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Hydrophilic Labeling Reagents of Dipyrrylmethene-BF_2 Dyes for Two-Photon Excited Fluorometry: Syntheses and Photophysical Characterization

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Recently introduced bioaffinity assay technology, ArcDia™TPX, is based on two-photon excited fluorescence (TPE) and it enables separation-free ultra-sensitive immunoassays from microvolumes. Here we present syntheses of novel two-photon excitable fluorescent labeling reagents which have been specially designed to be used as label molecules in the ArcDia™TPX assay technique. The labeling reagents are based on dipyrrylmetheneboron difluoride (dipyrrylmethene-BF_2) chromophore, which have been substituted with aryl, heteroaryl or arylalkenyl chemical groups to extend the π-electron conjugation. These substitutions results in a series of dipyrrylmethene-BF_2 fluorophores with different photophysical properties. Dipyrrylmethene-BF_2 fluorophores have been further substituted with a dipeptide linker unit and finally activated as succinimidyl esters to enable specific coupling with primary amino groups. The dipeptide linker serves as a spacer arm between the label and a target, and enhances the solubility of the label in aqueous solutions. Study of the chemical and photophysical performance of the new labeling reagents is described. The new labeling reagents exhibit high fluorescence quantum yields, and molar absorption coefficients. The results show that the new labels with the hydrophilic dipeptide linker unit provide large two-photon excitation cross-sections, high fluorescence quantum efficiency and good solubility in aqueous solutions. The results suggest that the novel dipyrrylmethene-BF_2 labels are highly applicable to bioaffinity assays based on two-photon excitation of fluorescence.

KEY WORDS: Two-photon excited fluorescence; dipyrrylmethene-BF_2; BODIPY; synthesis; labeling; ArcDia TPX.

INTRODUCTION

The dipyrrylmetheneboron difluoride dyes (dipyrrylmethene-BF_2 dyes) were introduced by Treibs and Kreuzer in the late 1960's [1]. Since then, dipyrrylmethene-BF_2 dyes have found various applications. A wide variety of dipyrrylmethene-BF_2 dyes are commercially available and are sold under trademark of BODIPY® (Molecular Probes). These fluorescent dyes have been used as tracers in fluorescence microscopy, in receptor binding assays and in flow cytometric analysis. As fluorescent label compounds dipyrrylmethene-BF_2 dyes have many desirable properties: High quantum efficiency, sharp absorption and emission bands and relatively high absorption coefficient [2].

Our previous good results with dipyrrylmethene-BF_2 dyes in the field of two-photon excitation [3,4] encouraged us to further investigate this group of fluorophores as two-photon excitable labels. In general, the two-photon excitation maximum is slightly blue-shifted compared to the one-photon excitation maximum [5]. Therefore the most optimal fluorophore for TPE using illumination of 1064 nm is supposed to be characterised with one-photon absorption maximum in the range of 540 to 600 nm instead

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of having excitation maximum exactly at the half-length of the illumination light (532 nm). Such red-shift in the position of absorption maximum can be brought about by extending the π-electron conjugation of the chromophore by means of unsaturated substituents. Such methodology has been earlier applied for dipyrrylmethene-BF_2 dyes by Haugland and Kang [6–9]. These authors have created a series of dipyrrylmethene-BF_2 dyes with absorption and emission maxima at increasing wavelength positions. In addition to the publications of Haugland and Kang very few scientific paper exists which deal with synthetic chemistry of dipyrrylmethene-BF_2 dyes with extended π-electron conjugation [10–14].

Most of the dipyrrylmethene-BF_2 dyes published so far, especially those with aryl substituents, suffer from intrinsic hydrophobicity that limits their use in labeling of biomolecules. As commonly known, hydrophobic interactions between a fluorophore and a target molecule often leads to decrease in photophysical performance of the fluorophore. In some cases a hydrophobic label can also result in a decrease of biological activity of the biomolecular target or even to precipitation of the label-conjugate. Hydrophilicity and solubility to aqueous solutions can be increased by appropriate substitution of a fluorophore. Commonly used water-solubilizing groups include carboxylic acid, sulfonic acid, hydroxyl and ammonium groups. Wories *et al*. [15] introduced a method for substitution of the dipyrrylmethene-BF_2 chromophore with sulfonic acid groups, thus to increase hydrophilicity of the chromophore. The authors report mono- and disulfonated dipyrrylmethene-BF_2 dyes with emission maximum below 520 nm. In our hands, however, this methodology was not applicable for aryl substituted dipyrrylmethene-BF_2 dyes, but resulted in a mixture of products with low synthetic yield.

The biological function of a labeled biomolecule is affected by the nature of the linker unit that combines the label to the biomolecule. Haptens, with a single epitope, are especially sensitive in this respect [16]. It has been demonstrated that an increase in the length of the linker unit between the biomolecule and the label reduces the interaction between the two components and serves as a simple means to restore the biological function of the biocomponent [17]. Also, it has been shown that a positive effect on photophysical properties of the label can be obtained by increasing the length of the spacer arm between the fluorescent label and the biomolecule [18–20].

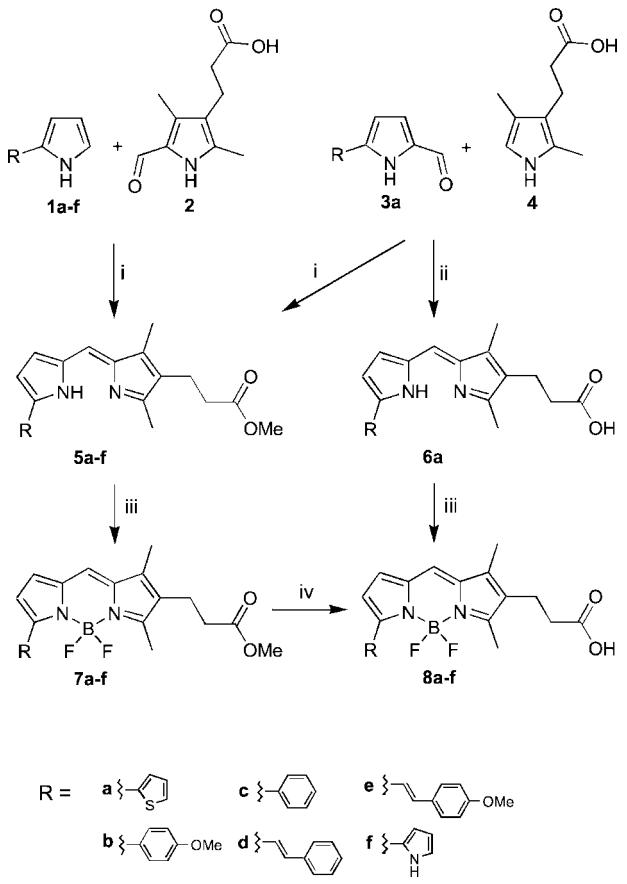
In this paper we describe a series of novel two-photon excitable fluorescent labeling reagents that are designed to be used as labels in bioaffinity assays according to the recently developed ArcDia™TPX assay technique [21]. These labeling reagents are derivatives of dipyrrylmethene-BF_2 dyes which have been substituted appropriately to provide fluorophores with increasing absorption and emission maxima. The fluorophores are further substituted with a hydrophilic linker unit that increases the hydrophilicity and aqueous solubility of the label, and serves as a spacer between the fluorophore and the biomolecular target. The performance of the new labeling reagents is studied with particular emphasis on the effect of the hydrophilic linker unit on the label performance.

