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Synthesis and spectroscopic characterisation of BODIPY® based fluorescent off–on indicators with low affinity for calcium

Nikola Basarić, Mukulesh Baruah, Wenwu Qin, Bert Metten, Mario Smet, Wim Dehaen and Noël Boens*

Department of Chemistry, Katholieke Universiteit Leuven, Celestijnenlaan 200F, 3001, Heverlee (Leuven), Belgium. E-mail: Noel.Boens@chem.kuleuven.be

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Two fluorescent off–on Ca^{2+} indicators based on APTRA (*o*-aminophenol-*N*,*N*,*O*-triacetic acid) as low-affinity ligand for Ca^{2+} and BODIPY® (4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene) as a fluorophore were synthesized. The new BODIPY®–APTRA compounds absorb in the visible spectrum, with absorption maxima from 505 nm to 570 nm, and have fluorescence spectra that span the visible spectrum, with emission maxima ranging from 525 nm to 625 nm dependent on the substituents at the α-positions to the nitrogen atoms. The indicators show a large increase of the fluorescence quantum yield upon increasing Ca^{2+} concentration. The ground-state dissociation constants K_d estimated at 20 °C in 100 mM KCl aqueous buffered solution, pH 7.20, for the two complexes with Ca^{2+} were found to be around 100 μM.

Introduction

Fluorescence spectroscopy is an attractive technique to measure (changes of) ion concentrations. Fluorescent chemosensors that show a spectral response upon binding Ca^{2+} are mostly derivatives of the Ca^{2+} chelators EGTA (ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), APTRA (*o*-aminophenol-*N*,*N*,*O*-triacetic acid)[1] and BAPTA [1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid].[2]

The choice of Ca^{2+} chelator depends on the Ca^{2+} concentration one intends to measure. The ground-state dissociation constant (K_d) of the formed Ca^{2+} complex must be well-matched with the Ca^{2+} concentration range of interest, because indicators have a usable fluorescence response in the range from approximately 0.1 K_d to 10 K_d. Therefore, high affinity Ca^{2+} probes, such as Fura-2 (K_d = 145 nM),[3] Indo-1 (K_d = 230 nM)[3] and Calcium Orange (K_d = 185 nM),[3] based on the tetracarboxylate Ca^{2+} ligand BAPTA,[2] become fully bound to Ca^{2+} at concentrations around and above 1 μM. Since the fluorescence response of high-affinity Ca^{2+} probes is saturated at these high Ca^{2+} concentrations, a series of indicators using the tricarboxylate chelator APTRA[1] with low affinity for Ca^{2+} (K_d for the Ca^{2+}–APTRA complex is around 30 μM) has been developed[1,3,4,5] to measure elevated intracellular Ca^{2+} levels[6] and for Ca^{2+} concentration measurements in the lumen of Ca^{2+} stores.[7] APTRA-based indicators are suitable for detecting Ca^{2+} levels between 1 μM and 100 μM. It must be noted that APTRA is a good ligand for several cations, *e.g.* Zn^{2+}.[8] While the low-affinity Ca^{2+} probes, such as mag-fura-2, mag-fura-5 and mag-indo-1,3, were originally designed to report intracellular Mg^{2+} levels (APTRA is sensitive to Mg^{2+} concentrations from 0.1 to 6 mM), these indicators have actually much higher affinity for Ca^{2+} than for Mg^{2+}. The moderate affinity of the APTRA based indicators for Ca^{2+} has been used to measure a whole range of Ca^{2+} mediated physiological processes.[3] On the other hand, typical physiological Ca^{2+} concentrations (10 nM–1 μM) do not usually interfere with Mg^{2+} changes because of the relatively low affinity of the APTRA-based indicators for Ca^{2+}.

There is still a need for new indicators with improved properties, especially those that absorb and emit light at wavelengths higher than 500 nm so that autofluorescence of cell components is avoided.

The well-known fluorophore BODIPY®[9] (4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene, borondipyrromethene, BDP) was chosen as fluorescent moiety because of its valuable qualities: high chemical stability, high photostability, relatively high fluorescence quantum yields and absorption coefficients.[10] Furthermore, BODIPY® dyes are excitable with visible light, have narrow emission bandwidths with high peak intensities and are amenable to structural modification.

The aim of this work is to synthesize and characterize fluorometrically new low-affinity calcium probes that absorb and emit light in the visible spectrum, with APTRA as the Ca^{2+} ligand and BODIPY® as the fluorescent group. Here we report the synthesis of two BODIPY®–APTRA indicators with a low affinity for Ca^{2+}, namely the caesium salts of 4,4-difluoro-8-(3-hydroxy-4-aminophenyl-*N*,*N*,*O*-triacetate)-3,5-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene and 4,4-difluoro-8-(3-hydroxy-4-aminophenyl-*N*,*N*,*O*-triacetate)-3,5-bis-(4-methoxyphenyl)-4-bora-3*a*,4*a*-diaza-*s*-indacene. In addition, we recorded their fluorescence emission and excitation spectra and investigated their Ca^{2+} binding properties *in vitro* by fluorometric titrations.

Results and discussion

Synthesis

Trimethyl 2-aminophenol-*N*,*N*,*O*-triacetate (**1**) was prepared according to our modified procedure.[11] Vilsmeier formylation afforded 4-APTRA carbaldehyde (**2**),[11] that was used in further condensations with adequately substituted pyrroles. In this way we transformed APTRA aldehyde **2** to the corresponding dipyrromethane *via* reaction with 2 eq. of 2-methylpyrrole.[12] The reaction was carried out in dichloromethane in the presence of trifluoroacetic acid (TFA). The formed dipyrromethane was oxidized *in situ* with *p*-chloranil to give dipyrromethene **3** which was isolated (Scheme 1).

