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DOTAGA–Anhydride: A Valuable Building Block for the Preparation of DOTA-Like Chelating Agents

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Abstract: A DOTA derivative that contains an anhydride group was readily synthesized by reacting DOTAGA with acetic anhydride and its reactivity was investigated. Opening the anhydride with propylamine led to the selective formation of one of two possible regioisomers. The structure of the obtained isomer was unambiguously determined by 1D and 2D NMR experiments, including COSY, HMBC, and NOESY techniques. This bifunctional chelating agent offers a convenient and attractive approach for labeling biomolecules and, more generally, for the synthesis of a large range of DOTA derivatives. The scope of the reaction was extended to prepare DOTA-like compounds that contained various functional groups, such as isothiocyanate, thiol, ester, and amino acid moieties. This versatile building block was also used for the synthesis of a bimodal tag for SPECT or PET/optical imaging.

Keywords: anhydrides · chelates · DOTA · imaging agents · protecting groups

Introduction

The use of so-called bifunctional chelating agents (BFCA) has been the most-reliable and most-commonly used method for attaching a metal ion (radiometal or paramagnetic metal) onto a biomolecule for molecular-imaging applications (PET, SPECT, MRI). Such an approach has been used for the development of metal-based imaging agents, which are essential for diagnostic and therapeutic applications.[1] DOTA (1,4,7,10-tetrakis(carboxymethyl)-1,4,7,10-tetraazacyclododecane) and its derivatives represent a major class of chelating agents for biomedical applications, owing to their excellent complexation properties towards many trivalent metal ions, and the high kinetic stability of the metal complexes thus formed.[1c,2]

In most cases, the attachment of a DOTA-fragment onto the biomolecule has involved one of the coordinating acetate pendant arms, thereby leading to DOTA–monoamide units (DOTA-MA).[3] However, the transformation of one of the carboxylic acid group into a carboxamide can decrease the stability of the complex by two or three orders of magnitude.[3d] To overcome this problem, DOTA-like BFCA, which contained four acetate side-arms, was synthesized through the preparation of C-functionalized DOTA macrocycles[4] or N-functionalized derivatives that contained an additional pendant arm.[1c] The DOTAGA derivative (GA = glutaric acid) was first synthesized by Maecke and co-workers[5] by the reaction of tris-*t*Bu-DOTA with a bromoglutaric diester derivative. Various DOTAGA bioconjugates were prepared and labeled with different radiometals (^{111}In, ^{68}Ga, ^{90}Y).[6] In most cases, the conjugation of DOTAGA onto a biomolecule was performed by using DOTAGA derivatives in which the four coordinating acetate arms were protected by *tert*-butyl ester groups.[5,7] The remaining carboxylic acid group on the glutaric acid moiety reacted with lysines in the presence of coupling reagents (i.e., HBTU, EDCI, etc.). However, the deprotection conditions that were used to cleave the ester groups were not suitable for fragile biomolecules, such as antibodies. To avoid this deprotection step, DOTAGA derivatives that contained four free acidic groups and an activated group, which was able to react specifically with an amino group, was present in the biomolecule function (i.e., a pentafluorophenyl ester or succinimidyl ester group) were developed.[8] The synthesis of such compounds was not straightforward and required protection/deprotection sequences and tedious chromatographic work-up. Gd-DOTAGA-conjugates were prepared by coupling the monohydrated Gd complex of DOTAGA onto a macromolecule with no need for a protection step because the four pendant arms of DOTA were involved in the coordination of the metal ion.[9] However, this

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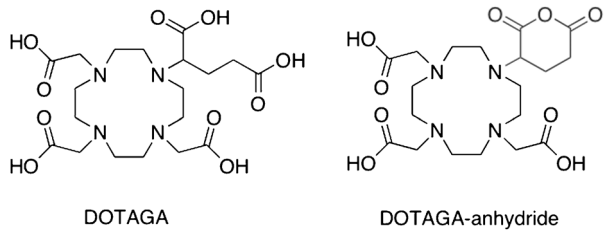
smart "metal-protection" approach could not be applied to radiolabeling because the complexation of the radiometal must be performed in the last step.

An alternative strategy that has not been explored so far is to combine the high reactivity and facile preparation of ligands that contain an anhydride group with the good chelation properties of DOTA and thus to prepare a new DOTA-like derivative, namely DOTAGA–anhydride (Scheme 1).



Scheme 1. DOTAGA and DOTAGA–anhydride.

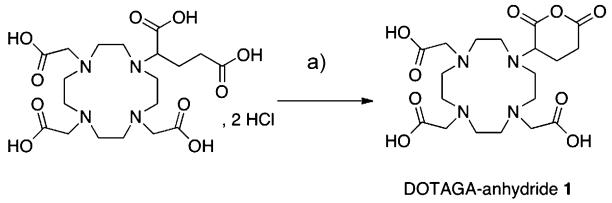
Indeed, this approach was very efficient in the case of a DTPA chelator. DTPA–bisanhydride has been successfully used for labeling antibodies (e.g., trastuzumab),[10] antibiotics,[11] enzymes,[12] and nanoparticles for MRI and therapy.[13]

We recently reported the preparation of ultrasmall nanoparticles that were decorated with DOTAGA units; the DOTAGA–anhydride precursor enabled facile decoration and their application for multi-imaging experiments.[14] ^{111}In-labeled DOTAGA–antibodies conjugates were also prepared in our group, which showed the usefulness of DOTAGA–anhydride for the efficient attachment of radiometals onto humanized mAb. Highly specific tumor targeting was observed in model human of breast tumors with overexpressed HER2/neu antigen.[15]

Herein, we report a comprehensive study on the reactivity of this new ligand, in particular concerning the selectivity of opening the anhydride with nucleophiles. Opening the anhydride group with propylamine was performed, which highlighted the formation of a single isomer. With the aim of extending the method and demonstrating its potential use, various nucleophiles were reacted with DOTAGA-anhydride. Finally, new SPECT or PET/optical imaging bimodal precursors were prepared by reacting DOTAGA–anhydride with a bodipy unit.

