.[5] To this end, receptors have been embedded in membranes[6] or sol-gel films.[7] RNA aptamers[8] represent a bioorganic solution to the problem of dopamine recognition. A less selective alternative is the fabrication of immobilized monotopic receptor structures with simple functional groups on electrodes, for example, in a polymer matrix,[9] on silica particles or other solids,[10] or on biofilms containing polyphenolases.[11] Recently a certain shape-selectivity has been achieved for the slim dopamine skeleton with designed cyclophanes.[12] However, almost none of the preceding examples are selective for catecholamines in complex mixtures that contain other amines, amino acids, and neurotransmitters. Herein we present a receptor that combines our bisphosphonate recognition motif, which is selective for amino alcohols,[13] with a boronic acid, which is highly selective for catechols. In a simple competition experiment with a catechol-containing dye, a color signal is induced that indicates the presence of catecholamines and allows an exact quantitative determination of the concentration of catecholamines in complex mixtures, for example, in urine samples.

The groups of Shinkai and Czarnik have used multiple boronic acid moieties extensively for the covalent recognition of *cis*-glycol units in sugars.[14,15] Numerous examples of derivatives with a secondary or tertiary benzylic amine functionality *ortho* to a phenylboronic acid have been reported,[16] and even in aqueous solution, the thermodynamically stable tetrahedral zwitterionic intermediate is formed almost quantitatively.[17] However, much less is known about the related formation of boronic acid esters with catechols, although the flat 1,2-arrangement of both acidic hydroxy groups should facilitate this condensation even more. Early investigations by Smith and co-workers described transport agents based on arylboronic acids for catecholamines.[18] Other groups combined the boronic acid moiety with binding motifs for other functionalities to synthesize ditopic receptor molecules with a high selectivity for the corresponding guests.[19] For example, Cooper and James covalently attached a phenylboronic acid to a crown ether through an anthracene spacer and obtained a fluorescent sensor for D-glucosamine hydrochloride.[19a,c]

In our rational design, we bridged a xylylene bisphosphonate unit for aminoalcohol recognition and a toluneboronic acid moiety for binding to catecholamine with a non-proteinogenic amino acid of appropriate length in the aim of using the amine group as an internal base. To this end, the *N*-acyl-protected amino acid was coupled to the readily available *m*-aminoxylylene bisphosphonate,[20] and the coupled product was then deprotected and monodealkylated at its phosphonate ester groups. In the final step, the dilithium salt was subjected to a reductive amination sequence with *o*-formylphenylboronic acid.[21] The resulting benzylamine, the desired receptor molecule, was obtained as a water-soluble, colorless solid (Scheme 1). Three different spacer groups were chosen: *m*-aminomethylbenzoic acid acts as a semirigid building block in **1**, whereas GABA (γ-aminobutyric acid) as the secondary and tertiary amine in **2** and **3,** respectively, provides a flexible alkyl chain.

Upon equilibration of the ditopic receptors with noradrenaline, a second set of signals were observed in the ^{1}H NMR spectrum which correspond to the catechol-boronate zwitterion (Figure 1). In our case, formation of the catechol-borane is slow on the NMR timescale. Distinct signals appeared for both the host (amino alcohol) and guest (catechol) parts. This effect was examined in buffered aqueous solution (NaH_2PO_4 (100 mM), pH 7.0). Recently a weak phosphate–boronic acid interaction was described which could also interfere in our system.[22] To verify this, a "model" NMR titration between a boronic acid derivative and catechol was carried out in the presence and absence of phosphate buffer. The corresponding binding constant was not affected by the presence of phosphate anions, so we conclude that the influence of coordination of the boronic



Scheme 1. Synthetic route with reductive amination as the key step to the ditopic adrenaline receptors **1**–**3**, which carry a boronate and a bisphosphonate group bridged by non-proteinogenic amino acids. PG = protecting group, Boc = *tert*-butyloxycarbonyl, Z = benzyloxycarbonyl, T3P = 1-propylphosphonic acid cyclic anhydride, NMM = *N*-methylmorpholine, TFA = trifluoroacetic acid.