Arylated pyrroles were prepared by Stille reaction of *N*-Boc-pyrrole tin derivative **5**.[13] The latter compound was prepared according to a described procedure[14] from *N*-Boc-pyrrole (**4**) by lithiation, using 2,2,6,6-tetramethylpiperidine lithium and subsequent quenching of the lithium derivative by tributyltin chloride. Stannane **5** was coupled with iodoanisole, giving *N*-Boc-2-(4-methoxyphenyl)pyrrole[15] (**6**), according to a published procedure.[16] The primary side-reaction that competes with the Stille reaction is Pd-catalyzed tin–carbon bond cleavage, giving *N*-Boc-pyrrole (**4**). When the reaction was carried out under anhydrous conditions only *N*-Boc-pyrrole (**4**) was obtained, regardless of the solvent polarity (THF or toluene). This C–Sn bond cleavage was partly suppressed by using a mixture

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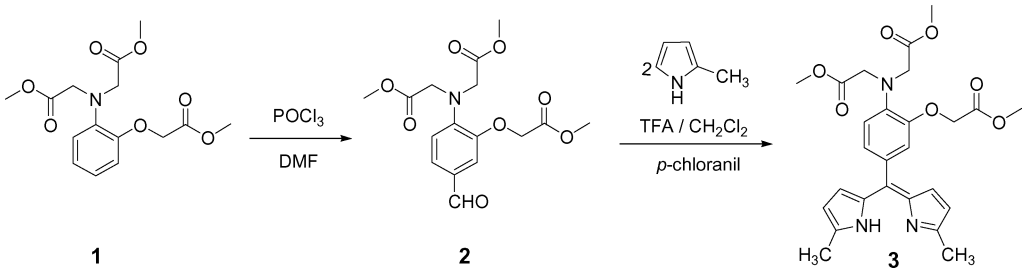
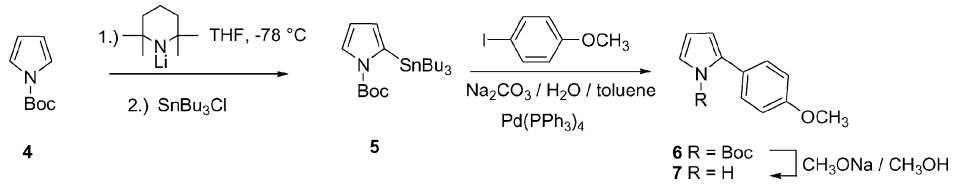


Scheme 1 Synthesis of dipyrromethene **3**.



Scheme 2 Synthesis of 2-substituted pyrrole compound **7**.

of toluene and an aqueous sodium carbonate solution as the solvent. However, the arylation reaction yield was restricted to a maximum of 40%. The Boc protective group was removed under basic conditions using sodium methoxide (Scheme 2).

Arylpyrrole **7** was transformed to dipyrromethane **8** and further oxidized to dipyrromethene **9** (Scheme 3).

The complexation was accomplished by stirring the dipyrromethenes **3** and **9** for 2 h with triethylamine and $BF_3{\cdot}OEt_2$ in dichloromethane. The synthesized APTRA esters **10** and **11** were readily transformed into the corresponding caesium salts **12** and **13** by saponification with caesium hydroxide (Scheme 4).

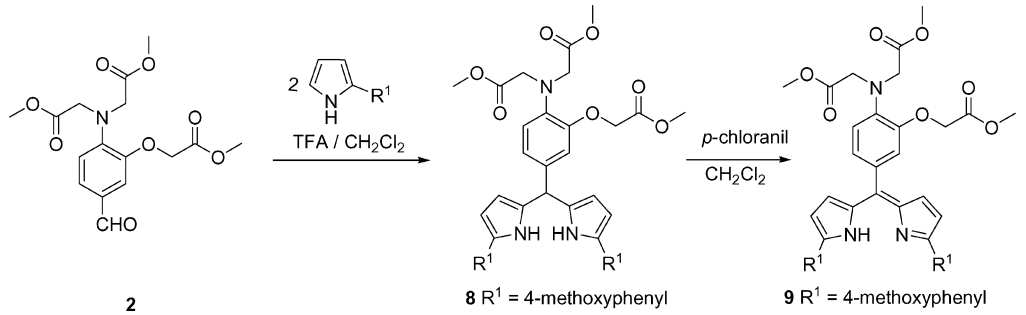
Table 1 Spectroscopic properties of the ester derivatives **10** and **11** in methanol

Compound	λ_{abs} (λ_{ex}) max/nm	$\varepsilon_{max}/M^{-1}\,cm^{-1}$	λ_{em} max/nm	ϕ_f
10	508	—	525	0.0046[a]
11	574	33000	618	0.039[b]

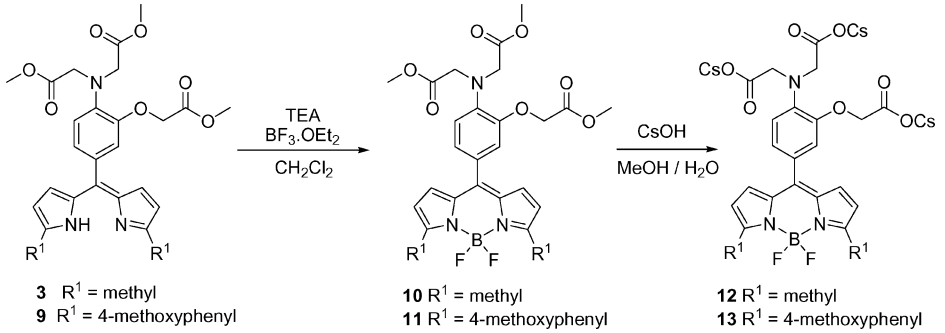
[a] The reference compound is rhodamine 6G in H_2O ($\lambda_{ex} = 488$ nm, $\phi_f = 0.76$). [b] The reference compound is cresyl violet acetate in MeOH ($\lambda_{ex} =$ 5 46 nm or $\lambda_{ex} = 578$ nm, $\phi_f = 0.55$).

Absorption and emission spectra

The spectroscopic properties of the synthesized BODIPY®–APTRA ester derivatives **10** and **11** in methanol were first investigated by recording their absorption, excitation and emission spectra. The data are summarized in Table 1. The synthesized compounds exhibit an absorption maximum in the wavelength region higher than 500 nm. For compound **10** the absorption (or excitation) maximum is at 508 nm and the emission maximum is at 525 nm. The absorption and emission spectra are of similar shape as those of the classical BODIPY® dyes.[17,18] Introduction of aryl substituents in positions 3 and 5 (in **11**) causes broadening of the typically sharp BODIPY® absorption (or excitation) and fluorescence bands and shifts the absorption (or excitation) and emission maxima bathochromically from 66 and 93 nm



Scheme 3 Synthesis of dipyrromethene **9**.



Scheme 4 Synthesis of the ester derivatives **10** and **11** and hydrolysis to the corresponding caesium salts **12** and **13**.