Results and Discussion

Formation of DOTAGA–anhydride 1: DOTAGA–anhydride **1** was synthesized in a one-step reaction starting from the DOTAGA macrocycle according to a literature procedure (Scheme 2).[9] The strategy was similar to that reported for the synthesis of DTPA–bisanhydride: the ammonium chloride salt of DOTAGA·2 HCl was reacted with acetic anhydride in the presence of pyridine at 65 °C to generate a cyclic anhydride. The desired product was isolated by filtration in 96 % yield. The resulting anhydride was quite stable and could be stored at 4 °C for several months without degradation. The formation of the anhydride was confirmed by MS (ESI; see the Supporting Information, Figure S3).



Scheme 2. Synthesis of DOTAGA–anhydride (**1**): a) acetic anhydride, pyridine, 65 °C, 18 h, 96 % yield.

Owing to its poor solubility in deuterated organic solvents, the ^{1}H NMR spectrum of compound **1** was recorded in deuterium oxide (Figure 1 a). The signal at $\delta = 4.45$ ppm was unambiguously assigned to the CH group of the anhydride group by 2D COSY analysis. Because of the fairly rapid hydrolysis of the anhydride moiety in D_2O, a mixture of DOTAGA and DOTAGA–anhydride was systematically obtained (65:45 ratio after 2 min at 293 K), thus confirming the high reactivity of the anhydride compound (see the Supporting Information, Figures S1 and S2). Hydrolysis of the anhydride could be followed by the disappearance of the CH signal at $\delta = 4.45$ ppm (Figure 1 b) and by the increase of a new peak at $\delta = 3.75$ ppm, which corresponded to the CH group in the hydrolyzed fragment.

Study of the regioselectivity of the anhydride-opening reaction: Even though we had previously demonstrated the high reactivity of the anhydride group towards nucleophilic

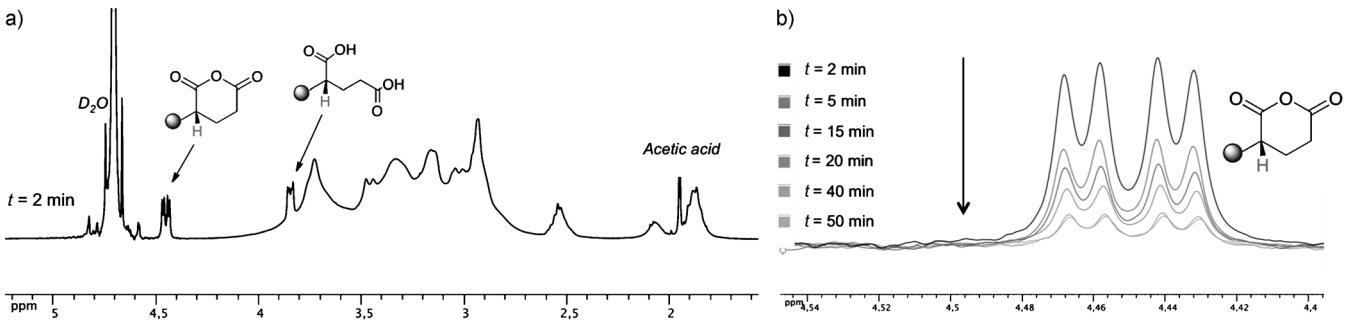
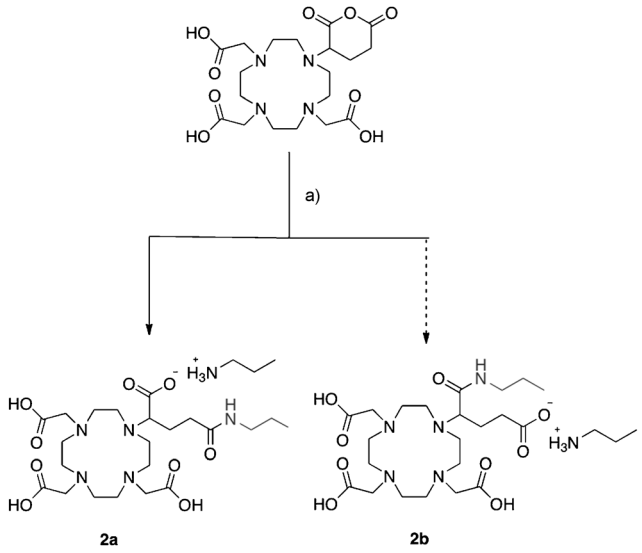


Figure 1. ^{1}H NMR spectra of compound **1** (293 K, 500 MHz, D_2O): a) after 2 min; b) change in the C*H* signal as a function of time.

agents, the selectivity of the reaction had not been elucidated. Indeed, because the anhydride group in compound **1** was asymmetrical, ring opening by a nucleophile could lead to two different isomers. To study this reaction in detail, we treated DOTAGA–anhydride with propylamine. As expected, HPLC analysis confirmed the formation of the amide in 98% yield (see the Supporting Information, Figure S4), but this result did not support the conclusion about the presence of isomers **2a**, **2b**, or both (Scheme 3).

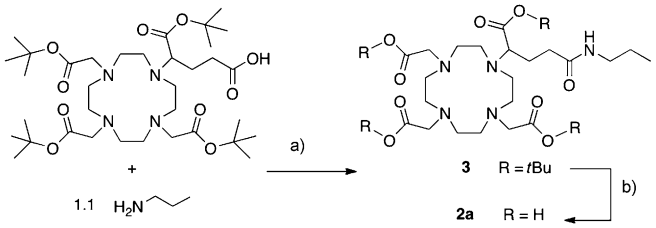


Scheme 3. Reaction of DOTAGA–anhydride (**1**) with 2 equiv of propylamine: a) propylamine (2 equiv), DMF, 50 °C, 2 h, 98% yield.

NMR experiments were carried out to determine the number of isomers that were formed during the reaction. ^{1}H NMR and ^{1}H–^{1}H COSY spectra were recorded in D_2O at different pD values (Figure 2; also see the Supporting Information, Figure S5).