RESULTS AND DISCUSSION

In this paper, we describe syntheses for a series of novel fluorescent labeling reagents. The labeling reagents are designed for two-photon excitation, and in particular for application of ArcDia™TPX bioaffinity assay technology [21]. This technology employs an inexpensive, passively Q-switched, diode pumped, micro-chip Nd:YAG laser as excitation light source. This laser produces nanosecond pulses at 1064 nm with ~20 kHz repetition rate and with average power of around 70 mW [22]. Using such inexpensive lasers for illumination, rather high two-photon excitation efficiencies, close to saturation, can be achieved for ordinary fluorescent dyes, such as Rhodamines. During the recent years, we have screened a number of fluorescent dyes as potential labels for Nd:YAG excitation, and found dipyrrylmethene-BF_2 fluorophores as the most potential group of compounds for this TPE application [3]. The purpose of the present work was to develop an optimal fluorescent label for two-photon excitation fluorometry, and for bioaffinity assay applications following the ArcDia™TPX assay methodology. In order to meet this goal, we decided to modify the basic core of dipyrrylmethene-BF_2 chromophore. Our strategy was to extend the π-electron conjugation of the dipyrrylmethene-BF_2 chromophore core, and thereby, to cause bathochromic and hyperchromic shifts in the absorption and emission bands. We prepared six fluorophore analogues (**7a–f**), which differed from each other by having different substituents (**a–f**) in the α-position of dipyrrylmethene-BF_2 complex.

Syntheses

The dipyrrylmethene-BF_2 dyes **7a–f** (Scheme 1) were synthesised from two pyrrole precursors, one of which contained a variable unsaturated substituent (**a–f**) while the other precursor served as a synthon for the constant part of dipyrrylmethene-BF_2 chromophore. The precursor for the constant part (compound **2** or

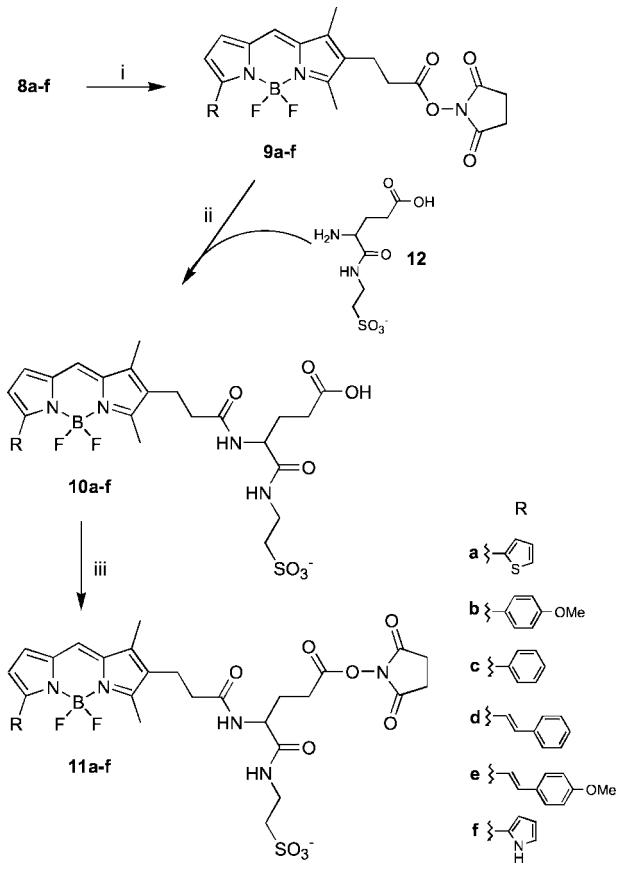


Scheme 1. Syntheses of dipyrrylmethene-BF_2 dyes **8a–f**. (i) $POCl_3$, CH_2Cl_2/MeOH 10:1 (v:v), RT, 18 hr; (ii) $POCl_3$, CH_2Cl_2, RT, 2 hr; (iii) DIPEA, BF_3*OEt_2, CH_2Cl_2, RT, 2 hr; (iv) HCl (0.5M in H_2O/THF), refl., 4 hr.

4) was synthesised from 2-ethoxycarbonyl-3,5-dimethyl-4-methoxycarbonylethylpyrrole [23] by applying the method of Lash *et al*. [24]. The free α-position of pyrrole **4** was formylated using Vilsmeier-Haack formylation to yield pyrrole **2**. Aryl pyrroles **1a–f** were prepared by applying the methods published in the literature. 2-phenyl- and 2-(4-methoxyphenyl)pyrroles (**1c** and **1b**) were synthesised via Suzuki coupling reaction [10]. Thienylpyrrole **1a** was synthesised by Paal-Knorr cyclisation [25] and formylated using the Vilsmeier-Haack method [26]. Pyrrolylpyrrole (bipyrrole) **1f** was prepared using the method described by Rapoport and Castagnoli [27]. The arylethenylpyrroles **1d** and **1e** were synthesised via Wittig reaction [7,9].

The α-free thienylpyrrole **1a** was found unstable even at decreased temperatures (-20°C) and therefore it was right away transformed to formylpyrrole **3a**. Formylation of the free α-position stabilises the pyrrole remarkably, and after formylation thienylpyrrole **3a** allowes storage at ambient temperature. Condensation of pyrrole **4** with thienylpyrrole **3a** was originally performed using phosphorus oxychloride as catalyst and dichloromethane as solvent to give dipyrrylmethene **6a**. After the condensation reaction, dipyrrylmethene **6a** was transformed in one pot to boron complex **8a** by addition of di-isopropylethyl amine (DIPEA) and boron trifluoride ethyl etherate. The product required chromatographic purification which was however quite difficult to perform due to impurities with equal chromatographic retention. The condensation reaction of compound **4** with thienylpyrrole **3a** was therefore modified by addition of methanol (10%) to the reaction mixture. Addition of methanol caused almost quantitative esterification and yielded dipyrrylmethene **5a**. After formation of dipyrrylmethene **5a** the mixture was evaporated to dryness to remove methanol, dissolved in dichloromethane followed by addition of DIPEA and boron trifluoride ethyl etherate. Chromatographic purification of the product **7a** was performed on silica gel using dichloromethane as eluent. This time the purification was easy and the product was obtained in pure form and in high yield. The other dipyrrylmethene BF_2-complexes **7b–f** were synthesised using the same reaction conditions as used for preparation of compound **7a**. The condensation reactions for preparation of compounds **5b–f** were, however, chosen to obey different synthetic strategy than that for compound **5a**. This was because α-free pyrroles **1b–f** were found to be more stable than the α-free thienylpyrrole **1a**, and therefore formylation of these pyrroles was not necessary. In this approach, the dipyrrylmethenes **5b–f** were prepared by condensation reaction of α-free aryl-, or arylethenyl pyrroles **1b–f** and pyrrole **2**. In general, the condensation reaction and the subsequent formation of the boron complexes (**7a–f**) proceeded with high synthetic yields (53–93%). Formation of boron complexes required relatively large molar excess of boron trifluoride ethyl etherate and DIPEA. Addition of these reagents was done stepwise by addition first 5 equivalents of DIPEA and then 5 equivalents of boron trifluoride ethyl etherate. The stepwise addition was continued until the mixture became fluorescent. Generally, bright fluorescence was observed from the reaction mixture after addition of 20 to 30 boron trifluoride ethyl etherate and DIPEA equivalents.

Thienyl substituted dipyrrylmethene-BF_2 complex **7a** was used as a model compound to study hydrolysis of the methyl ester derivatives. Hydrolysis of the methyl ester derivative **7a** was performed using either phosphoric acid [9] or hydrochloric acid as catalyst. Both methods yielded the product **8a** with reasonable yields. However, hydrochloric acid was found as more convenient catalyst by affording product of higher purity. Hydrolysis of methyl ester derivatives **7b–f** was therefore



Scheme 2. Synthesis of hydrophilic labeling reagents of aryl-, heteroaryl-, and arylethenyl-substituted dipyrrylmethene boron difluorides. (i) DCC, NHS, DMF, RT, 20 hr; (ii) DMF, TEA, RT, 30 min; (iii) DCC, NHS, DMF, RT, 48 hr.

performed using hydrochloric acid as catalyst. The carboxylic acid residue of compounds **8a–f** was transformed to succinimidyl ester by treatment with 1.5 equivalents of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 3 equivalents of *N*-hydroxysuccinimide (NHS) in anhydrous DMF (Scheme 2.). NHS was used in excess to ensure fast conversion of the activated carboxylic acid to corresponding succinimidyl ester and thus to minimise the formation of acylurea by-product. The desired dipyrrylmethene-BF_2 succinimidyl esters **9a–f** were obtained in high yield (72–92%) after column chromatography on silica gel.