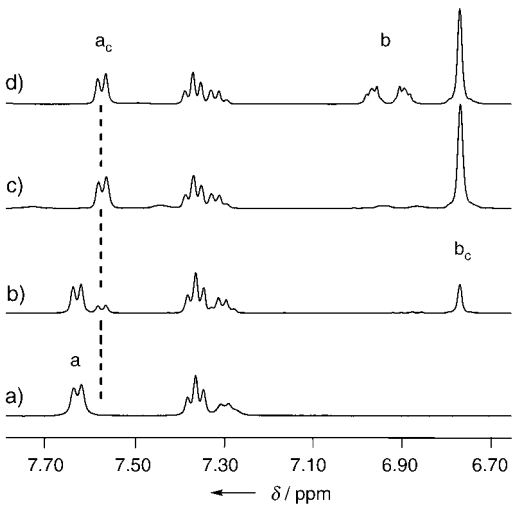
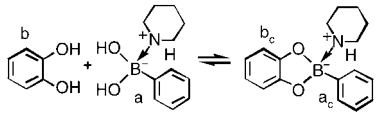


Figure 1. Model binding ^{1}H NMR experiment for the quantitative determination of the K_a value between phenylboronic acid (host), precomplexed with piperidine, and catechol. a) Free host, b)–d) host in the presence of 0.6 (b), 1.0 (c), and 1.6 equivalents (d) of catechol. A new set of signals appear for the protons of the host ($\delta > 7$ ppm) and the guest ($\delta < 7$ ppm). Binding constants (K_a) can be calculated from the ratio of free:complexed host (b) or free:complexed guest (d).

Table 1: Association constants between amines and/or diols and ditopic receptors **1**–**3**.[a]



Guest	**1** K_a [м$^{-1}$][b]	**2** K_a [м$^{-1}$][b]	**3** K_a [м$^{-1}$][b]
catechol	50	150	350
adrenaline	190	200	550
DOPA[c]	–	–	590
dopamine	180	230	630
noradrenaline	190	340	690
tyrosine ester	<10	<10	<10
phenylalanine ester	<10	<10	<10

[a] Determined from ^{1}H NMR titrations in buffered aqueous solution (Na_2HPO_4/NaH_2PO_4 (100 мм)) at 20 °C. [b] Error values were calculated as standard deviations from three independent measurements; all values were between 4% and 12%. [c] DOPA = 3,4-dihydroxyphenylalanine.

acid to phosphate ions is negligible. Analysis of the ratio between free host, guest, and complex revealed binding constants (K_a) in the order of 200–700 м$^{-1}$ for adrenaline derivatives, but significantly smaller values for catechol itself ($K_a \approx 50$–300 м$^{-1}$).[23] Together with distinct changes in the chemical shifts in the bisphosphonate and amino alcohol region, these findings indicate a simultaneous cooperative participation of the ammonium-boronate and the bisphosphonate in the binding of catecholamine. Relative to conventional NMR titrations, this is a very economic and convenient procedure for the accurate determination of K_a values: by variation of the host:guest stoichiometry, each mixture gives an association constant with very small deviations (see Table 1).

Probably owing to its C-4 linker, the rigid benzoic acid spacer in **1** produced only a relatively weak adrenaline receptor and gave a K_a value of 190 м$^{-1}$ upon association with noradrenaline. A little more effective was the flexible C-3 alkyl chain of the GABA spacer in **2** with a K_a value of 340 м$^{-1}$, which was further improved by introduction of a second *N*-alkyl group to produce a superior electron-pair donor in the form of the tertiary amine bridge in **3**. The resulting association constant of **3** with noradrenaline almost reached 700 м$^{-1}$, while with its *N*-isopropyl derivative isoproterenol, K_a reached almost 800 м$^{-1}$.[24] Although the affinities towards adrenaline derivatives in buffered aqueous solution are still moderate, the new receptor family is extremely selective for catecholamines. Simple biogenic amines such as phenylethylamine are not bound at all, and aliphatic diols such as glucose are likewise rejected. Even more impressive is the phenylalanine, tyrosine, and DOPA series, which shows exclusive interaction between the ditopic receptors and the dihydroxy amino acid. Other neurotransmitters are also neglected, such as glycine, glutamate, GABA, and acetylcholine.