Whatever the pD value, we were able to recognize two sets of signals that were attributed to the hydrogen atoms of two propyl fragments: the first set was assigned to the propylamide formed and the second set corresponded to the propyl ammonium counterion. All of the NMR spectra showed a complex signal in the region above $\delta = 2.8$ ppm, which corresponded to the diastereotopic hydrogen atoms of the methylene groups on the macrocyclic backbone, as well as the CH_2 protons on the three carboxymethyl groups and the CH proton ($CHCH_2CH_2CO$). The chemical shifts were strongly dependent on the pD value, owing to the presence of many protonation sites on the macrocycle (tertiary amine functions and carboxylic acid groups). From ^{1}H–^{1}H COSY experiment that were performed at pD = 8.5, the signals at $\delta = 1.75$–1.85 ppm and $\delta = 2.15$–2.20 ppm were attributed to protons Hb/Hb′ and Ha/Ha′, respectively. The cross-peak on the COSY spectrum, which corresponded to the correlation between the multiplet that was assigned to the Hb/Hb′ protons and the CH proton, was particularly dependent on the pD value (Figure 2). However, only one cross-peak was observed whatever the pD value. This result confirmed that only one product had formed during the reaction. Indeed, even if the isochrony of the CH protons of two isomers could accidentally occur for one pD value, it could not be the case at four different pD values.

At this stage, the regioselectivity of the reaction had been demonstrated but it remained unclear which isomer (**2a** or **2b**) had formed. Thus, RM1 semi-empirical molecular-orbital modeling of DOTAGA–anhydride **1** was performed (Figure 3). The model showed a higher accessibility of carbon atom C1 than C2, which suggested that the sole product of the reaction was isomer **2a**, which arose from exclusive nucleophilic attack on the carbonyl C1 atom. DFT energies of final products **2a** and **2b** were also calculated (see the Supporting Information). No significant difference was noted and the steric hindrance around the C2 atom was probably the main reason for the selective formation of isomer **2a**. To gain further insight into the regioselectivity of the reaction, we unequivocally prepared isomer **2a** from DOTAGA(tBu)$_4$ macrocycle according to a modified literature procedure.[5] A coupling reaction with propylamine in the presence of HBTU and HOBt, followed by treatment with concentrated hydrochloric acid, afforded the ammonium chloride salt of the desired deprotected product in 63% yield (Scheme 4). The ^{1}H NMR spectra of this compound and isomer **2a** were superimposable, which confirmed the assignment of the isomer that had formed during the reaction (see the Supporting Information, Figure S11).



Scheme 4. Synthesis of isomer **2a** from DOTAGA(tBu)$_4$: a) propylamine (1.1 equiv), HOBt, HBTU, EDCI, CH_2Cl_2, room temperature, 12 h, 64% yield; b) HCl, room temperature, 30 min, 98% yield. HOBt = 1-hydroxybenzotriazole, HBTU = *O*-(benzotriazol-1-yl)-tetramethyluronium hexafluorophosphate, EDCI = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide monochloride.

To confirm these results, we carried out NOESY and HMBC experiments on compound **2a**, which was obtained by opening compound **1** with propylamine. These NMR spectra were recorded after elution on an ion-exchange resin to remove the propylammonium cation (see the Supporting Information, Figures S7 and S8). [D_6]DMSO could be used as a solvent for NMR analysis in this case. The ^{13}C NMR high-frequency region of the HMBC spectrum is shown in Figure 4a. The signal that corresponded to carbonyl atom C1 was identified from the cross-peak with the NH proton. Atom C1 also showed correlations with Ha/Ha′ and Hc/Hc′, thereby corresponding to coupling constants ^2J(Ha/a′,C1) and ^3J(Hc/c′,C1). Importantly, no cross-peak was ob-