In order to increase solubility of the dipyrrymethane-BF_2 dyes in aqueous solutions, a hydrophilic dipeptide linker unit (**12**) was attached to carboxyl side chain of compounds **9a–f**. This dipeptide linker compound was a derivative of glutamic acid with taurine substituent at the α-carboxyl position. This linker compound also serves as 5 atom long spacer and increases the distance between the label and the biomolecular target. The dipeptide linker **12** was reacted with active ester **9a–f** to produce compounds **10a–f** in high yields. The products **10a–f** were isolated by phenol extraction. The product transferred to phenol phase, while the unreacted fraction of dipeptide linker and the released NHS remained in water phase. Addition of a few volume equivalents of ether allowed reversed extraction of the product to aqueous phase whereas the unreacted starting material remained in the organic phase. The final product was isolated by precipitation from dichloromethane with carbon tetrachloride and dried in vacuo over phosphorus pentoxide to ensure moisture-free product. The carboxylic acid residue of compounds **10a–f** was finally transformed to succinimidyl ester by treatment with DCC and NHS in anhydrous DMF.

The dipyrrylmethene-BF_2 dyes **10a–f** and the dyes **11a–f** were found to be highly soluble in water and hygroscopic. The hygroscopic nature resulted problems in handling, especially in case of the succinimidyl esters derivatives. It was found that even short-term storage at open atmosphere (relative humidity <30%) was sufficient to turn products from solid powder to sludge. Therefore, storage of these compounds under desiccation is required. The effect of the linker unit on the solubility of the dipyrrylmethene-BF_2 dyes was found dramatic. Compounds **10a–f** and **11a–f** were readily soluble in water even in millimolar concentration whereas the corresponding compounds without the linker unit (**8a–f** and **9a–f**) were hardly soluble in aquoeus solutions other than mixtures with polar organic solvents.

The synthetic yields of the compounds **11a–f** were determined photometrically using the same molar absorption coefficients as for compounds **10a–f**. The synthetic products were characterized either by ^{1}H NMR or MS techniques. Dipyrrylmethene-BF_2 dyes **10a–f** and the corresponding labeling reagents **11a–f** were analysed also with reversed phase HPLC. The HPLC analysis of compounds **10a–f** showed single peaks with retention times between 2.7 and 5.6 min. These retention times correlated well with the size of the hydrophobic substituent **a–f**. The HPLC analysis of compounds **11a–f** showed, besides the product peak, also a smaller peak corresponding to starting materials **10a–f**. The retention times of the compounds **11a–f** were between 6.2 and 7.8 min. Thus, these peaks were readily distinguishable from the peaks of the corresponding starting materials **10a–f**. The HPLC analysis for compounds **11b,c** and **f** revealed a small peak of unknown impurity with retention time slightly longer than that of the main product. This impurity was, however, not possible to be removed by reversed

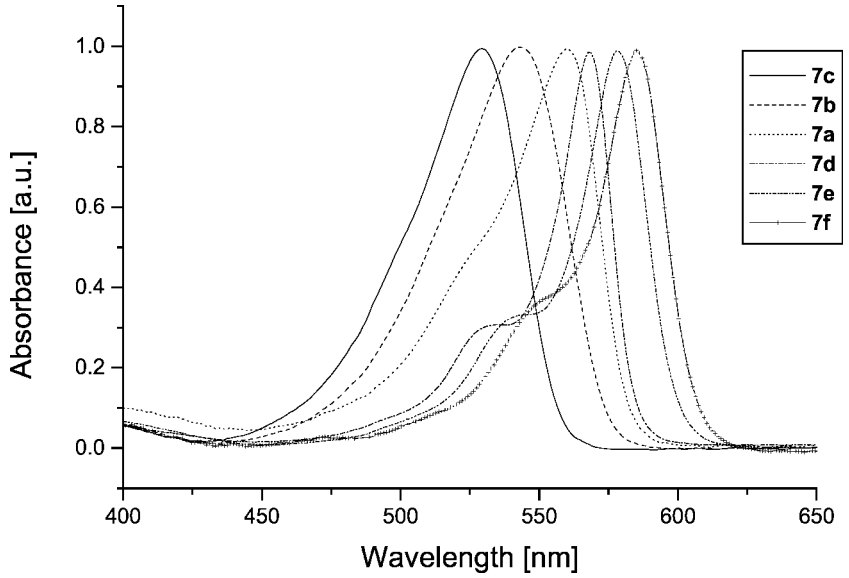


Fig. 1. Absorption spectra of dipyrrylmethene-BF_2 dyes **7a–f** in methanol.

phase chromatography due to sensitivity of the products to hydrolysis.

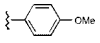
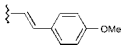
Absorption and Fluorescence Spectroscopy

Absorption spectra of the dipyrrylmethene-BF_2 complexes **7a–f**, were measured in methanol and are presented in Fig. 1. The spectra for compounds **8a–f** and **9a–f** were also measured and found practically identical in shape to the parent compounds **7a–f**. Molar absorption coefficients of methyl and succinimidyl derivatives (compounds **7a–f** and **9a–f**) were, however, somewhat higher than those of corresponding carboxylic acid derivatives (**8a–f**). The six fluorophore analogues showed absorption maxima in the range of 530 nm and 600 nm. The effect of the substituents (**a–f**) on the position of absorption peak maxima followed the general trend by being dependent on the length of π-electron conjugation and on the strength of electron donation character. The main absorption peak of the chromophores with aryl substituents **b** and **c** were found broader than of the others. The main absorption peak of heteroaryl (**a** and **f**) and arylethenyl (**d** and **e**) substituted dyes were correspondingly narrower but exhibited prominent shoulder at blue edge of the main absorption peak.

Fluorescence emission spectra of dipyrrylmethene-BF_2 complexes **7a–f** were measured in equimolar methanol solutions using 514 nm or 531 nm laser lines of argon-krypton laser for illumination. Fluorescence maxima of the compounds located between 550 nm and 610 nm. The fluorescence spectrum of each dipyrrylmethene-BF_2 dye was integrated and the integral was divided by molar absorptivity. Of the resulting values fluorescence quantum yields were calculated using Rhodamine B as a standard. The absorption and fluorescence data for compounds **7a–f** are summarised in Table I. Dipyrrylmethene-BF_2 dyes are, in general, characterized by narrow absorption and emission bands, and short Stokes shifts. The compounds investigated in this study exhibited the same features by having Stokes shifts between 6 and 25 nm. The longest Stokes shifts were shown by the analogues with phenyl and 4-methoxyphenyl substituents (**7c** and **b**) while the shortest Stokes shift was exhibited by the analogue with phenylethenyl substituent **7d**.

The absorption and emission spectra of the hydrophilic dyes **10a–f** were measured both in methanol and in aqueous solution of Triton X-100 (TX-100, Fig. 2). In methanol the absorption and emission spectra were equal to those of methyl ester derivatives (**7a–f**). The absorption coefficients, however, were on average 10% smaller than those for the corresponding methyl esters derivatives (**7a–f**, Table I). The absorption and emission maxima in TX-100 solution were few nanometers red-shifted and the quantum yields were somewhat lower than those measured in methanol. Although the dipyrrylmethene-BF_2 dyes **10a–f** and **11a–f** are highly soluble in aqueous solutions, it was found that in the absence of detergent both the absorptivity and the fluorescence quantum efficiency were remarkably decreased (data not shown). We account this

Table I. Absorption and Fluorescence Data of Dipyrrylmethene-BF_2 Dyes **7a–f** (Measured in Methanol) and **10a–f** (Measured in 0.1% TX-100, aq.)