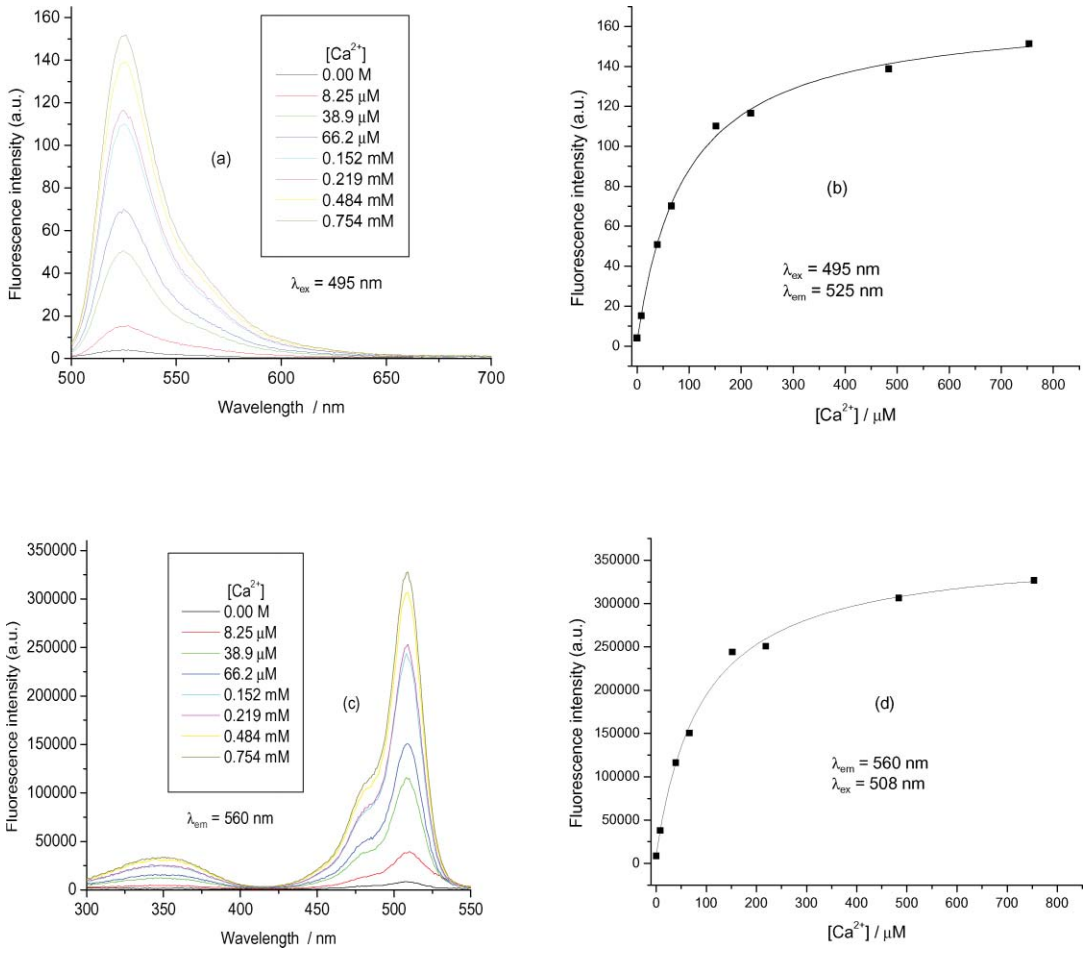


Fig. 1 Fluorescence emission spectra of compound **12** as a function of Ca^{2+} concentration (λ_{ex} = 495 nm): (a) fluorescence titration curve (λ_{ex} = 495 nm, λ_{em} = 525 nm); (b) obtained from Fig. 1a. Excitation spectra of compound **12** as a function of Ca^{2+} concentration (λ_{em} = 560 nm); (c) fluorescence titration curve (λ_{ex} = 508 nm, λ_{em} = 560 nm); (d) obtained from Fig. 1c. The solid lines in Fig . 1b and Fig. 1d represent the best fits of eqn. (1), with $n = 1$, to the fluorometric titration data of **12**.

to 574 and 618 nm, respectively. Compound **10** has the lowest fluorescence quantum yield ϕ_f (0.0046). Compared to **10**, ϕ_f for **11** increases by a factor of about 10 (0.039). The rather low ϕ_f values for **10** and **11** may be explained by an efficient quenching by a photoinduced electron transfer (PET) process in the excited state from APTRA to the BODIPY® unit, analogous to the process in the *N*,*N*-dimethylanilino BODIPY® derivative described in the literature.[17]

Calcium binding properties

The ground-state dissociation constants K_d of the complexes between Ca^{2+} and the probes were determined in a buffered aqueous solution (pH 7.20) by fluorometric titration as a function of Ca^{2+} concentration, using the fluorescence excitation and/or emission spectra. Nonlinear fitting of eqn. (1)[19] to the steady-state fluorescence data F, recorded as a function of $[Ca^{2+}]$, yields values of K_d, the fluorescence signals F_{min} and F_{max} at minimal and maximal $[Ca^{2+}]$, respectively (corresponding to the free and Ca^{2+} bound forms of the probe, respectively), and n (the number of calcium ions bound per probe). Eqn. (1) assumes that the absorbance of the sample is small (<0.1) and that Ca^{2+} complex formation in the excited state is negligible.[19]

$$F = \frac{F_{max}\left[Ca^{2+}\right]^n + F_{min}K_d}{K_d + \left[Ca^{2+}\right]^n} \quad (1)$$

Fitting eqn. (1) to the steady-state fluorescence data F with n, K_d, F_{min} and F_{max} as freely adjustable parameters always gave values of n close to 1, indicating that one calcium ion is bound per fluorescent indicator. Therefore, n was kept fixed at 1 in the final fittings of eqn. (1) to the fluorescence excitation or emission spectral data, from which the estimated values of K_d, F_{min} and F_{max} are reported. Plots of the fluorometric titrations of **12** are displayed in Fig . 1b and Fig. 1d for emission and excitation data, respectively. Fig. 2b and Fig. 2d show the corresponding curves for **13**.