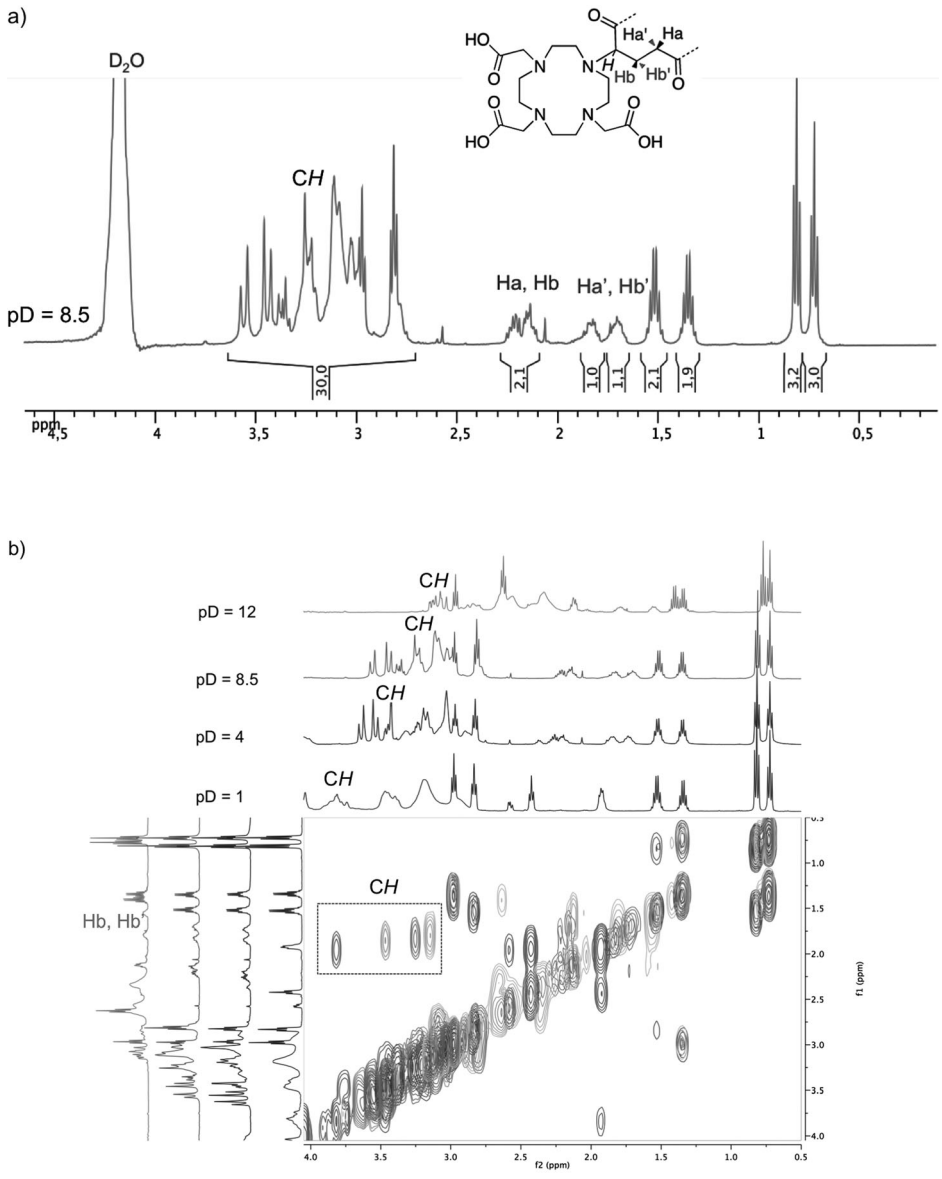


Figure 2. a) ^{1}H NMR spectrum of compound **2** (340 K, 500 MHz, D_2O, pD = 8.5); b) ^{1}H–^{1}H *g*-COSY 2D correlations of compound **2** at pD = 1, 4.2, 8.5, and 12 (340 K, 500 MHz, D_2O). The cross-peaks that corresponded to the correlation between the CH and CH_2 peaks are indicated by black dashed lines. The solvent peak was omitted for clarity (for a color version, see the Supporting Information, Figure S6).

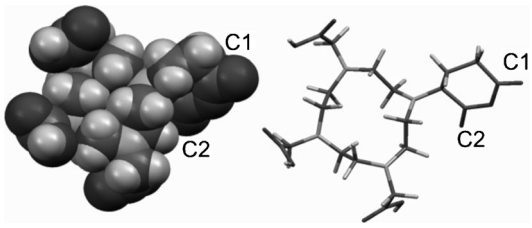


Figure 3. RM1 semi-empirical molecular-orbital model of DOTAGA–anhydride **1** (CPK).

served between the carbonyl atom of the amide moiety and the CH proton, unlike what might have been expected for isomer **2b**. To further confirm this result, a NOESY spectrum was recorded under the same conditions. Figure 4b shows the region of cross-peaks that corresponded to correlations that involved proton NH. Through-space interactions were identified between the NH proton and Ha/Ha′, Hc/Hc′, and Hd/Hd′.

Taken together, these studies unambiguously demonstrated that compound **2a** was obtained as the sole product in the reaction of compound **1** with propylamine.

Synthesis of the DOTAGA building blocks: We investigated the use of DOTAGA–anhydride for the preparation of new building blocks. The regioselectivity of the anhydride-opening reaction allowed the selective introduction of various functional groups that were suitable for bioconjugation.

First, we focused on the opening reaction of the anhydride with commercially available amines. Owing to the poor solubility of DOTAGA–anhydride in organic solvent, the reaction was performed at high temperature. For instance, compound **1** was reacted with 4-nitrobenzylamine hydrochloride in DMF at 65 °C in the presence of three equivalents of triethylamine (Scheme 5a). The crude product, which was about 85% pure by NMR spectroscopy (integration of the aromatic protons), was purified by column chromatography on a C_{18} reverse-phase column to remove the starting material, thereby affording compound **4** in 72% yield (96% purity, as measured by HPLC analysis).

To design DOTA precursors for attachment to a cysteine group, which is an attractive target for labeling peptides or antibodies,[16] and to gold nanoparticles,[17] we introduced a thiol group onto the DOTAGA backbone (Scheme 5b). We used the same conditions as those described for the synthesis of compound **4** to prepare compound **5** in 45% yield. This low conversion was presumably due to the formation of dimers (through a disulfide bridge).

We also considered the opening of the anhydride with alcohols, such as EtOH, to afford their corresponding esters (Scheme 5c). Owing to the lower reactivity of alcohols than

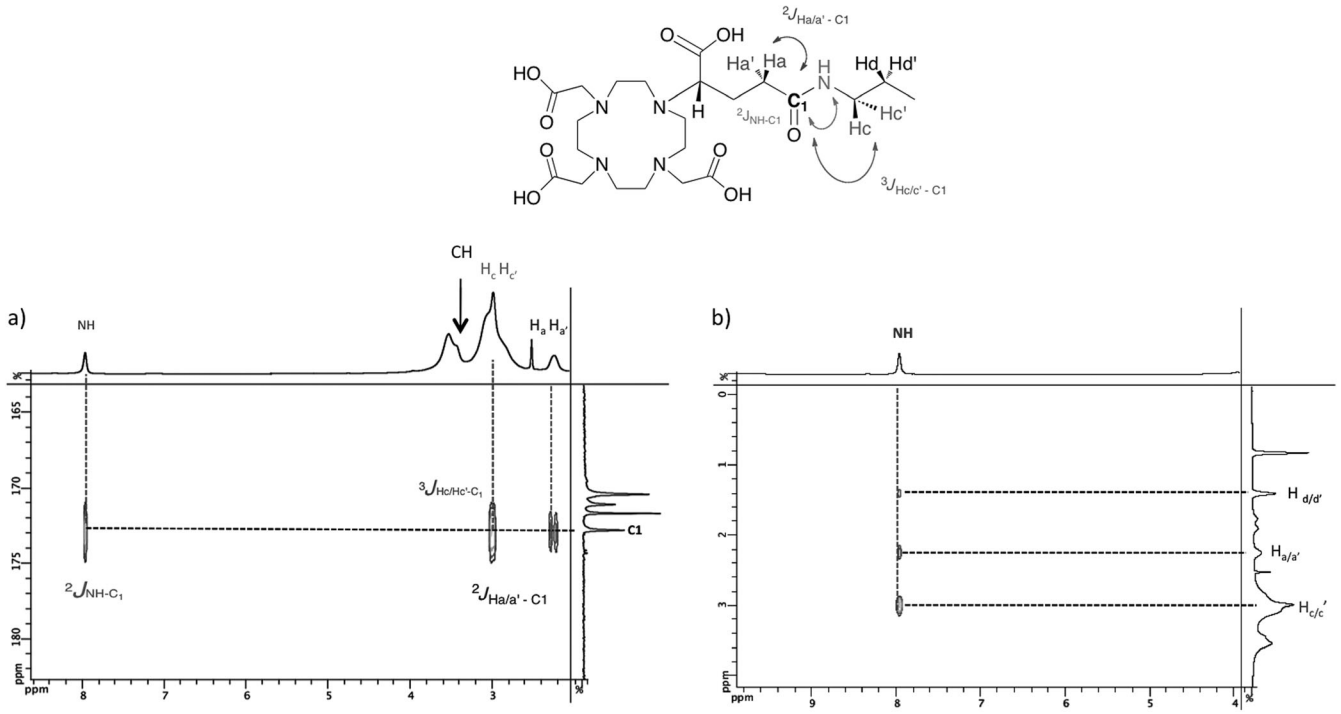
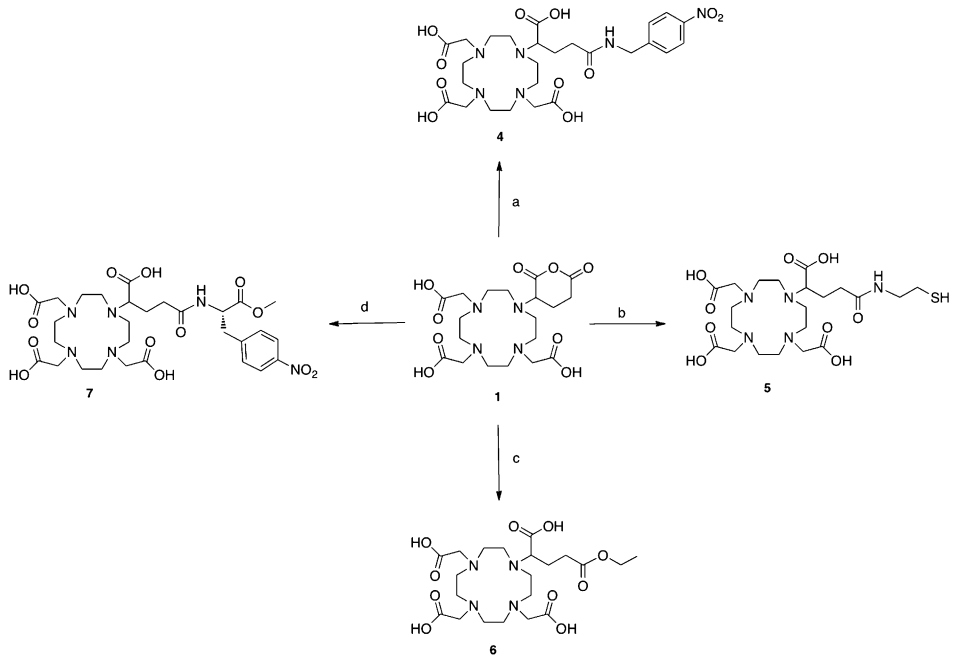