Substituent	Compound	λ_{Abs} (nm)	$\varepsilon(cm^{-1}M^{-1})$	λ_{fluor} (nm)	ϕ_{fluor}	TPE (a.u.)	$\delta(GM)^a$
	7a	560	83000	576	0.82	—	—
	**10a**	565	69000	579	0.65	265	100
	7b	544	66000	569	0.91	—	—
	**10b**	547	64000	574	0.78	220	70
	7c	530	64000	552	0.72	—	—
	**10c**	533	54000	557	0.61	194	80
	7d	569	128000	575	0.85	—	—
	**10d**	574	118000	582	0.57	200	90
	7e	579	126000	591	0.79	—	—
	**10e**	584	124000	598	0.41	62	40
	7f	585	85000	600	0.47	—	—
	**10f**	589	74000	603	0.18	21	30

$^a 1\ GM = 1 \times 10^{-50}\ cm^4 s$.

phenomenon as consequence of amphiphilic nature of the dye, which can cause dimer and aggregate formation. Addition of detergent prevents formation of dye dimers, and thus increases both the absorptivity and the fluorescence quantum efficiency.

The two-photon excited fluorometry was carried out with ArcDia™TPX-microfluorometer. The instrument was equipped with an emission filter that enables measurements in the range of 530–610 nm. The two-photon excited fluorescence yields and cross-sections for compounds **10a–f** are presented in Table I. The TPE cross-sections were determined relative to R-phycoerythrin standard [28]. The cross-sections of compounds **10a–f** were found to be between 30 and 100 GM units. These cross-section values are in good agreement with the values for other dipyrrylmethene-BF_2 dyes previously reported in the literature [3,5]. The highest TPE efficiency was obtained for thienyl substituted dye (**10a**) while pyrrolyl substituted dye (**10f**) provided the lowest TPE fluorescence efficiency.

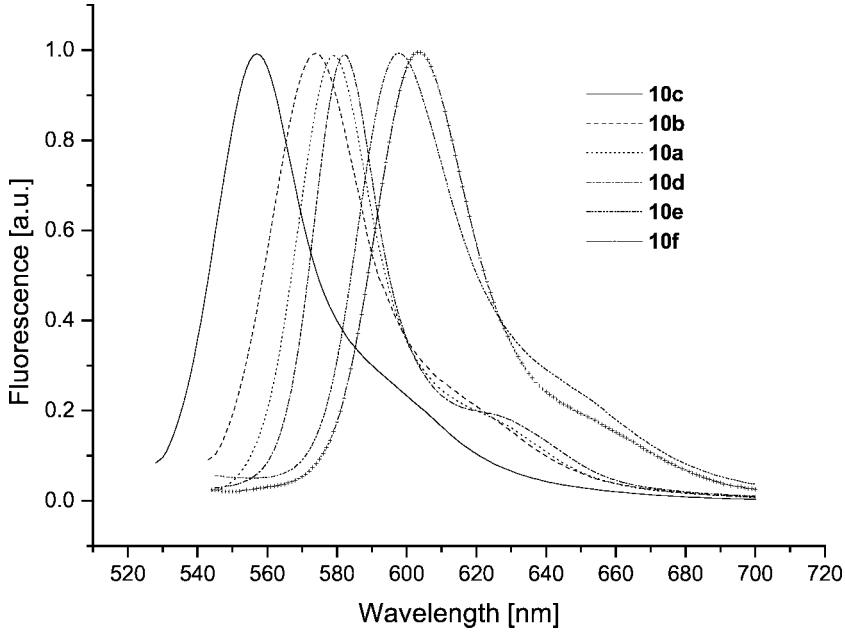


Fig. 2. Fluorescence emission spectra of dipyrrylmethene-BF_2 dyes **10a–f** in water (0.1% TX-100). Excitation with argon–krypton laser at 514 nm (compound **10c**) or at 531 nm (compounds **10a**, **b**, **d–f**).

Labeling of Immunoglobulin G

Performance of the new labeling reagents was tested by labeling of monoclonal mouse IgG. The labeling was performed under various reaction conditions. It was found that the labeling reagents exhibit good reactivity under typical conditions for succinimidyl esters. In order to study the effect the hydrophilising dipeptide linker unit on the labeling performance, labeling studies were carrier out also for reactive derivatives **9a–f**. It was found that the labeling reagents with linker unit exhibit somewhat higher reactivity than those without the hydrophilising linker unit. For example, the reactive derivatives of the fluorophore containing thienyl substituent, compound **9a** and **11a**, provided conjugation degrees of 2.5 and 3.2 labels per IgG, respectively. Conjugates of labels without the linker unit, however, turned out not to be stable but gave complete precipitation in few days of storage. Conjugates of labels with the dipeptide linker unit, in turn, exhibited excellent stability and no precipitation or aggregation was observed even in extended time of storage. More comprehensive studies about the performance of compounds **11a–f** as two-photon excitable fluorescent labels were recently carried out in our laboratory and the results will be published later.

EXPERIMENTAL

Materials

The reagents for syntheses were purchased from either from Fluka, J.T. Baker, Novabiochem, or Merck and used without further purification. The solvents were p.a. grade either from J.T. Baker, Lab-Scan or Merck and used as received unless otherwise stated. *N*,*N*-dimethylformamide (DMF) from Lab-Scan was dried over molecular sieves (4Å). Monoclonal mouse IgG against human α-fetoprotein (hAFP, clone 5108) was purchased from Medix Biochemica (Finland). Rhodamine B was purchased from Merck and R-phycoerythrin (RPE) from Molecular Probes.

Chromatography

Analytical thin-layer chromatography (TLC) was performed on silica gel Si 60 F_{254} plates (Merck). Preparative absorption chromatography was performed on silica gel 60 (Merck). The reversed phase absorption chromatographic analysis was carried out under the following conditions: Column: LiChroCART 125x3, Purosphere RP-18e. Eluent system: Acetonitrile:water, linear gradient (v:v) from 15:85 to 70:30 in 14 min. Gel filtration was performed on disposable NAP-5 gelfiltration columns (Amersham Pharmacia Biotech, Uppsala, Sweden).

Instrumentation

^{1}H NMR spectra were recorded either on Bruker AM200, JEOL JNM-LA400 or Bruker Avance DRX 500 spectrometer using deuterated chloroform or dimethylsulfoxide as a solvent and tetramethylsilane as internal standard. Mass spectra (MS) were recorded either on Voyager DE-PRO MALDI TOF (Perseptive Biosystems) using α-cyano-4-hydroxy-cinnamic acid as matrix, or on ZABSpec-oaTOF (Fisons Instruments). The UV–Vis spectra were recorded on SD-2000 Ocean Optics single beam fiber optic diode array spectrophotometer. The fluorescence emission spectra were recorded by using in-house constructed spectrofluorometer which employed argon-krypton laser as illumination source (laser line at 514 nm or 531 nm was used for excitation). The fluorescence emission spectra were recorded in wavelength range of 525–700 nm. The two-photon excited fluorometry was performed with ArcDia™TPX microfluorometer [22]. This fluorometer employed Nd:YAG microchip laser (average power 70 mW, repetition rate 17 kHz, nominal pulse length 1 ns) as illumination source and BG39 short pass emission filter.