The high selectivity of our new receptor molecules for catecholamines prompted us to develop a color assay for their detection in complex mixtures. Recently a simple dopamine sensor which comprised a boronic acid tethered to a fluorescent reporter group was reported.[5] Secor and Glass very recently reported a related system that featured a coumarin aldehyde for chromophoric response.[25] Our approach relies on precomplexation of alizarin complexone ((3,4-dihydroxy-2-anthraquinonylmethyl)iminodiacetic acid) by the receptor and its subsequent displacement by the guest of choice. This concept has been introduced in several elegant contributions by Anslyn and co-workers who used competition experiments for citrate and tartrate ion sensing.[26] The dye compound carries a relatively acidic catechol moiety incorporated into its anthraquinone chromophore, and—in its zwitterionic form—also an ammonium ion.[27] In a similar approach, the groups of Wang and James established a fluorometric and colorimetric system using the alizarine red S–boronic acid interaction.[28] ^{1}H NMR titrations of a mixture of alizarin complexone and increasing amounts of **2** reveal a rather high binding constant of $K_a \approx 1700$ м$^{-1}$. Formation of the complex was also accompanied by a strong bathochromic shift in the absorption maximum of the dye. Figure 2 illustrates the resulting UV/Vis absorption curves obtained, as well as a clear isosbestic point and the concomitant color change from deep red (bordeaux) to orange.[29] As soon as a competing catecholamine is added, the dye is successively replaced and released into the free solution, and the color change is reverted. A large excess of the guest finally restores the original spectrum of the dye.

From the quantitative evaluation of the decrease in intensity at the new absorption maximum at 460 nm, binding constants were calculated for the guest molecules discussed above and lie in exactly the same relative order as those determined by NMR titrations, but remain somewhat lower (Figure 3a, Table 2).[30] Upon changing the solvent from pure

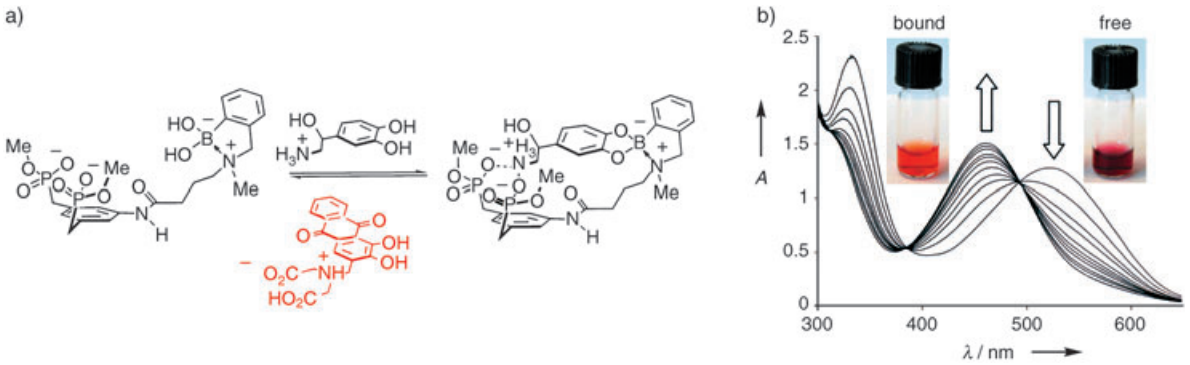


Figure 2. Competitive color assay that relies on the displacement of precomplexed alizarin complexone with concomitant color change. a) Lewis acidic/basic structures of the host **3**. b) Corresponding UV/Vis absorption curves of alizarin complexone with **2**, with deep red (bordeaux) and orange colors indicating the free and complexed states, respectively, of the alizarin dye. The isosbestic points indicate the direct interconversion of both states.