Figure 4. a) 2D-HMBC spectrum of compound **2** (300 K, 500 MHz, [D_6]DMSO). Only the region of cross-peaks that corresponded to the correlation between the C1 and H atoms (labeled) is represented for clarity. Correlations are indicated by dashed lines. b) 2D-NOESY spectrum (300 K, 500 MHz, [D_6]DMSO).

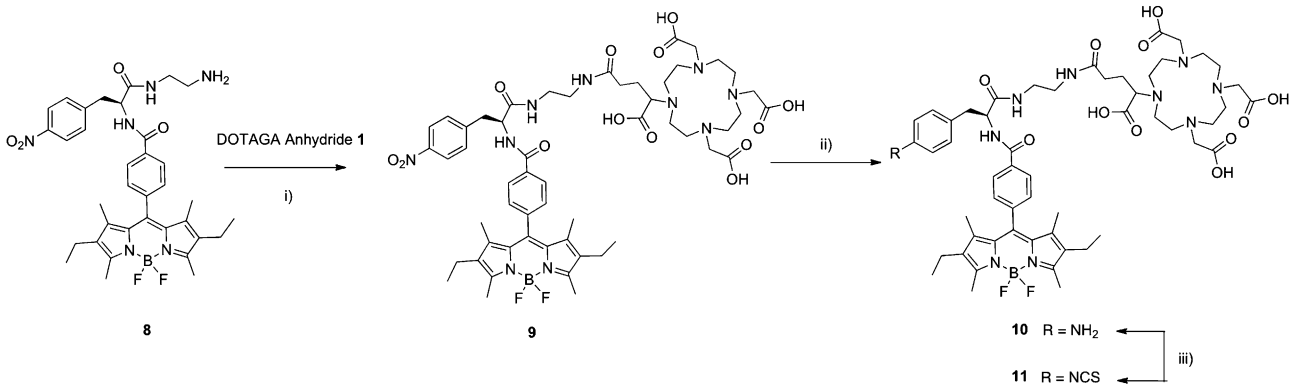


Scheme 5. Reaction of compound **1** with various nucleophiles: a) 4-nitrobenzylamine hydrochloride (1 equiv), NEt_3 (3 equiv), DMF, 70 °C, 2 h, 72 % yield; b) 2-aminoethanethiol hydrochloride (1 equiv), NEt_3 (3 equiv), DMF, 70 °C, 4 h, 45 % yield; c) EtOH, NEt_3 (3 equiv), DMF, 45 °C, 12 h, 35 % yield; d) nitrophenylalanine monoester hydrochloride (1 equiv), NEt_3 (3 equiv), DMF, 70 °C, 12 h, 68 % yield.

primary amines, a large excess of EtOH was used. The reaction was performed in refluxing DMF, and purification by column chromatography on a C_{18} reverse-phase column gave the desired compound (**6**) in 35 % yield.

This reaction was also extended to the coupling of an amino acid onto the DOTAGA unit (Scheme 5 d). For this purpose, a protected DOTAGA–nitrophenylalanine derivative was prepared by reacting 1 equiv of the appropriate nucleophile with the DOTAGA–anhydride. After purification by column chromatography on a C_{18} reverse-phase column, the desired compound (**7**) was obtained in 68 % yield and characterized by LCMS (see the Supporting Information, Figure S19). The presence of two chiral centers led to the formation of a mixture of diastereoisomers. This valuable building block could then be inserted at any position on a peptide or PNA sequence.