To study the binding of Ca^{2+} in an environment similar to the intracellular one, the caesium salts **12** and **13** were dissolved in Milli-Q water containing 0.1 M KCl and 0.01 M MOPS (3-[*N*-morpholino]propanesulfonic acid), pH 7.20. Fluorescence emission and excitation spectra measured as a function of Ca^{2+} concentration are shown in Fig. 1 and Fig. 2 for **12** and **13**, respectively. The free Ca^{2+} concentrations were adjusted by use of the calcium-nitrilotriacetic acid buffer.[20] In the absence of Ca^{2+}, very weak fluorescence intensity was observed in both excitation and emission spectra. Upon increasing the Ca^{2+} concentration, a large increase of the fluorescence intensity and fluorescence quantum yield was observed, without a change of the position of the maxima. It is known that the lone electron pair of the amino group of APTRA is involved in the binding of Ca^{2+}.[21] Consequently, binding of calcium partly blocks the PET process. This is observed as an increase of the intensity of fluorescence (and ϕ_f). The data obtained by fluorometric titrations for the indicators **12** and **13** are summarized in Table 2. The dissociation constants $K_d(Ca^{2+})$ are estimated for these indicators to be ~100 µM, indicating that the structure modification in positions 3 and 5 of the BODIPY® fluorophore only slightly changes the Ca^{2+} binding properties of APTRA. However, the spectral properties of the BODIPY® fluorophore are quite different

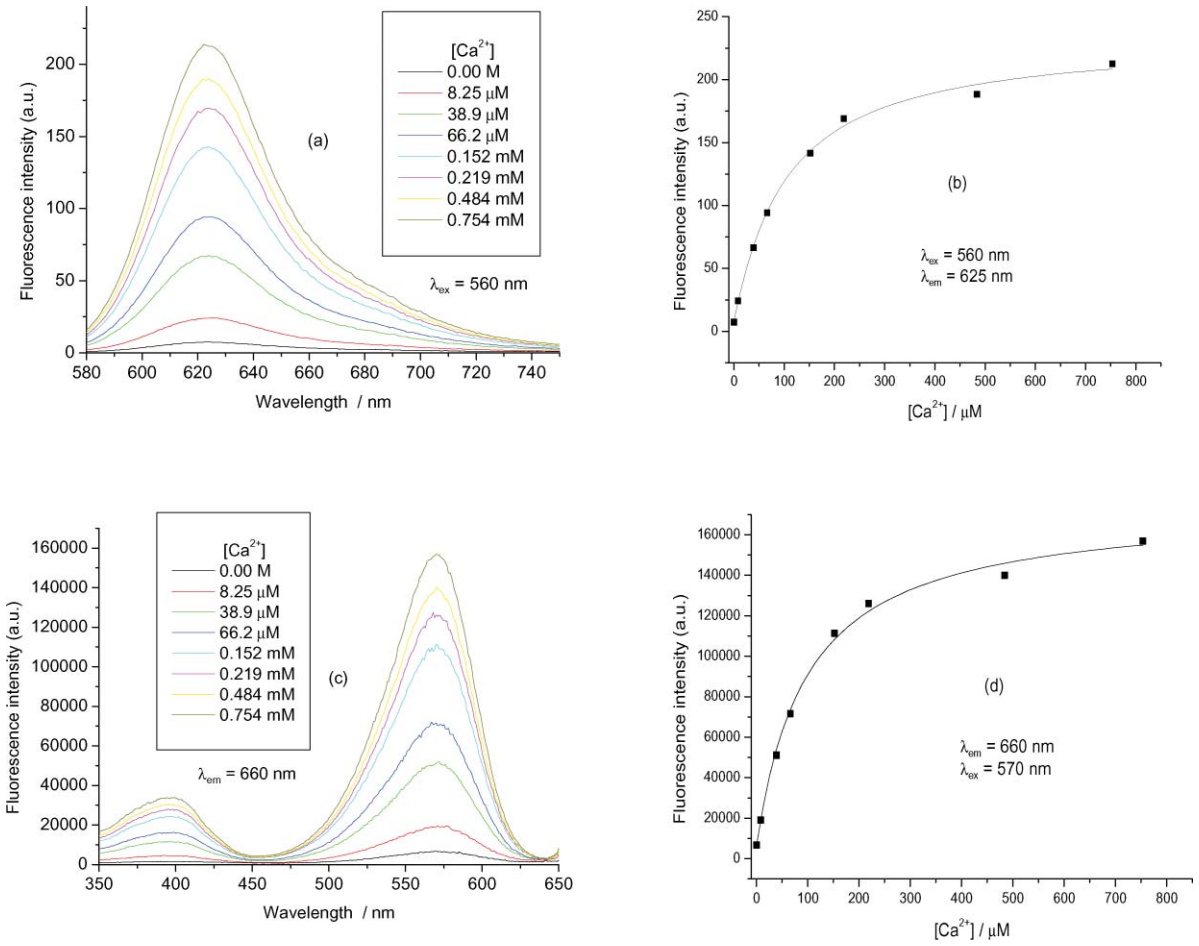


Fig. 2 Fluorescence emission spectra of compound **13** as a function of Ca^{2+} concentration (λ_{ex} = 560 nm): (a) fluorescence titration curve (λ_{ex} = 560 nm, λ_{em} = 625 nm); (b) obtained from Fig. 2a. Excitation spectra of compound **13** as a function of Ca^{2+} concentration (λ_{em} = 660 nm); (c) fluorescence titration curve (λ_{ex} = 570 nm, λ_{em} = 660 nm); (d) obtained from Fig. 2c. The solid lines in Fig. 2b and Fig. 2d represent the best fits of eqn. (1), with $n = 1$, to the fluorometric titration data of **13**.

Table 2 Spectroscopic properties of the caesium salts (**12** and **13**) in Milli-Q water containing 0.1 M KCl and 0.01 M MOPS (pH 7.20). The reported $K_d(Ca^{2+})$ values were estimated by fitting eqn. (1) (with $n = 1$) to the fluorescence emission and/or excitation data F

Compound	λ_{ex} max/nm	λ_{em} max/nm	$K_d(Ca^{2+})$ μM	ϕ_f (free)	ϕ_f (Ca^{2+} bound)	F_{max}/F_{min}
12	505	525	96 ± 8	0.004	0.14[a]	40
13	570	625	103 ± 11	0.003	0.13[b]	30

[a] The reference compound is rhodamine 6G in H_2O (λ_{ex} = 488 nm, ϕ_f = 0.76). [b] The reference compound is cresyl violet acetate in MeOH (λ_{ex} = 546 nm or λ_{ex} = 578 nm, ϕ_f = 0.55).

so that two BODIPY®–APTRA dyes with excitation and fluorescence spectra in the visible spectrum can be made.

The measured $K_d(Ca^{2+})$ values of the commercially available indicators mag-fura-2, mag-fura-5 and mag-indo-1 are 25, 28 and 35 μM,[3] respectively. The arylthiophene–APTRA probe Thio-H[4] has a $K_d(Ca^{2+})$ value of 45 μM, while for dioxopyrrolopyrrole probes[5] $K_d(Ca^{2+})$ values in the 10–20 μM range have been reported.