Syntheses

*General Procedure for Preparation of Compounds **7a–f***

*Synthesis of 4,4-Difluoro-5-(2-thienyl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid Methyl Ester (**7a**).* 2-Formyl-5-(2-thienyl)pyrrole (**3a**) (150 mg, 0.84 mmol) and 2,4-dimethyl-3-carboxyethylpyrrole (**4**) (140 mg, 0.84 mmol) were dissolved in dichloromethane:methanol (10:1, v:v, 30 mL). Phosphorus oxychloride (77 μL, 0.84 mmol) was added and solution was stirred at room temperature for 18 hr. Reaction mixture was evaporated to dryness and the residue was dissolved in dichloromethane (150 mL). 5 equivalents of di-isopropylethylamine (DIPEA) (0.72 mL, 4.2 mmol) was added to the reaction mixture followed by addition of 5 equivalents of boron trifluoride ethyl etherate (0.53 mL, 4.2 mmol). Additional 5 equivalents of DIPEA and boron trifluoride ethyl etherate were added. Strong orange fluorescence was observed immediately after the second addition of boron trifluoride ethyl etherate. The reaction mixture was washed with water, dried with sodium sulphate and evaporated to dryness. The crude product was purified with column chromatography on silica gel using dichloromethane as eluent. Fractions containing the

desired product were pooled and evaporated to dryness yielding 304 mg (93%) of compound **7a**. ^{1}H NMR (Bruker AM200, $CDCl_3$, 200 MHz, δ ppm): 8.04 (1H, d, $J = 2.8$ Hz, thienyl-H), 7.40 (1H, d, $J = 4.3$ Hz, thienyl-H), 7.15 (1H, dd, $J = 3.8$ Hz, thienyl-H), 7.05 (1H, s, meso-H), 6.91 (1H, d, $J = 3.4$ Hz, pyrrole-H), 6.70 (1H, d, $J = 3.2$ Hz, pyrrole-H), 3.68 (3H, s, $COOCH_3$), 2.74 (2H, t, $J =$ 7.4 Hz, CH_2CH_2COO), 2.59 (3H, s, pyrrole-CH_3), 2.47 (2H, t, $J = 7.5$ Hz, CH_2CH_2COO), 2.21 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max}(MeOH)=560 nm, $\varepsilon = 83\,000$ M^{-1} cm^{-1}.

*Synthesis of 4,4-Difluoro-5-(4-methoxyphenyl)-1, 3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid Methyl Ester (**7b**)*. Compound **7b** was prepared according to the general procedure using 2-(4-methoxyphenyl)pyrrole (**1b**) and 2-formyl-3,5-dimethyl-4-(2-carboxyethyl)pyrrole (**2**) as starting materials. The product (**7b**) was obtained in 78% yield. ^{1}H NMR Bruker Avance DRX500 ($CDCl_3$, δ ppm): 7.87 (2H, m, 2*Ph-H), 7.09 (1H, s, meso-H), 6.97 (2H, m, 2*Ph-H), 6.96 (1H, d, $J = 3.8$ Hz, pyrrole-H), 6.54 (1H, d, $J = 4.0$ Hz, pyrrole-H), 3.86 (3H, s, OCH_3), 3.67 (3H, s, $COOCH_3$), 2.73 (2H, t, $J = 7.5$ Hz, CH_2CH_2COO), 2.53 (3H, s, pyrrole-CH_3), 2.45 (2H, t, $J = 8.0$ Hz, CH_2CH_2COO) and 2.22 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 544 nm, $\varepsilon =$ 66 000 M^{-1} cm^{-1}.

*Synthesis of 4,4-Difluoro-5-phenyl-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid Methyl Ester (**7c**)*. Compound **7c** was prepared according to the general procedure using 2-phenylpyrrole (**1c**) and 2-formyl-3,5-dimethyl-4-(2-carboxyethyl)pyrrole (**2**) as starting materials. The product (**7c**) was obtained in 65% yield. ^{1}H NMR Bruker Avance DRX500 ($CDCl_3$, δ ppm): 7.95 (2H, m, 2*Ph-H), 7.53 (2H, m, 2*Ph-H), 7.47 (1H, m, Ph-H), 7.20 (1H, s, meso-H), 7.04 (1H, d, $J = 4.3$ Hz, pyrrole-H), 6.62 (1H, d, $J = 4.3$ Hz, pyrrole-H), 3.75 (3H, s, $COOCH_3$), 2.81 (2H, t, $J = 7.5$ Hz, CH_2CH_2COO), 2.61 (3H, s, pyrrole-CH_3), 2.53 (2H, t, $J = 8.0$ Hz, CH_2CH_2COO) and 2.31 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 530 nm, $\varepsilon = 64000$ M^{-1} cm^{-1}.

*Synthesis of 4,4-Difluoro-5-((E)-2-phenylethen-1-yl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid Methyl Ester (**7d**)*. Compound **7d** was prepared according to the general procedure using 2-((E)-2-phenylethen-1-yl)pyrrole (**1d**) and 2-formyl-3,5-dimethyl-4-(2-carboxyethyl)pyrrole (**2**) as starting materials. The product (**7d**) was obtained in 52% yield. ^{1}H NMR Bruker Avance DRX500 ($CDCl_3$, δ ppm): 7.62 (1H, d, $J = 16.4$ Hz, ethenyl-H), 7.58 (2H, d, $J =$ 7.3 Hz, 2*Ph-H), 7.37 (2H, t, $J = 7.1$ Hz, 2*Ph-H), 7.30 (1H, d, $J = 7.3$ Hz, Ph-H), 7.24 (1H, d, $J =$ 16.0 Hz, ethenyl-H), 7.02 (1H, s, meso-H), 6.92 (1H, d, $J = 4.1$ Hz, pyrrole-H), 6.84 (1H, d, $J = 4.1$ Hz, pyrrole-H), 3.68 (3H, s, $COOCH_3$), 2.74 (2H, t, $J =$ 7.5 Hz, CH_2CH_2COO), 2.59 (3H, s, pyrrole-CH_3), 2.47 (2H, t, $J = 8.0$ Hz, CH_2CH_2COO) and 2.21 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 569 nm, $\varepsilon =$ 128000 M^{-1} cm^{-1}.

*Synthesis of 4,4-Difluoro-5-((E)-2-(4-methoxyphenyl)ethen-1-yl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid Methyl Ester (**7e**)*. Compound **7e** was prepared according to the general procedure using 2-((E)-2-(4-methoxyphenyl)ethen-1-yl)pyrrole (**1e**) and 2-formyl-3,5-dimethyl-4-(2-carboxyethyl)pyrrole (**2**) as starting materials. The product (**7e**) was obtained in 62% yield. ^{1}H NMR Bruker Avance DRX500 ($CDCl_3$, δ ppm): 7.54 (2H, d, $J = 8.8$ Hz, 2*Ph-H), 7.50 (1H, d, $J = 16.3$ Hz, ethenyl-H), 7.24 (1H, d, $J = 16.2$ Hz, ethenyl-H), 7.00 (1H, s, meso-H), 6.92 (1H, d, $J = 4.1$ Hz, pyrrole-H), 6.90 (2H, d, $J = 8.8$ Hz, 2*Ph-H), 6.82 (1H, d, $J =$ 4.1 Hz, pyrrole-H), 3.84 (3H, s, OCH_3), 3.68 (3H, s, $COOCH_3$), 2.74 (2H, t, $J = 7.5$ Hz, CH_2CH_2COO), 2.58 (3H, s, pyrrole-CH_3), 2.46 (2H, t, $J = 8.0$, CH_2CH_2COO) and 2.20 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 579 nm, $\varepsilon = 126000$ M^{-1} cm^{-1}.

*Synthesis of 4,4-Difluoro-5-(2-pyrrolyl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid Methyl Ester (**7f**)*. Compound **7f** was prepared according to the general procedure using 2,2'-bipyrrole (**1f**) and 2-formyl-3,5-dimethyl-4-(2-carboxyethyl)pyrrole (**2**) as starting materials. The product (**7f**) was obtained in 55% yield. ^{1}H NMR Bruker AM200 ($CDCl_3$, δ ppm): 10.34 (1H, s, pyrrole-NH), 7.08 (1H, m, pyrrole-H), 6.97 (1H, s, meso-H), 6.95 (1H, d, $J = 4.2$ Hz, pyrrole-H), 6.89 (1H, m, pyrrole-H), 6.78 (1H, d, $J = 4.2$ Hz, pyrrole-H), 6.34 (1H, m, pyrrole-H), 3.69 (3H, s, $COOCH_3$), 2.75 (2H, t, $J = 7.7$ Hz, CH_2CH_2COO), 2.56 (3H, s, pyrrole-CH_3), 2.46 (2H, t, $J = 7.6$ Hz, CH_2CH_2COO) and 2.20 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 585 nm, $\varepsilon =$ 85000 M^{-1} cm^{-1}.