SPECT- and PET/optical-imaging bimodal precursors: Finally, this convenient method was used for the preparation of a new dual-mode probe that was capable of simultaneous detection by optical imaging and PET or SPECT scintigraphy.[18] There are no reports of bi-



Scheme 6. Synthesis of DOTAGA–bodipy, starting from bodipy system **8**:[19] a) DOTAGA–anhydride (**1**), NEt_3 (3 equiv), DMF, 70 °C, 12 h, 35 % yield; b) H_2, Pd/C, H_2O/EtOH (96:4), room temperature, 12 h, 61 % yield; b) thiophosgene (3 equiv), CH_2Cl_2/H_2O (1:1), room temperature, 1 h, 86 % yield.

modal agents that contain DOTAGA units in the literature, presumably owing to the difficulty in synthesizing this type of compounds. Recently, we reported the synthesis of second-generation water-soluble DOTA–bodipy derivatives,[19] by coupling DOTA–NHS with a bodipy moiety, to afford a fluorescent probe that exhibited strong fluorescent properties and high stability.[20] The advantage of our approach was in the possibility of introducing any kind of macrocyclic chelating agents in the last step. Similar systems that contain DOTAGA units could be easily prepared by opening the anhydride group of compound **1** (Scheme 6). The free primary amine group in bodipy **8**[19] was reacted with anhydride **1** at 70 °C in the presence of triethylamine to afford the desired bimodal system (**9**) in one step. The "ready to be grafted" final product (**11**) was obtained by a classical procedure, that is, the reduction of the nitro group into an aniline moiety, followed by transformation into an isothiocyanate group.[21] As expected, the presence of the four carboxylic pendant arms on the DOTAGA moiety led to water-soluble systems, without the need to add any hydrophilic groups onto the bodipy core.[22] Preliminary photophysical measurements were performed for water-soluble bimodal systems **9** and **10**. Absorption data of compound **11** were not recorded owing to the low stability of the activated NCS moiety in aqueous solution. Absorption spectra were recorded in MeOH, water, and under simulated physiological conditions (phosphate buffer solution (PBS), pH 7.4; see Figure 5 and the Supporting Information, Figure S23). All of the systems considered exhibited the typical absorption features of a bodipy system,[23] that is, a strong absorption transition in the range 520–530 nm, which was attributed to the S0–S1 transition of the bodipy unit, and a smaller transition in the range 340–420 nm, which corresponded to the S0–S2 transition. The molar absorption coefficients in PBS were in the range 15000–30000 $M^{-1}cm^{-1}$, which were lower than those in organic solvent, but were in good agreement with those reported previously for water-soluble bodipy units.[19,22k]

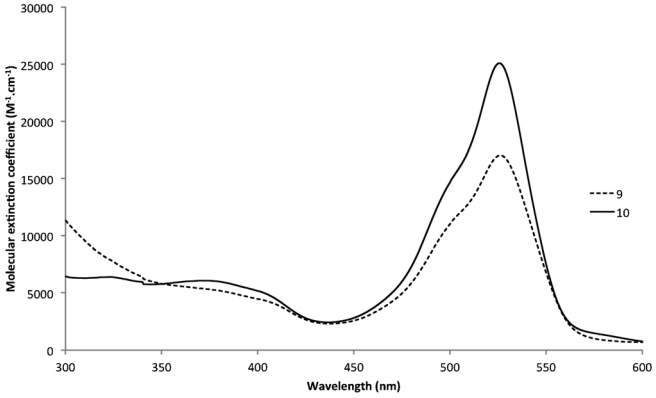


Figure 5. Absorption spectra of compounds **9** and **10** in PBS 0.1 M, pH 7.4, at 25 °C.

Conclusion

Bifunctional chelator DOTAGA–anhydride was readily prepared thanks to a key cyclic-anhydride formation. The high reactivity of the anhydride group and the regioselectivity of the anhydride-opening reaction, as demonstrated by various NMR experiments, made this compound a highly valuable precursor for labeling biomolecules. Indeed, the reaction with amines or other nucleophilic agents provided easy access to bifunctional chelating agents by the introduction of various functional groups that were suitable for bioconjugation, such as isothiocyanate or thiol groups. This approach did not require the protection of the three acetate groups. Moreover, when compared to other activated acid derivatives, such as NHS or phenolic esters, the formation of the amide bond between the anhydride and the amine groups generated no side-product that needed to be separated from the conjugate. The extension of the method for the preparation of chelators that contain other functional groups, such as primary amines, through the reactions of DOTAGA–anhydride with ethylendiamine or maleimide derivatives that are suitable for attachment to cysteine, is underway. The incorporation of an additional fluorescent moiety gave rise to

a promising new class of SPECT/optical bimodal imaging agents. The conjugation of such bimodal agent onto peptides or antibodies through thiourea bond formation is also currently under investigation.

Experimental Section

All of the chemicals that were used to prepare the title compounds, including propylamine, 4-hydroxybenzotriazole, and *N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate, were purchased from Acros and Aldrich and were used without further purification. 5-(*tert*-Butoxy)-5-oxo-4-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl) pentanoic acid (DOTAGA(tBu)$_4$), DOTAGA, and DOTA-NHS were obtained from CheMatech® and used without further purification. Bodipy precursor **8** was prepared according to a literature procedure.[19] Organic solvents were removed under reduced pressure on a rotary evaporator. Water was removed by lyophilization.

Synthesis of ligand 1: Acetic anhydride (20 mL) was added to a suspension of DOTAGA·2HCl (10 g, 18.5 mmol) in pyridine (8.5 mL, 0.1 mol, 6 equiv) under a nitrogen atmosphere. The mixture was heated at 65 °C for 18 h, then stirred at room temperature for 30 min. The suspension was filtered, washed with acetic anhydride (30 mL), MeCN (50 mL), and Et_2O (80 mL). The precipitate was dried under vacuum to afford compound **1** as a gray solid (9.5 g, 96 %). Compound **1** was used without further purification. M.p. 108(±2) °C; HRMS (ESI): m/z: calcd for $C_{19}H_{30}N_4O_9$+Na^+: 481.19050; found: 481.18922.