Conclusion

Two BODIPY®–APTRA derivatives were synthesized, namely, the caesium salts of 4,4-difluoro-8-(3-hydroxy-4-aminophenyl-*N*,*N*,*O*-triacetate)-3,5-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene (**12**) and 4,4-difluoro-8-(3-hydroxy-4-aminophenyl-*N*,*N*,*O*-triacetate)-3,5-bis-(4-methoxyphenyl)-4-bora-3*a*,4*a*-diaza-*s*-indacene (**13**). Absorption, excitation and emission spectra of these indicators were measured at 20 °C in 100 mM KCl aqueous buffered solution, pH 7.20, as a function of Ca^{2+} concentration. All the indicators absorb light of wavelengths higher than 500 nm with absorption maxima from 505 to 575 nm. In the absence of Ca^{2+}, the quite weak fluorescence emission (low ϕ_f values) may be due to the PET process from APTRA to the excited-state BODIPY® moiety. Binding of calcium ions blocks this electron transfer and causes a large increase of the fluorescence intensity (or ϕ_f).

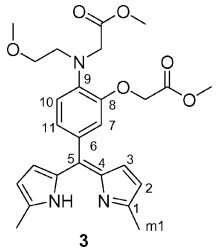
Experimental

General methods

^1H, ^{13}C and DEPT NMR spectra were recorded on a Bruker-300 Avance instrument operating at a frequency of 300 MHz for ^1H and 75 MHz for ^{13}C. All spectra were recorded in $CDCl_3$. The ^1H chemical shifts are reported in ppm relative to tetramethylsilane (0.00 ppm), using the residual solvent signal as an internal reference and ^{13}C shifts with $CDCl_3$ (77.67 ppm) as internal standard. Chemical shift multiplicities are reported as s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet. Low resolution mass spectra were obtained with a Hewlett Packard 5989A MS instrument (E. I. and C. I. mode). High resolution mass data were obtained with a KRATOS MS50TC instrument. Melting points were determined with a Reichert Thermovar

apparatus and are uncorrected. Trimethyl 2-aminophenol-*N*,*N*,*O*-triacetate (**1**) and 4-APTRA carboxaldehyde (**2**) were prepared according to our modified procedure.[11] The solvent (MeOH, spectroscopic grade, Aldrich) used for spectroscopic measurements was used as received. Potassium chloride (KCl, 99.999%), caesium hydroxide monohydrate ($CsOH{\cdot}H_2O$, 99.97%), calcium chloride anhydrous ($CaCl_2$, 99.99%) and MOPS (>99.5%) were obtained from Sigma-Aldrich and were used as such. Nitrilotriacetic acid (NTA, >99%) was purchased from Fluka and was used as received. The absorption measurements were performed on a Perkin Elmer Lambda 40 UV–VIS spectrophotometer. Corrected steady-state excitation and emission spectra were recorded on a SPEX Fluorolog. Fluorescence quantum yields ϕ_f of the indicators were determined using rhodamine 6G in water or cresyl violet acetate in methanol. The ϕ_f values reported in this work are the average values of multiple (generally four) independent measurements. Correction for the refractive index was applied when necessary. Fluorescence quantum yields were taken to be 0.76 (λ_{ex} = 488 nm) for rhodamine 6G and 0.55 (λ_{ex} = 546 nm or λ_{ex} = 578 nm) for cresyl violet acetate.[22] The spectra of the BODIPY®–APTRA esters were recorded in methanol, while their corresponding caesium salts were measured in buffered aqueous (Milli-Q water) solutions. Dissociation constants K_d of the complexes with Ca^{2+} were determined by fluorometric titration. For that purpose solutions were prepared to mimic the intracellular environment of mammalian cells containing 100 mM KCl, 10 mM MOPS and were adjusted to pH 7.20 with KOH and HCl. The free Ca^{2+} concentrations were adjusted with calcium-nitrilotriacetic acid buffers, as described by Fabiato and Fabiato.[20] Free Ca^{2+} concentrations were computed by the CHELATOR program developed by Schoenmakers *et al.*[23] All the solutions for the fluorescence measurements were adjusted to have an absorbance lower than 0.1. All measurements were performed at 20 °C.

Trimethyl 1,9-dimethyl-5-(3-hydroxy-4-aminophenyl-*N*,*N*,*O*-triacetate)dipyrro-methene (3). 4-APTRA carboxaldehyde (**2**) (116 mg, 0.33 mmol) and 2-methyl pyrrole (73 mg, 0.9 mmol) were dissolved in nitrogen flushed, dry dichloromethane (40 mL). One drop of TFA was then added through a syringe under nitrogen and the mixture was stirred overnight at rt. When TLC showed disappearance of APTRA aldehyde, a solution of *p*-chloranil (80 mg, 0.33 mmol) in dry dichloromethane (15 mL) was added. The reaction mixture was still stirred for 0.5 h and then quenched with water (80 mL), extracted with dichloromethane (200 mL), dried over $MgSO_4$ and concentrated by rotary evaporator. The crude product was purified through a neutral alumina column using hexane–ethyl acetate (7 : 3, v/v) as eluent to afford 81 mg (51%) of product **3**.



3

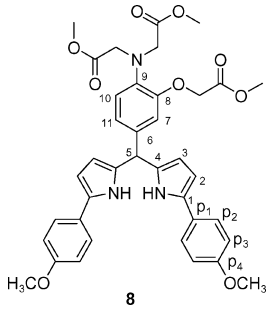
Reddish powder, mp 79 °C;^{1}H NMR ($CDCl_3$) δ 7.04 (dd, 1H, J = 1.8 Hz, J = 8.8 Hz, H-11), 6.89 (d, 1H, J = 2.1 Hz, H-7), 6.83 (d, 1H, J = 8.8 Hz, H-10), 6.50 (d, 2H, J = 4.4 Hz, H-2/3), 6.15 (d, 2H, J = 4.4 Hz, H-2/3), 4.63 (s, 2H, OCH_2), 4.28 (s, 4H, NCH_2), 3.78 (s, 9H, OCH_3), 2.44 (s, 6H, m1-H), NH not seen; ^{13}C NMR ($CDCl_3$) δ 171.51 (s, $NCH_2C{=}O$), 169.32 (s, $OCH_2C{=}O$), 153.95 (s, C-1/4), 148.41 (s, C-8), 140.26 (s, C-1/4), 138.23 (s, C-9), 131.55 (s, C-6), 129.35 (d, C-10), 125.90 (d, C-11), 118.32 (d, C-7), 117.71 (d, C-3), 108.46 (d, C-2), 66.15 (t, CH_2O), 54.01 (t, CH_2N), 52.28 (q, OCH_3), 16.65(q, C-m1); MS (EI 70 eV) m/z 497 (M^+, 9), 496 (M^+, 35), 495 (M^+, 81), 436 (40), 423 (28), 422 (100), 406 (32), 349 (26), 348 (23), 335 (25).