*General Procedure for Preparation of Compounds **8a–f***

*Synthesis of 4,4-Difluoro-5-(2-thienyl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid (**8a**)*

a) Compound **7a** (107 mg, 0.27 mmol) was dissolved in THF:water (3:2, 50 mL). Phosphoric acid (85%, 3 mL) was added and the mixture was refluxed for 5 days. The reaction mixture was diluted with water (50 mL) and the product was extracted with dichloromethane (2 × 50 mL). The organic phase was washed with water (50 mL), dried with sodium sulphate and evaporated to dryness. The

crude product was purified with column chromatography on silica gel using dichloromethane:methanol (10:1) as eluent. Fractions containing the desired product were pooled and evaporated to dryness. The product was crystallised from dichloromethane:petroleum ether yielding 68 mg (66%) of compound **8a**.

b) Compound **7a** (112 mg, 0.29 mmol) was dissolved in THF:water (3:2, 50 mL). Hydrochloric acid (conc., 2 mL) was added and the mixture was refluxed for 6 hr. The product was isolated as described above yielding 77 mg (71%) of compound **8a**. ^{1}H NMR Bruker AM200 (DMSO-d_6, δ ppm): 7.88 (1H, d, J = 3.7 Hz, thienyl-H), 7.73 (1H, d, J = 4.9 Hz, thienyl-H), 7.69 (1H, s, meso-H), 7.19 (1H, dd, J = 3.8 Hz, thienyl-H), 7.11 (1H, d, J = 4.1 Hz, pyrrole-H), 6.80 (1H, d, J = 4.1 Hz, pyrrole-H), 2.63 (2H, t, J = 7.5 Hz, CH_2CH_2COO), 2.51 (3H, s, pyrrole-CH_3), 2.40 (2H, t, J = 7.3 Hz, CH_2CH_2COO), 2.22 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 560 nm, ε = 79000 M^{-1} cm^{-1}.

*Synthesis of 4,4-Difluoro-5-(4-methoxyphenyl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid (**8b**)*. Compound **8b** was synthesised according to the general procedure (procedure b). The product (**8b**) was obtained in 50% yield. ^{1}H NMR Bruker Avance DRX500 ($CDCl_3$, δ ppm): 7.88 (2H, d, J = 8.9 Hz, 2*Ph-H), 7.09 (1H, s, meso-H), 6.98 (2H, d, J = 9.1 Hz, 2*Ph-H), 6.96 (1H, d, J = 4.0 Hz, pyrrole-H), 6.54 (1H, d, J = 4.2 Hz, pyrrole-H), 3.86 (3H, s, OCH_3), 2.75 (2H, t, J = 8.0 Hz, CH_2CH_2COO), 2.54 (3H, s, pyrrole-CH_3), 2.51 (2H, t, J = 8.0 Hz, CH_2CH_2COO) and 2.20 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 543 nm, ε = 55 000 M^{-1} cm^{-1}.

*Synthesis of 4,4-Difluoro-5-phenyl-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid (**8c**)*. Compound **8c** was synthesised according to the general procedure (procedure b). The product (**8c**) was obtained in 56% yield. ^{1}H NMR Bruker Avance DRX500 ($CDCl_3$, δ ppm): 7.88 (2H, m, 2*Ph-H), 7.44 (2H, m, 2*Ph-H), 7.41 (1H, m, Ph-H), 7.13 (1H, s, meso-H), 6.97 (1H, d, J = 4.1 Hz, pyrrole-H), 6.55 (1H, d, J = 4.1 Hz, pyrrole-H), 2.74 (2H, t, J = 7.6 Hz, CH_2CH_2COO), 2.54 (3H, s, pyrrole-CH_3), 2.51 (2H, t, J = 7.9 Hz, CH_2CH_2COO) and 2.23 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 530 nm, ε = 54 000 M^{-1} cm^{-1}.

*Synthesis of 4,4-Difluoro-5-((E)-2-phenylethen-1-yl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid (**8d**)*. Compound **8d** was synthesised according to the general procedure (procedure b). The product (**8d**) was obtained in 52% yield. ^{1}H NMR Bruker Avance DRX500 ($CDCl_3$, δ ppm): 7.70 (1H, d, J = 16.2 Hz, ethenyl-H), 7.66 (2H, d, J = 7.8, 2*Ph-H), 7.44 (2H, t, J = 7.8 Hz, 2*Ph-H), 7.38 (1H, d, J = 6.4 Hz, Ph-H), 7.36 (1H, d, ethenyl-H, shielded by solvent peak), 7.10 (1H, s, meso-H), 7.00 (1H, d, J = 4.6 Hz, pyrrole-H), 6.92 (1H, d, J = 4.6 Hz, pyrrole-H), 2.83 (2H, t, J = 7.6 Hz, CH_2CH_2COO), 2.67 (3H, s, pyrrole-CH_3), 2.60 (2H, t, J = 7.6 Hz, CH_2CH_2COO) and 2.29 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 569 nm, ε = 102000 M^{-1} cm^{-1}.

*Synthesis of 4,4-Difluoro-5-((E)-2-(4-methoxyphenyl)ethen-1-yl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid (**8e**)*. Compound **8e** was synthesised according to the general procedure (procedure b). The product (**8e**) was obtained in 52% yield. ^{1}H NMR Bruker Avance DRX500 ($CDCl_3$, δ ppm): 7.54 (2H, d, J = 8.9 Hz, 2*Ph-H), 7.50 (1H, d, J = 16.2 Hz, ethenyl-H), 7.24 (1H, d, J = 16.5 Hz, ethenyl-H), 6.99 (1H, s, meso-H), 6.92 (1H, d, J = 4.5 Hz, pyrrole-H), 6.90 (2H, d, J = 8.9 Hz, 2*Ph-H), 6.82 (1H, d, J = 4.1 Hz, pyrrole-H), 3.84 (3H, s, OCH_3), 2.75 (2H, t, J = 7.9 Hz, CH_2CH_2COO), 2.59 (3H, s, pyrrole-CH_3), 2.52 (2H, t, J = 8.2 Hz, CH_2CH_2COO) and 2.21 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 579 nm, ε = 102000 M^{-1} cm^{-1}.

*Synthesis of 4,4-Difluoro-5-(2-pyrrolyl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid (**8f**)*. Compound **8f** was synthesised according to the general procedure (procedure b). The product (**8f**) was obtained in 55% yield. ^{1}H NMR Bruker Avance DRX500 (DMSO-d_6, δ ppm): 11.21 (1H, s, NH), 7.48 (1H, s, meso-H), 7.20 (1H, m, pyrrole-H), 7.18 (1H, d, J = 4.4 Hz, pyrrole-H), 7.14 (1H, m, pyrrole-H), 7.00 (1H, d, J = 4.4 Hz, pyrrole-H), 6.27 (1H, m, pyrrole-H), 2.63 (2H, t, J = 7.8 Hz, CH_2CH_2COO), 2.48 (3H, s, CH_3), 2.35 (2H, t, J = 7.8 Hz, CH_2CH_2COO) and 2.20 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 585 nm, ε = 75000 M^{-1} cm^{-1}.

*General Procedure for Preparation of Compounds **9a–f***

*Synthesis of 4,4-Difluoro-5-(2-thienyl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid Succinimidyl Ester (**9a**)*. Compound **8a** (57 mg, 0.15 mmol) was dissolved in anhydrous DMF (3 mL). *N*,*N*′-dicyclohexylcarbodiimide (47 mg, 0.23 mmol) and *N*-hydroxysuccinimide (52 mg, 0.45 mmol) were added and the mixture was stirred at room temperature for 20 hr. The urea precipitate was filtrated off and the filtrate was evaporated to dryness. The residue was purified with column chromatography on silica gel using dichloromethane:acetone:acetic acid (100:8:1, v:v:v) as eluent. Fractions containing the product were combined and evaporated to dryness. The product was further dried

in a vacuum desiccator over silica gel yielding 35 mg (75%) of compound **9a**. ^{1}H NMR Bruker AM200 ($CDCl_3$, δ ppm): 8.06 (1H, d, J = 3.5 Hz, thienyl-H), 7.41 (1H, d, J = 4.4 Hz, thienyl-H), 7.15 (1H, dd, J = 4.3 Hz, thienyl-H), 7.08 (1H, s, meso-H), 6.93 (1H, d, J = 3.8 Hz, pyrrole-H), 6.72 (1H, d, J = 3.7 Hz, pyrrole-H), 2.86 (4H, s, COCH_2CH_2CO), 2.74–2.82 (4H, 2t, J = 8.0 Hz, CH_2CH_2COO), 2.61 (3H, s, pyrrole-CH_3), 2.23 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 560 nm, ε = 82000 M^{-1} cm^{-1}.