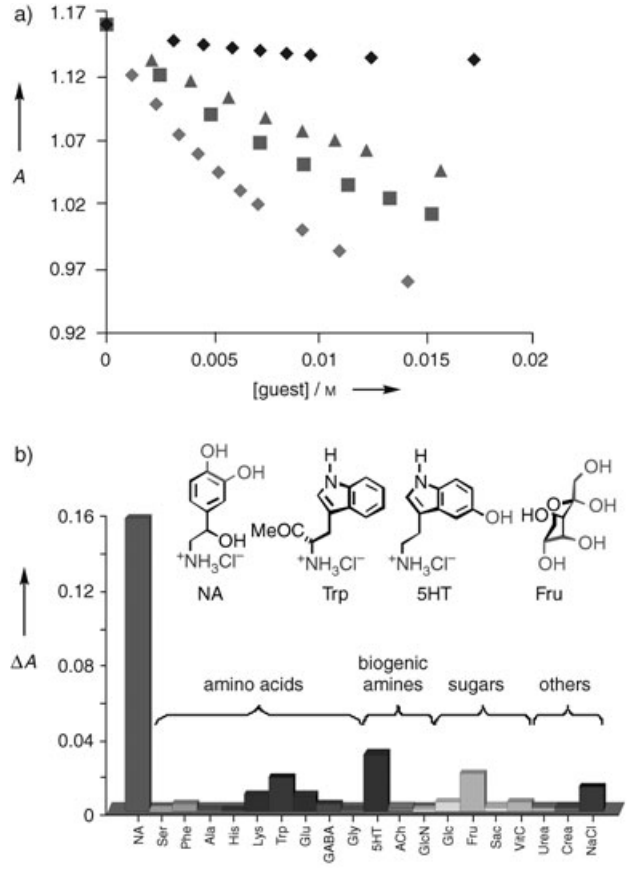


Figure 3. a) Binding curves obtained from the UV/Vis titrations of receptor **3** (1 mM) with various analytes by indirect visualization of their affinities through the respective decrease in the absorbance at $\lambda = 460$ nm (A_{460}) of the receptor-precomplexed alizarin dye (1 mM). Methoxytyrosine hydrochloride (black rhombus); fructose (gray triangle); catechol (gray square); noradrenaline (light-gray rhombus). b) Selectivity of the new ditopic receptor **3** for catecholamines as illustrated by changes in absorption ΔA_{460} at the absorption maximum of the dye–**3** complex (1 mM each) after the addition of 5 equivalents of guest in the color assay. NA = noradrenaline, Ser = serine, Phe = phenylalanine, Ala = alanine, His = histidine, Lys = lysine, Trp = tryptophan, Glu = glutamate, GABA = γ-aminobutyric acid, Gly = glycine, 5HT = serotonin, ACh = acetylcholine, GlcN = glucosamine, Glc = glucose, Fru = fructose, Sac = saccharose, VitC = vitamin C, Crea = creatinine.

Table 2: Association constants between amines and/or diols and ditopic receptors **1**–**3**.[a]

Guest	**1** K_a [M^{-1}][b]	**2** K_a [M^{-1}][b]	**3** K_a [M^{-1}][b]
catechol	60	150	100
adrenaline	160	210	310
DOPA	–	–	270
dopamine	170	220	280
noradrenaline	180	250	350
alizarin complexone	2050	1620	3440

[a] Determined by UV/Vis spectroscopy from competition experiments in buffered aqueous solution (NaH_2PO_4 (100 mM)) at 20 °C. [b] Error values were calculated as standard deviations from three independent measurements; all values were between 4% and 10%.

water to a 3:1 mixture of methanol/water (HEPES (*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid) buffer (150 mM), pH 7.0), the K_a value for alizarin complexone increases from 3300 M^{-1} to 7000 M^{-1}. However, the expected rise in noradrenaline binding could not be confirmed: the determined association constant remained at 330 M^{-1} in this system. In general, catecholamines are again bound in the range of 300–400 M^{-1}, catechol somewhat less tightly with 200 M^{-1} and simple amines such as phenylethylamine are not bound at all. With the latter, no color change whatsoever could be detected which underlies the extreme selectivity of the receptor for catecholamines. Again a large number of other potential guests were examined, with the satisfying result that only catecholamines and no other amino acids, sugars,[31] neurotransmitters, or biogenic amines produced any positive responses (Figure 3b).

Consequently, we measured the decrease in absorption, brought about by noradrenaline during the competition experiment, at varying concentrations of analyte in the linear range of the Beer–Lambert law, between 10^{-4} M and 10^{-2} M, and obtained a straight calibration line. Extrapolation of the ΔA values measured from unknown samples of this neurotransmitter led to concentrations which were in excellent agreement with the amounts weighed (Figure 4). These values were exactly reproduced (less than 15% difference) in complex mixtures with up to 10 different competing amines or

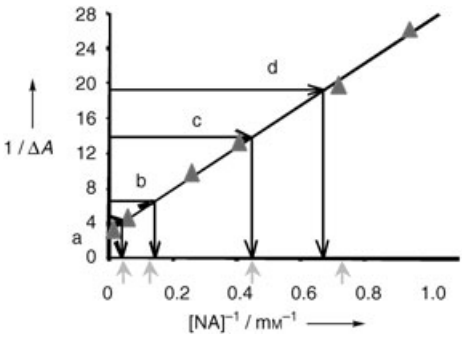


Figure 4. Benesi–Hildebrandt plot of the linear relationship between reciprocal absorption and reciprocal concentration of noradrenaline (NA).[32] a–d) Analytical samples of unknown concentration of noradrenaline which were determined by extrapolation from the observed $1/\Delta A_{460}$ value to the 1/[NA] axis. Such calibration curves were used for all subsequent adrenaline determinations.