Trimethyl 1,1′-bis-(4-methoxyphenyl)-5-(3-hydroxy-4-aminophenyl-*N*,*N*,*O*-triacetate)dipyrromethane (8)

Preparation of *N*-Boc-2-(4-methoxyphenyl)pyrrole (6). *N*-Boc-pyrrolyl stannane (**5**)[13] (1 g, 2.20 mmol) and 4-iodoanisole (520 mg, 2.20 mmol) were dissolved in toluene (15 mL). To the mixture a 1 M aqueous solution of sodium carbonate (15 mL) was added and the solution was purged with argon. A spatula-point of palladium tetrakis(triphenylphosphin) catalyst was added and the reaction mixture heated under argon at reflux temperature for 1 d. The layers were separated and the water layer extracted three times with dichloromethane. The combined organic layers were dried over magnesium sulfate. The solvent was evaporated and the brown residue chromatographed on a column with silica gel using hexane–dichloromethane (2 : 1) as eluent. In the first fraction 350 mg of *N*-Boc-pyrrole (**4**) was isolated, followed by 250 mg of product **6**.

Removal of the Boc group. *N*-Boc-2-(4-methoxyphenyl)-pyrrole (**6**) (350 mg, 1.28 mmol) was dissolved in 50 mL of methanol. A solution of freshly prepared sodium methoxide (prepared by reacting 120 mg Na, 5.2 mmol, with 10 mL of methanol) was added and the reaction mixture heated under reflux for 24 h. The solvent was evaporated and the residue treated with water and extracted with dichloromethane. The organic extracts were dried over $MgSO_4$, the solvent was evaporated and the residue chromatographed on a column with silica gel using hexane–dichloromethane (2 : 1) as eluent. In the second fraction 180 mg of product **7** was isolated.

Condensation with aldehyde. APTRA aldehyde (**2**) (180 mg, 0.51 mmol) and 2-(4-methoxyphenyl)pyrrole (**7**) (180 mg, 1.04 mmol) were added to dried dichloromethane (30 mL). The solution was purged with argon and a drop of TFA was added. The reaction mixture was stirred for 24 h under argon, after which 50 mL of an aqueous solution of sodium bicarbonate was added. The layers were separated and the water layer extracted with dichloromethane. The collected organic extracts were dried over $MgSO_4$, the solvent was evaporated and the residue chromatographed on a column with silica gel using dichloromethane as solvent. In the first fraction 50 mg of 2-(4-methoxyphenyl)pyrrole (**7**) was isolated, followed by 140 mg (42%) of product **8**.



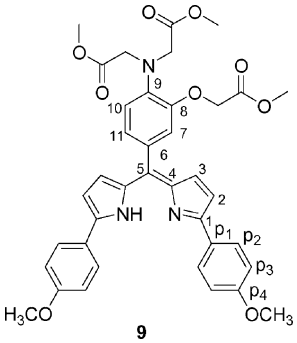
8

Bluish crystals, mp 70–71 °C; ^{1}H NMR ($CDCl_3$) δ 8.22 (broad s, 2H, NH), 7.34 (d, 4H, J = 8.8 Hz, H–P_2), 6.87 (d, 4H, J = 8.8 Hz, H–P_3), 6.85 (m, 2H, H-10, H-11), 6.74 (s, 1H, H-7), 6.32 (dd, 2H, J = 2.9, J = 2.9 Hz, H-3), 5.94 (dd, 2H, J = 2.1, J = 2.9 Hz, H-2), 5.41 (s, 1H, H-5), 4.60 (s, 2H, OCH_2), 4.21 (s, 4H, NCH_2), 3.81 (s, 6H, $PhOCH_3$), 3.72 (s, 6H, OCH_3(N)), 3.64 (s, 3H, OCH_3(O)); ^{13}C NMR ($CDCl_3$) δ 172.20 (s, $NCH_2C{=}O$), 169.66 (s, $OCH_2C{=}O$), 158.48 (s, C–P_4), 149.87 (s, C-8), 138.72 (s, C-9), 136.32 (s, C-1/4), 133.36 (s, C-1/4), 132.19 (s, C–P_1), 126.19 (s, C-6), 125.43 (d, C–P_2), 122.88 (d, C-11), 120.05 (d, C-7), 115.22 (d, C-10), 114.46 (d, C–P_3), 109.42 (d, C-3), 105.26 (d, C-2), 66.18 (t, CH_2O), 55.72 (q, $PhOCH_3$), 53.90 (t, CH_2N), 52.47 (q, OCH_3(O)), 52.19 (q, OCH_3(N)), 44.04 (d, C-5); MS (EI 70 eV) m/z 679 (M^+ − 2H, 20), 509 (80), 451 (70), 435 (80), 377 (40), 206 (80), 173 (100), 158 (100).

Oxidation of dipyrromethane 8 to dipyrromethene 9

To a solution of 1 eq. of dipyrromethane **8**, a solution of 1 eq. of *p*-chloranil in dichloromethane was added. The reaction mixture was stirred for 1 h, the solvent removed by distillation and the residue chromatographed on silica using dichloromethane and gradually increasing the polarity by adding ethyl acetate (up to 10% (v) EtOAc).

Trimethyl 1,9-bis-(4-methoxyphenyl)-5-(3-hydroxy-4-aminophenyl-*N*,*N*,*O*-triacetate)dipyrromethene (9)