Synthesis of 4,4-Difluoro-5-(4-methoxyphenyl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid Succinimidyl Ester (***9b****).* Compound **9b** was synthesised according to the general procedure in 88% yield. ^{1}H NMR Bruker Avance DRX500 ($CDCl_3$, δ ppm): 7.88 (2H, d, 2*Ph-H), 7.12 (1H, s, meso-H), 6.98-6.97 (3H, m, 2*PhH and pyrrole-H), 6.56 (1H, d, J = 4.1, pyrrole-H), 3.86 (3H, s, OCH_3), 2.86-2.83 (6H, m, COCH_2CH_2CO and CH_2CH_2COO), 2.73 (2H, m, CH_2CH_2COO), 2.55 (3H, s, pyrrole-CH_3) and 2.23 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 544 nm, ε = 58000 M^{-1} cm^{-1}.

Synthesis of 4,4-Difluoro-5-phenyl-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid Succinimidyl Ester (***9c****).* Compound **9c** was synthesised according to the general procedure in 92% yield. ^{1}H NMR Bruker Avance DRX500 ($CDCl_3$, δ ppm): 7.90-7.87 (2H, m, 2*Ph-H), 7.46-7.40 (3H, m, 3*Ph-H), 7.16 (1H, s, meso-H), 6.99 (1H, d, J = 4.2 Hz, pyrrole-H), 6.57 (1H, d, J = 4.0 Hz, pyrrole-H), 2.86-2.83 (6H, m, COCH_2CH_2CO and CH_2CH_2COO), 2.74 (2H, t, J = 7.8 Hz, CH_2CH_2COO), 2.55 (3H, s, pyrrole-CH_3) and 2.24 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 530 nm, ε = 64000 M^{-1} cm^{-1}.

Synthesis of 4,4-Difluoro-5-((E)-2-phenylethen-1-yl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid Succinimidyl Ester (***9d****).* Compound **9d** was synthesised according to the general procedure in 92% yield. ^{1}H NMR Bruker Avance DRX500 ($CDCl_3$, δ ppm): 7.71 (1H, d, J = 16.9, ethenyl-H), 7.67 (2H, d, J = 7.3 Hz, 2*Ph-H), 7.45 (2H, t, J = 7.3 Hz, 2*Ph-H), 7.38 (1H, d, J = 7.3 Hz, Ph-H), 7.35 (1H, d, J = 17.6 Hz, ethenyl-H), 7.12 (1H, s, meso-H), 7.03 (1H, d, J = 4.5 Hz, pyrrole-H), 6.94 (1H, d, J = 4.3 Hz, pyrrole-H), 2.93 (2H, t, J = 7.3 Hz, CH_2CH_2COO), 2.92 (4H, m, COCH_2CH_2CO), 2.83 (2H, t, J = 7.8 Hz, CH_2CH_2COO), 2.68 (3H, s, pyrrole-CH_3) and 2.30 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 568 nm, ε = 132000 M^{-1} cm^{-1}.

Synthesis of 4,4-Difluoro-5-((E)-2-(4-methoxyphenyl)ethen-1-yl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid Succinimidyl Ester (***9e****).* Compound **9e** was synthesised according to the general procedure in 81% yield. ^{1}H NMR Bruker Avance DRX500 ($CDCl_3$, δ ppm): 7.61 (2H, d, J = 8.8 Hz, 2*Ph-H), 7.58 (1H, d, J = 16.4 Hz, ethenyl-H), 7.32 (1H, d, ethenyl-H, shielded by solvent peak), 7.09 (1H, s, meso-H), 7.02 (1H, d, J = 4.7 Hz, pyrrole-H), 6.98 (2H, d, J = 8.8 Hz, 2*Ph-H), 6.91 (1H, d, J = 4.2 Hz, pyrrole-H), 3.92 (3H, s, OCH_3), 2.93 (6H, m, COCH_2CH_2CO and CH_2CH_2COO), 2.82 (2H, t, J = 8.1 Hz, CH_2CH_2COO), 2.67 (3H, s, pyrrole-CH_3) and 2.29 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 579 nm, ε = 122000 M^{-1} cm^{-1}.

Synthesis of 4,4-Difluoro-5-(2-pyrrolyl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic Acid Succinimidyl Ester (***9f****).* Compound **9f** was synthesised according to the general procedure in 72% yield. ^{1}H NMR Bruker Avance DRX500 (DMSO-d_6, δ ppm): 11.21 (1H, s, NH), 7.51 (1H, s, meso-H), 7.22 (1H, m, pyrrole-H), 7.19 (1H, d, J = 4.3 Hz, pyrrole-H), 7.15 (1H, m, pyrrole-H), 7.03 (1H, d, J = 4.3 Hz, pyrrole-H), 6.28 (1H, m, pyrrole-H), 2.86 (2H, t, J = 8.2 Hz, CH_2CH_2COO), 2.81 (4H, s, COCH_2CH_2CO), 2.77 (2H, t, J = 7.9 Hz, CH_2CH_2COO), 2.50 (3H, s, pyrrole-CH_3, shielded by solvent peak) and 2.22 (3H, s, pyrrole-CH_3). UV–Vis: λ_{max} (MeOH) = 585 nm, ε = 83000 M^{-1} cm^{-1}.

General Procedure for Coupling of Dipeptide Linker Unit (Compounds ***10a–f****)*

Compound ***10a****.* Compound **9a** (200 mg, 0.42 mmol) and dipeptide linker (**12**) (163 mg, 0.64 mmol) were dissolved in anhydrous DMF (10 mL). Anhydrous triethylamine (176 μL, 1.27 mmol) was added and the reaction mixture was stirred at room temperature for 1 hr. The reaction mixture was evaporated to dryness and the residue was dissolved in water. The solution was acidified with concentrated hydrochloric acid (pH < 3) and the product was extracted with phenol (3 × ∼30 mL). The organic extracts were combined and diethyl ether (300 mL) was added. The product was extracted from organic solution with water (5 × 30 mL). The aqueous fractions were combined and washed with diethyl ether (2 × 50 mL) and evaporated under reduced pressure. The product was precipitated from dichloromethane/carbon tetrachloride yielding 205 mg (68%) of compound **10a**.

MS (MALDI TOF, negative mode): Calculated 609.15 (M–1), Found 609.88 (M–1). UV–Vis: λ_{max} (H_2O, 0.1% TX-100) = 565 nm, ε = 69000 M^{-1} cm^{-1}. HPLC: t_R = 4.16 min.

Compound ***10b.*** Compound **10b** was synthesised according to the general procedure. MS (MALDI TOF, negative mode): Calculated 633.21 (M–1), Found 633.33

(M–1). UV–Vis: λ_{max} (H_2O, 0.1% TX-100) = 547 nm, $\varepsilon = 64000\ M^{-1}\ cm^{-1}$. HPLC: $t_R = 4.28$ min.

*Compound **10c***. Compound **10c** was synthesised according to the general procedure. MS (MALDI TOF, negative mode): Calculated 603.20 (M–1), Found 603.32 (M–1). UV–Vis: λ_{max} (H_2O, 0.1% TX-100) = 533 nm, $\varepsilon = 54000\ M^{-1}\ cm^{-1}$. HPLC: $t_R = 3.60$ min.