Table 3: Quantitative detection of noradrenaline (NA) in a complex mixture of 11 different analytes in approximately equimolar concentrations, as well as detection in the millimolar regime of noradrenaline and total catecholamine (Cat) in a sample of human urine.

11-compound mixture		Urine			
$[NA]_{calcd}$ [mM]	$[NA]_{found}$ [mM]	$[NA]_{calcd}$ [mM]	$[NA]_{found}$ [mM]	$[Cat]_{calcd}$ [mM]	$[Cat]_{found}$ [mM]
1.3	1.5	2.7	3.3	6.4	7.6
2.2	2.5	7.5	7.9	9.5	11.3
7.9	6.7	8.8	9.8	13.0	14.6
23.2	22.5	14.9	17.6	–	–

diols in fivefold molar excess (Table 3). Human urine contains large amounts of urea, uric acid, creatinine, amino acids, organic acids, and various salts, as well as the yellow dye urobilin. Remarkably, even in this highly competitive environment, our color assay works and gives reliable results (less than 20% difference) with the simple calibration curve taken from the initial competition experiment with pure noradrenaline (Table 3). As all catecholamines give comparable quantitative effects, the total catecholamine concentration can be calculated from microliter quantities of such a sample, which contains dopamine, noradrenaline, adrenaline, and DOPA (Table 3), for example. Together with the simple competition measurement by UV/Vis spectroscopy, these features characterize the new method as a highly economic and fast assay for the determination of catecholamines in urine samples.[33] The selectivity of our new ditopic receptor molecules for catecholamines in highly competitive environments renders them useful in situations normally reserved for antibodies. There is only one drawback: the average level of catecholamine in human urine is much lower, in the micromolar concentration range. We are currently increasing the sensitivity of our assay, on one hand, by optimizing the receptor affinity for catecholamines and, on the other hand, by transferring the experiment to fluorescence techniques.

Experimental Section

General Procedure for Colorimetric Titrations: *Determination of the complexation constant of alizarin complexone with hosts **1–3***: A solution of alizarin complexone (1 mM) was prepared in phosphate buffer (150 mM, pH 6.9). Solutions of hosts **1–3** (≈20 mM) were prepared from this buffered solution of alizarin complexone to avoid dilution of the dye during the titration. An aliquot of the solution of alizarin complexone (400 μL) was taken in a cuvette (2-mm inner diameter) and treated stepwise with the hosts **1–3** (0–6 equiv). The reference cuvette was filled with phosphate buffer (150 mM; 400 μL). After every addition of host, the mixture was equilibrated for 30 s before the corresponding spectrum was measured. The binding constant was determined by nonlinear regression of the curve of ΔA versus [host] at a specific wavelength. In all cases, the absorption at 460 nm (λ_{max} of the alizarin complexone–host complex) was used as the maximum changes in the absorption were observed at this specific wavelength.

*Determination of the complexation constants for the guests catechol, adrenaline, dopamine, and noradrenaline with hosts **1–3** by competitive UV/Vis titration*: The solution of alizarin complexone (1 mM in phosphate buffer (150 mM)) prepared above was treated with host **1–3**, such that a 1:1 mixture was obtained. The stock solutions of guests were prepared using the solution of alizarin complexone–host complex, again to avoid dilution of the dye during the titration. An aliquot of the solution of alizarin complexone–host (1 mM each in phosphate buffer (150 mM)) complex (400 μL) was filled in a cuvette (2-mm inner diameter; reference: phosphate buffer) and treated stepwise with the guests (0–33 equiv). The ΔA_{460} values were used in the calculation of the corresponding binding constants (see Supporting Information for the detailed procedure).

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[30] For algorithms for competitive equilibria, see: K. A. Connors, *Binding Constants, The Measurement of Molecular Complex Stability*, Wiley, New York, **1987**.

[31] An exception is fructose, which is a *cis*-diol and binds to the receptor **3** with $K_a = 170\,\text{M}^{-1}$.[16a]

[32] H. A. Benesi, J. H. Hildebrandt, *J. Am. Chem. Soc.* **1949**, *71*, 2703.

[33] Earlier examples of indicators in competition with the desired analyte were mainly focused on biological systems, see: a) M. A. Roberts, R. A. Durst, *Anal. Chem.* **1995**, *67*, 482 – 491; b) C. Beyer, I. H. Alting, *Clin. Chem.* **1996**, *42*, 313 – 318.