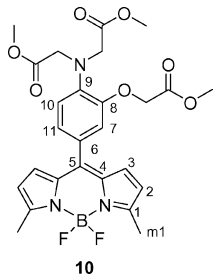
9

Deep blue crystals, 100 mg (90%); mp 53 °C; ^{1}H NMR ($CDCl_3$) δ 7.86 (d, 4H, $J = 8.8$ Hz, H–P_2), 7.15 (dd, 1H, $J = 1.4$, $J = 8.0$ Hz, H-11), 7.02 (d, 4H, $J = 8.8$ Hz, H–P_3), 6.99 (d, 1H, $J = 1.4$ Hz, H-7), 6.90 (d, 1H, $J = 8.0$ Hz, H-10), 6.77 (d, 2H, $J = 4.4$ Hz, H-3), 6.73 (d, 2H, $J = 4.4$ Hz, H-2), 4.67 (s, 2H, OCH_2), 4.32 (s, 4H, NCH_2), 3.89 (s, 6H, $PhOCH_3$), 3.80 (s, 6H, OCH_3(N)), 3.78 (s, 3H, OCH_3(O)), NH is not seen; ^{13}C NMR ($CDCl_3$) δ 172.20 (s, $NCH_2C{=}O$), 169.37 (s, $OCH_2C{=}O$), 160.64 (s, C–P_4), 153.86 (s, C-1/4), 148.49 (s, C-8), 141.57 (s, C-1/4), 140.52 (s, C-9), 131.40 (s, C-5), 130.26 (d, C-3), 128.00 (d, C–P_2), 126.45 (s, C–P_1), 126.05 (d, C-11), 125.29 (s, C-6), 118.39 (d, C-7), 117.37 (d, C-10), 115.24 (d, C-2), 114.84 (d, C–P_3), 66.15 (t, CH_2O), 55.83 (q, $PhOCH_3$) 54.04 (t, CH_2N), 52.61 (q, OCH_3(O)), 52.35 (q, OCH_3(N)); MS (EI 70 eV) *m*/*z* 679 (M^+, 90), 680 (M^+, 40), 606 (60), 547 (40), 510 (50), 474 (70), 451 (75), 310 (50), 206 (100).

Preparation of BODIPYs®

General procedure. A dichloromethane solution of 1 eq. of dipyrromethene was purged with argon. To the solution was added 10 eq. of triethylamine and the solution stirred for 0.5 h, then BF_3 etherate was added and the reaction mixture was stirred for 1 h. The reaction was quenched by addition of 1 M aqueous solution of sodium hydroxide. The layers were separated and the water layer extracted with dichloromethane. The combined organic layers were dried over $MgSO_4$, the solvent was evaporated and the residue chromatographed on silica. For **10**, hexane–ethyl acetate 7/3, v/v, was used as eluent. For **11**, dichloromethane was used as eluent and gradually ethyl acetate was added (up to 10% (v) EtOAc).

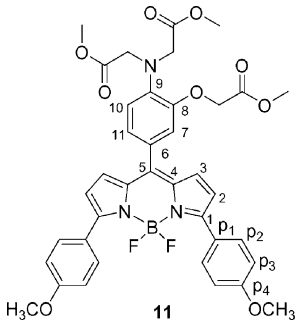
Trimethyl 4,4-difluoro-8-(3-hydroxy-4-aminophenyl-*N*,*N*,*O*-triacetate)-3,5-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene (10)



10

Orange–red needles, 30 mg (54%); mp 169 °C; recrystallized three times from chloroform–cyclohexane mixture; ^{1}H NMR ($CDCl_3$) δ 7.12 (dd, 1H, $J = 1.8$ Hz, $J = 8.4$ Hz, H-11), 6.92 (d, 1H, $J = 2.1$ Hz, H-7), 6.89 (d, 1H, $J = 8.8$ Hz, H-10), 6.76 (d, 2H, $J = 4.0$ Hz, H-2/3), 6.27 (d, 2H, $J = 4.0$ Hz, H-2/3), 4.65 (s, 2H, OCH_2), 4.28 (s, 4H, NCH_2), 3.79 (s, 9H, OCH_3), 2.65 (s, 6H, H-m1); ^{13}C NMR ($CDCl_3$) δ 171.92 (s, $NCH_2C{=}O$), 169.05 (s, $OCH_2C{=}O$), 157.40 (s, C-1/4), 148.68 (s, C-8), 141.72 (s, C-9), 134.68 (s, C-1/4), 130.58 (d, C-7), 127.82 (s), 125.63 (d, C-10), 119.52 (d, C-11), 118.57 (d, C-2), 116.89 (d, C-3), 66.13 (t, CH_2O), 54.05 (t, CH_2N), 52.64 (q, OCH_3(O)), 52.3 (q, OCH_3(N)), 15.24 (q, m1-C); MS (EI, 70 eV) *m*/*z* 544 (M^+, 11), 543 (M^+, 38), 542 (M^+, 9), 511 (3), 485 (28), 484 (100), 483 (24), 470 (2). HRMS (EI+): calcd for $C_{26}H_{28}BF_2N_3O_7$ (M^+) 543.19884; found 543.19774.

Trimethyl 4,4-difluoro-8-(3-hydroxy-4-aminophenyl-*N*,*N*,*O*-triacetate)-3,5-bis-(4-methoxyphenyl)-4-bora-3*a*,4*a*-diaza-*s*-indacene (11)



11

Deep blue crystals, 80 mg (74%); mp 80 °C; recrystallized three times from chloroform–cyclohexane mixture; ^{1}H NMR ($CDCl_3$) δ 7.87 (d, 4H, $J = 8.8$ Hz, H–P_2), 7.18 (dd, 1H, $J = 1.4$, $J = 8.0$ Hz, H-11), 7.00 (d, 1H, $J = 1.4$ Hz, H-7), 6.98 (d, 4H, $J = 8.8$ Hz, H–P_3), 6.92 (d, 1H, $J = 8.0$ Hz, H-10), 6.89 (d, 2H, $J = 4.4$ Hz, H-3), 6.01 (d, 2H, $J = 4.4$ Hz, H-2), 4.68 (s, 2H, OCH_2), 4.32 (s, 4H, NCH_2), 3.86 (s, 6H, $PhOCH_3$), 3.80 (s, 9H, OCH_3(N), OCH_3(O)); ^{13}C NMR ($CDCl_3$) δ 171.99 (s, $NCH_2C{=}O$), 169.17 (s, $OCH_2C{=}O$), 161.05 (s, C–P_4), 158.28 (s, C-1/4), 148.69 (s, C-8), 142.44 (s, C-1/4), 141.72 (s, C-9), 136.44 (s, C-5), 131.49(d, C–P_2), 130.68 (d, C-3), 128.21 (s, C–P_1), 125.88 (d, C-11), 125.69 (s, C-6), 120.68 (d, C-7), 118.56 (d, C-10), 116.99 (d, C-2), 114.19 (d, C–P_3), 66.10 (t, CH_2O), 55.69 (q, $PhOCH_3$) 54.09 (t, CH_2N), 52.70 (q, OCH_3(O)), 52.43 (q, OCH_3(N)); MS (EI 70 eV) *m*/*z* 726 (M^+, 25), 727 (M^+, 100), 728 (M^+, 40), 668 (30), 595 (70), 576 (80), 521 (50), 334 (60). HRMS (EI+): calcd for $C_{38}H_{36}BF_2N_3O_9$ (M^+) 727.26272; found 727.26654.