Synthesis of compound 2a (route A): Propylamine (46.5 μL, 0.56 mmol, 2 equiv) were added to a suspension of compound **1** (150 mg, 0.28 mmol) in DMF (2 mL). The mixture was stirred at 50 °C for 2 h. The solvent was evaporated to dryness to give compound **2a** (+$CH_3CH_2CH_2NH_3^+$) as a yellow solid (160 mg, 98 %). ^{1}H NMR (500 MHz, D_2O, 340 K, pD = 4.2): δ = 0.72 (t, ^3J(H,H) = 7.4 Hz, 3H), 0.81 (t, ^3J(H,H) = 7.4 Hz, 3H), 1.35 (qt, ^3J(H,H) = 7.4 Hz, ^3J(H,H) = 7.1 Hz, 2H), 1.52 (qt, ^3J(H,H) = 7.4 Hz, ^3J(H,H) = 7.1 Hz, 2H), 1.68–1.78 (m, 1H), 1.79–1.90 (m, 1H), 2.15–2.23 (m, 1H), 2.24–2.31 (m, 1H), 2.82 (t, ^3J(H,H) = 7.1 Hz, 2H), 2.97 (t, ^3J(H,H) = 7.1 Hz, 2H), 2.76–2.37 (m, 17H), 3.40–3.67 ppm (m, 6H); MS (ESI): m/z: 518.28 [M+H]$^+$, 540.26 [M+Na]$^+$; HPLC (A: CH_3CN, B: H_2O+0.4 % HCOOH; B: 98→0 %): t_R = 6.3 min.

Compound **2a** was deprotonated on an amberlite ion-exchange resin (eluent: CH_3COOH/water, 5 %). ^{1}H NMR (500 MHz, DMSO, 300 K): δ = 0.81 (t, ^3J(H,H) = 7.4 Hz, 3H), 1.39 (qt, ^3J(H,H) = 7.4 Hz, ^3J(H,H) = 7.1 Hz, 2H), 1.69–1.80 (m, 1H), 1.82–1.94 (m, 1H), 2.15–2.30 (m, 2H), 2.71–3.19 (m, 17H), 3.31–3.59 (m, 8H), 7.95 (brs, 1H; NH), 9.16–13.40 ppm (m, 4H; COOH); ^{1}H NMR (500 MHz, D_2O, 300 K, pD = 3): δ = 0.88 (t, ^3J = 7.4 Hz, 3H), 1.51 (qt, ^3J = 7.4 Hz, ^3J = 7.1 Hz, 2H), 1.87–2.08 (m, 2H), 2.39–2.59 (m, 2H), 2.93–4.02 ppm (m, 25H); ^{13}C{^{1}H} NMR (125 MHz, DMSO, 300 K): δ = 11.4 (CH_3), 22.4, 32.4, 40.4, 46.8, 49.4, 50.8, 51.1, 54.7, 51.1 (CH_2), 62.5 (CH), 170.4, 171.1, 171.6, 172.8 ppm (C=O); elemental analysis calcd (%) for $C_{22}H_{39}N_5O_9$·3.1 H_2O: C 46.08, H 7.95, N 11.74; found: C 46.62, H 8.26, N 12.21.

General procedure for the opening reaction of compound 1 with a primary amine: Ligands **4**, **5**, and **7** were prepared by mixing a solution of DOTAGA–anhydride **1** in DMF at 70 °C with a solution of the amine salt (1 equiv) in DMF at 70 °C in the presence of triethylamine (3 equiv). The mixture was heated for a minimum of 2 h until the two starting compounds had completely solubilized. After the evaporation of DMF, the crude solid was purified by reverse phase column chromatography on C_{18}. After evaporation of MeCN, the aqueous solution was lyophilized to afford a white solid in 35–72 % yield.

Compound 4: M.p. 165(±2) °C; ^{1}H NMR (300 MHz, DMSO, 300 K): δ = 1.78 (m, 1H), 1.91 (m, 1H), 2.27 (m, 1H), 2.51 (m, 1H), 2.56–3.16 (m, 15H), 3.24–3.62 (m, 8H), 4.32 (dd, ^3J(H,H) = 6.2 Hz, ^2J(H,H) = 16.0 Hz, AB system, 1H; CH_2ArNO_2), 4.41 (dd, ^3J(H,H) = 6.2 Hz, ^2J(H,H) = 16.0 Hz, AB system, 1H; CH_2ArNO_2), 7.54 (d, ^3J(H,H) = 8.6 Hz, 2H), 8.17 (d, ^3J(H,H) = 8.6 Hz, 2H), 9.06 ppm (t, ^3J(H,H) = 6.2 Hz, 1H; NH); ^{1}H NMR (500 MHz, D_2O, 300 K) δ = 1.94 (m, 1H), 2.05 (m, 1H), 2.48 (m, 1H), 2.61 (m, 1H), 2.82–4.05 (m, 20H), 4.51 (d, ^2J(H,H) = 16.0 Hz, AB system, 1H; CH_2ArNO_2), 4.55 (d, ^2J(H,H) = 16.0 Hz, AB system, 1H; CH_2ArNO_2), 7.55 (d, ^3J(H,H) = 8.6 Hz, 2H), 8.26 ppm (d, ^3J(H,H) = 8.6 Hz, 2H); IR (KBr): $\tilde{\nu}$ = 1389 (N=O), 1517 (N=O), 1626 (C=O), 3097 (C–H), 3394 cm^{-1} (O–H); MS (ESI): m/z: 611.26 [M+H]$^+$, 633.24 [M+Na]$^+$; HRMS (ESI): m/z: calcd for $C_{26}H_{38}N_6O_{11}$+H: 611.26713; found: 611.26886; elemental analysis calcd (%) for $C_{26}H_{39}N_6O_{11}$·H_2O: C 49.68, H 6.41, N 13.37; found: C 49.50, H 6.14, N 13.59;

Compound 5: M.p. 144(±1) °C; ^{1}H NMR (600 MHz, D_2O, 300 K): δ = 1.81–2.07 (m, 2H), 2.30–2.56 (m, 2H), 2.69 (t, ^3J(H,H) = 6.7 Hz, 1H; SH), 2.83–2.96 (m, 2H), 3.06–3.70 (m, 16H), 3.70–3.99 ppm (m, 9H); HRMS (ESI): m/z: calcd for $C_{21}H_{37}N_5O_9SNa$: 588.22042; found: 588.22076; HPLC (A: CH_3CN, B: H_2O+0.4 % HCOOH; B: 98→0 %): t_R = 5.05 min.

Compound 7: M.p. 155(±1) °C; ^{1}H NMR (600 MHz, D_2O, 300 K): δ = 1.73–1.88 (m, 2H), 2.36–2.54 (m, 2H), 2.93–3.59 (m, 18H), 3.61–3.71 (m, 2H), 3.77 (s, 3H; OCH_3), 3.77–3.87 (m, 5H), 4.79–4.81 (m, 1H; CHCOOMe), 7.51 (d, ^3J(H,H) = 8.8 Hz, 2H), 8.23 ppm (d, ^3J(H,H) = 8.8 Hz, 2H); LCMS: (A: H_2O+0.4 % HCOOH, B: CH_3CN+0.4 % HCOOH; B: 2→100 %): t_R = 5.8 min, 683.8 [M+H]$^+$.