*Compound **10d***. Compound **10d** was synthesised according to the general procedure. MS (MALDI TOF, negative mode): Calculated 629.21 (M–1), Found 629.39 (M–1). UV–Vis: λ_{max} (H_2O, 0.1% TX-100) = 574 nm, $\varepsilon = 118000\ M^{-1}\ cm^{-1}$. HPLC: $t_R = 5.48$ min

*Compound **10e***. Compound **10e** was synthesised according to the general procedure. MS (MALDI TOF, negative mode): Calculated 659.22 (M–1), Found 659.21 (M–1). UV–Vis: λ_{max} (H_2O, 0.1% TX-100) = 584 nm, $\varepsilon = 124000\ M^{-1}\ cm^{-1}$. HPLC: $t_R = 5.63$ min.

*Compound **10f***. Compound **10f** was synthesised according to the general procedure. MS (MALDI TOF, negative mode): Calculated 592.19 (M–1), Found 592.60 (M–1). λ_{max} (H_2O, 0.1% TX-100) = 589 nm, $\varepsilon = 74000\ M^{-1}\ cm^{-1}$. HPLC: $t_R = 2.68$ min.

*General Procedure for Preparation of Hydrophilic Labeling Reagents **11a–f***

*Compound **11a***. Compound **11a** (100 mg, 0.14 mmol), *N*-hydroxysuccinimide (48 mg, 0.42 mmol) and DCC (87 mg, 0.42 mmol) were dissolved in anhydrous DMF (3 mL). The reaction mixture was stirred at room temperature for 24 hr. The urea precipitate was filtrated off and the filtrate was evaporated to dryness. The product was precipitated from dichloromethane/carbon tetrachloride and dried in vacuum desiccator over phosphorus pentoxide. Yield 95% (photometric determination). MS (MALDI TOF, negative mode): Calculated 706.17 (M–1), Found 706.00 (M–1). HPLC: $t_R = 6.75$ min (>95%).

*Compound **11b***. Compound **11b** was synthesised according to the general procedure. MS (MALDI TOF, negative mode): Calculated 730.22 (M–1), Found 730.44 (M–1). HPLC: $t_R = 7.14$ min (∼85%), minor peaks at 4.30 min (∼10%) and 7.54 min.

*Compound **11c***. Compound **11c** was synthesised according to the general procedure. MS (MALDI TOF, negative mode): Calculated 700.21 (M–1), Found 700.35 (M–1). HPLC: $t_R = 6.96$ min (>90%), minor peak at 7.44 min.

*Compound **11d***. Compound **11d** was synthesised according to the general procedure. MS (MALDI TOF, negative mode): Calculated 726.23 (M–1), Found 726.47 (M–1). HPLC: $t_R = 7.82$ min (>90%), minor peak at 5.50 min.

*Compound **11e***. Compound **11e** was synthesised according to the general procedure. MS (MALDI TOF, negative mode): Calculated 756.24 (M–1), Found 756.35 (M–1). HPLC: $t_R = 7.78$ min (>90%), minor peak at 5.70 min.

*Compound **11f***. Compound **11f** was synthesised according to the general procedure. MS (MALDI TOF, negative mode): Calculated 689.20 (M–1), Found 689.90 (M–1). HPLC: $t_R = 6.19$ min (>90%), minor peaks at 4.11 min and 6.88 min.

*Synthesis of Glutamic Acid-Taurine Linker (**12**)*

A glutamic acid derivative, BOC-Glu(OtBu)-OSu (500 mg, 1.25 mmol) was dissolved in anhydrous DMF (5 mL). Taurine (782 mg, 6.25 mmol) was dissolved in a solution of triethylamine (1.20 mL, 8.75 mmol) and water (10 mL). This solution was added to the solution of BOC-Glu(OtBu)-OSu and the reaction mixture was stirred at room temperature for 30 min. Ethanol (30 mL) was added and the taurine precipitate was filtrated off and the filtrate was evaporated to dryness. The residue was dissolved in trifluoroacetic acid (2 mL) and stirred at room temperature for 2 hr. The reaction mixture was evaporated to dryness and the residue was dissolved in dichloroethane:methanol (1:1, v:v). The product was precipitated as a white solid upon slow evaporation of methanol in a rotary evaporator. The glutamic acid-taurine linker (**12**) was obtained in 64% yield (204 mg). MS (FAB): Calculated 255 (M + 1), Found 255 (M + 1).

Photometry

A small amount of dipyrrylmethene-BF_2 dye (**7–10**) was weighed and dissolved in DMF. DMF stock solution was diluted by factor of 1000 either with methanol (compounds **7–10**) or with TX-100 (aq., 0.1%) (compounds **9a–f**). UV–Vis spectrum was recorded followed by calculation of molar extinction coefficient. Taking into account the possible errors in weighting and in pipetting the precision of the molar extinction coefficients are estimated to be ±5%.

Fluorescence Spectroscopy

The same DMF stock solutions that were used for photometry were used also for measurement of fluorescence emission spectra and for two-photon excitation fluorometry. The stock solutions were diluted with methanol or with TX-100 (aq., 0.1%) to concentration of 100 nM. The fluorescence emission spectra were recorded in wavelength range of 525–700 nm. The excitation wavelengths were 514 nm (compound **6c** and **9c**) and 531 nm (compounds **6a,b,d–f** and **9a,b,d–f**).The fluorescence spectrum of each dipyrrylmethene-BF_2 dye was integrated and the integral was divided by molar absorptivity (at excitation

wavelength). The resulting values were compared to a value obtained for the reference compound (Rhodamine B in EtOH [29]), and used for calculation of fluorescence quantum yields. Two-photon excited fluorescence efficiency was measured for compounds **9a–f** in the wells of 384-well plate (μClear SV-plate, code: 788096, lot: 01490126, Greiner BIO-ONE GmbH). The resulting TPE values were compared to the value obtained for a reference compound (RPE) [28], and used for calculation of two-photon excitation cross-sections.

Labeling of Immunoglobulin G

To the solution of monoclonal mouse IgG (200 μg, 1.25 nmol) in 56 μL phosphate buffered saline (Na_2HPO_4 10mM, NaCl 150 mM, pH 7.4) 10 fold molar excess of labeling reagent **8a** or **10a** in anhydrous DMF was added. 5.6 μL of $NaHCO_3$ (1 M, aq.) was added and the mixture was incubated at room temperature for 3 hr. The product was purified with NAP-5 gelfiltration column using phosphate buffered saline (50 mM/150 mM, 10 mM NaN_3, pH 7.4) as eluent. The fast moving red fraction was collected. The labeling degrees of the IgG conjugates were determined photometrically using the following ε-values: 82000 cm^{-1} M^{-1} (**9a**) and 69000 $cm^{-1}M^{-1}$ (**11a**) at 560 nm and 210000 $cm^{-1}M^{-1}$ at 280 nm for IgG.

CONCLUSIONS

In this paper we have described a series of novel dipyrrylmethene-BF_2 labeling reagents with extended π-electron conjugation pathway and with enhanced hydrophilicity and solubility to aqueous solutions. The enhanced solubility was achieved by introduction of a hydrophilic dipeptide linker between the chromophore and the reactive succinimidyl ester group. The new labels exhibit high fluorescence quantum yields in aqueous solutions and molar absorption coefficients between 54000 and 124000 $cm^{-1}M^{-1}$. The effect of hydrophilicity of the label on the biomolecule labeling was tested by labeling of monoclonal mouse IgG. Compared to labels without the linker unit, the labeling reagent with the hydrophilic linker provided higher substitution degree and better stability of the labeled protein. With hydrophilic label the IgG conjugate exhibited excellent stability, whereas the hydrophobic label resulted in almost complete precipitation of the labeled protein. Two-photon excited fluorescence was measured with a two-photon excitation microfluorometer equipped with a 1064 nm Nd:YAG microchip laser. Large two-photon excitation cross-section, good solubility to aqueous solutions and high fluorescence quantum efficiency, suggests that the novel dipyrrylmethine-BF_2 labels are highly applicable as fluorescent reporters in bioaffinity assays, particularly in assays which are based on two-photon excited fluorescence.

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