Hydrolysis of the BODIPY®–APTRA ester derivatives

To investigate the Ca^{2+} binding properties of the new indicators in aqueous solution, the water-insoluble BODIPY®–APTRA ester derivatives **10** and **11** were saponified to yield the corresponding water-soluble carboxylate salts **12** and **13**. A methanol solution of the ester derivative **10** (or **11**) was mixed with a very concentrated Milli-Q water solution of 10 eq. of spectroscopic grade $CsOH{\cdot}H_2O$. The reaction mixture was refluxed for 16 h under argon. When TLC showed the disappearance of the starting compound, the mixture was allowed to cool down to rt. More Milli-Q water was added and the solution washed with spectroscopic grade $CHCl_3$ to extract any residual unreacted starting compound. The separated water layer was evaporated to dryness in a rotary evaporator. The residue was then used to prepare stock solutions of the indicator for further fluorescence measurements. Comparison of the absorption and fluorescence emission spectra of the esters in methanol and the caesium salts in water indicates that the fluorophore structure remains intact for the compounds **10**, **12** and **11**, **13**.

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References

1 P. A. Otten, R. E. London and L. A. Levy, *Bioconjugate Chem.*, 2001, **12**, 76–83.
2 R. Y. Tsien, *Biochemistry*, 1980, **19**, 2396–2404.
3 R. P. Haugland, *Handbook of Fluorescent Probes and Research Products*, Molecular Probes, Eugene (Oregon), 2002.
4 E. Cielen, A. Stobiecka, A. Tahri, G. J. Hoornaert, F. C. De Schryver, J. Gallay, M. Vincent and N. Boens, *J. Chem. Soc., Perkin Trans. 2*, 2002, 1197–1206.
5 N. Avcibaşi, M. Smet, B. Metten, W. Dehaen, F. C. De Schryver, G. Bultynck, G. Callewaert, H. De Smedt, L. Missiaen and N. Boens, *Int. J. Photoenergy*, 2004, **6**, 159–167.
6 (*a*) I. Parker, J. Choi and Y. Yao, *Cell Calcium*, 1996, **20**, 105–121; (*b*) J. Eilers, G. Callewaert, C. Armstrong and A. Konnerth, *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 10272–10276; (*c*) S. Rajdev and I. J. Reynolds, *Neurosci. Lett.*, 1993, **162**, 149–152; (*d*) M. Konishi, S. Hollingworth, A. B. Harkins and S. M. Baylor, *J. Gen. Physiol.*, 1991, **97**, 271–301; (*e*) M. Zhao, S. Hollingworth and S. M. Baylor, *Biophys. J.*, 1996, **70**, 896–916.
7 (*a*) K. Hirose and M. Iino, *Nature*, 1994, **372**, 791–794; (*b*) F. H. van de Put, J. J. De Pont and P. H. Willems, *J. Biol. Chem.*, 1994, **269**, 12438–12443; (*c*) T. Sugiyama and W. F. Goldman, *Am. J. Physiol. Cell Physiol.*, 1995, **269**, C698–C705; (*d*) V. A. Golovina and M. P. Blaustein, *Science*, 1997, **275**, 1643–1648.
8 (*a*) O. Reany, T. Gunnlaugsson and D. Parker, *J. Chem. Soc., Perkin Trans. 2*, 2000, 1819–1831; (*b*) O. Reany, T. Gunnlaugsson and D. Parker, *Chem. Commun.*, 2000, 473–474.
9 BODIPY® is a registered trademark of Molecular Probes, Inc., Eugene, OR, USA.
10 (*a*) J. Karolin, L. B.-A. Johansson, L. Strandberg and T. Ny, *J. Am. Chem. Soc.*, 1994, **116**, 7801–7806; (*b*) F. Bergström, I. Mikhalyov, P. Hägglöf, R. Wortmann, T. Ny and L. B.-A. Johansson, *J. Am. Chem. Soc.*, 2002, **124**, 196–204; (*c*) R. W. Wagner and J. S. Lindsey, *Pure Appl. Chem.*, 1996, **68**, 1373–1380; (*d*) K. Rurack, M. Kollmannsberger and J. Daub, *Angew. Chem., Int. Ed.*, 2001, **40**, 385–387; (*e*) A. C. I. García-Moreno, C. Gomez, R. Sastre, F. Amat-Guerri, M. Liras, F. López Arbeloa, J. Bañuelos Prieto and I. López Arbeloa, *J. Phys. Chem. A*, 2002, **106**, 7736–7742.
11 B. Metten, M. Smet, N. Boens and W. Dehaen, *Synthesis*, DOI: 10.1055/5-2005-869900.
12 J. W. Cornforth and M. E. Firth, *J. Chem. Soc.*, 1958, 1091–1099.
13 N. Basarić, Ž. Marinić and M. Šindler-Kulyk, *Tetrahedron Lett.*, 2003, **44**, 7337–7340.
14 S. Martina, E. Enkelmann, G. Wegner and A.-D. Schlüter, *Synthesis*, 1991, 613–615.
15 L. H. Thoresen, H. Kim, M. B. Welch, A. Burghart and K. Burgess, *Synlett*, 1998, 1276–1278.
16 L. Groenendaal, H. W. I. Peerlings, E. Havinga, J. A. J. M. Vekemans and E. Meijer, *Synth. Met.*, 1995, **69**, 467–470.
17 M. Kollmannsberger, K. Rurack, U. Resch-Genger and J. Daub, *J. Phys. Chem. A*, 1998, **102**, 10211–10220.
18 T. López Arbeloa, F. López Arbeloa, I. López Arbeloa, I. García-Moreno, A. Costela, R. Sastre and F. Amat-Guerri, *Chem. Phys. Lett.*, 1999, **299**, 315–321.
19 E. Cielen, A. Tahri, K. Ver Heyen, G. J. Hoornaert, F. C. De Schryver and N. Boens, *J. Chem. Soc., Perkin Trans. 2*, 1998, 1573–1580.
20 A. Fabiato and F. Fabiato, *J. Physiol. (Paris)*, 1979, **75**, 463–505.
21 A. Delabie, E. Cielen, N. Boens and K. Pierloot, *J. Chem. Soc., Perkin Trans. 2*, 2001, 468–475.
22 J. Olmsted, *J. Phys. Chem.*, 1979, **83**, 2581–2584.
23 T. J. M. Schoenmakers, G. J. Visser, G. Flik and A. P. R. Theuvenet, *Biotechniques*, 1992, **12**, 870–879.