Compound 6: EtOH (4 mL) was added to a suspension of compound **1** (200 mg, 0.43 mmol) in DMF (20 mL) and the mixture was warmed at 45 °C for 12 h. After evaporation of the solvent, the crude solid was purified by column chromatography on a C_{18} reverse-phase column (H_2O; A, t_R = 4 min). The fraction was collected and the solution was lyophilized to give compound **6** as a white solid (80 mg, 35 %). ^{1}H NMR (300 MHz, D_2O, 300 K): δ = 1.30 (t, ^3J(H,H) = 7.1 Hz, 3H; OCH_2CH_3), 1.89–2.01 (m, 1H), 2.02–2.15 (m, 1H), 2.51–2.76 (m, 2H), 2.94–3.28 (m, 6H), 3.31–4.00 (m, 14H), 4.01–4.10 (m, 1H, CH), 4.23 ppm (q, ^3J(H,H) = 7.1 Hz, 2H; OCH_2CH_3); MS (ESI): m/z: 525.15 [M−2H+Na]$^-$, 541.17 [M−2H+K]$^-$, 563.16 [M−3H+K+Na]$^-$; elemental analysis calcd (%) for $C_{21}H_{36}N_4O_{10}$·H_2O·HCl: C 45.12, H 7.03, N 10.02; found: C 44.73, H 7.86, N 10.08; HPLC (A: CH_3CN, B: H_2O+0.4 % HCOOH; B: 98→0 %): t_R = 6.3 min.

Compound 9: DOTAGA–anhydride (**1**; 383 mg, 0.967 mmol, 1.1 equiv) and NEt_3 (527 μL, 4.39 mmol, 5 equiv) were added to a solution of bodipy–ethylenediamine (**8**; 500 mg, 0.879 mmol, 1 equiv) in DMF (50 mL), then the mixture was stirred 12 h at 70 °C. After the evaporation of DMF, the crude product was purified by column chromatography on silica gel (Cl_2Cl_2/EtOH/NH_4OH, 2:7:1) to give compound **9** as a mixture of two diastereoisomers as a red solid (360 mg, 40 %). M.p. > 200 °C; ^{1}H NMR (300 MHz, MeOD, 300 K): δ = 0.99 (t, ^3J(H,H) = 7.5 Hz, 6H), 1.30 (s, 6H), 1.97–2.11 (m, 2H), 2.35 (q, ^3J(H,H) = 7.5 Hz, 4H), 2.48 (s, 6H), 2.61–2.82 (m, 2H), 2.82–3.02 (m, 2H), 3.03–3.30 (m, 14H), 3.34–3.87 (m, 16H), 5.03 (m, 1H), 7.41 (d, ^3J(H,H) = 8.1 Hz, 2H), 7.59 (d, ^3J-(H,H) = 8.5 Hz, 2H), 7.97 (d, 2H, ^3J(H,H) = 8.1 Hz), 8.12 ppm (d, ^3J-(H,H) = 8.5 Hz, 2H); MS (ESI): m/z: 1115.52 [M−H]$^-$, 1137.50 [M+Na−2H]$^-$, 1159.48 [M+2Na−3H]$^-$; UV/Vis (MeOH): λ_{max} (ε) = 523 (53100), 377 (sh, 6300), 324 nm (sh, 7600 M^{-1}cm^{-1}); UV/Vis (0.1 M PBS): λ_{max} (ε) = 526 (17000), 406 (sh, 4300), 275 nm (sh, 1520 M^{-1}cm^{-1}).

Compound 10: Compound **9** (200 mg, 0.179 mmol, 1 equiv) was dissolved in a solution of H_2O/EtOH (96:4, 10 mL), activated palladium (30 mg, 0.028 mmol, 0.15 equiv) was added to the bodipy and the mixture was stirred under a hydrogen atmosphere overnight. The solution was filtered through Clarcel®, and the solvents were evaporated. The crude product was purified by column chromatography on silica gel (EtOH/CH_2Cl_2/NH_4OH, 7:2:1) to afford the two diastereoisomers as a red powder (120 mg, 61 %). M.p. > 200 °C; ^{1}H NMR (300 MHz, MeOD, 300 K) δ = 1.02 (t, ^3J(H,H) = 7.5 Hz, 6H), 1.35 (s, 6H), 1.84–1.98 (m, 2H), 2.04–2.32 (m, 6H), 2.38 (q, ^3J(H,H) = 7.5 Hz, 4H), 2.50 (s, 6H), 2.57–3.30 (m, 21H), 3.36–3.70 (m, 7H), 4.78 (m, 1H), 6.71 (d, ^3J(H,H) = 8.1 Hz, 2H), 7.08 (d, ^3J(H,H) = 8.1 Hz, 2H), 7.46 (d, ^3J(H,H) = 8.1 Hz, 2H), 7.99 ppm (d, ^3J(H,H) = 8.1 Hz, 2H); MS (ESI): m/z: 1107.51 [M+Na−2H]$^-$, 1123.49 [M+K−2H]$^-$, 1145.47 [M+Na+K−3H]$^-$; UV/Vis (MeOH): λ_{max} (ε) = 523 (43100), 377 (sh, 5500), 321 nm (sh, 4800 M^{-1}cm^{-1}); UV/Vis (0.1 M PBS): λ_{max} (ε) = 526 (20500), 373 (sh, 4700), 322 (sh, 5200 M^{-1}cm^{-}).

Compound 11: Compound **10** (50 mg, 0.046 mmol) was dissolved in a mixture of water and CH_2Cl_2 (5 mL). Then, a solution of thiophosgene

(10.6 μL, 0.14 mmol, 3 equiv) in CH_2Cl_2 (2 mL) was added and the mixture was stirred for 1 h at room temperature. The solvents were evaporated and the product was dried under vacuum to give compound **11** as a pink powder (45 mg, 86%). MS (ESI): *m*/*z*: 1129.5 $[M+H]^+$, 1151.5 $[M+Na]^+$, 1173.5 $[M+2Na-H]^+$.

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