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Bis(phosphonate)-Building Blocks Modified with Fluorescent Dyes

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ABSTRACT: *Preparation of two benzylic bis(phosphonic acids) modified with primary amine or carboxylic acid groups on the benzene ring is described. These compounds were prepared and characterized in the form of both bis(phosphonate) tetraesters and corresponding free acids. The phosphonic acid esters are suitable for further derivation, mainly for conjugation through the amide bond. Mild conversion of the bis(phosphonate) esters to free acids using trimethylsilylbromide allowed to work with functional groups sensitive to conditions of acidic and/or alkaline hydrolysis. Three bis(phosphonate)-containing fluorescent probes were prepared from the building blocks, utilizing amide and sulfamide bonds as spacers. Dyes containing the dansyl group, rhodamine B, and fluorescein were chosen due to their common availability and low cost. The prepared bis(phosphonate)-building blocks and modified fluorescent probes were used for adsorption studies with hydroxyapatite, the commonly used model of bone tissue. Sorption ability of the prepared bis(phosphonate) compounds was similar to that of pamidronate.* © 2013 Wiley Periodicals, Inc. Heteroatom Chem 24:413–425, 2013; View this article online at wileyonlinelibrary.com. DOI 10.1002/hc.21108

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INTRODUCTION

Geminal bis(phosphonates) are a widely studied family of compounds as the group shows very high affinity to hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)_2$), which is the main inorganic component of bones [1–3]. This affinity is a basis for important applications in the treatment of diseases associated with the disorder of calcium metabolism, including osteoporosis, Paget's disease, and cancer [1–4]. Owing to the affinity to the surface of other materials, bis(phosphonates) are also used as corrosion inhibitors or as complexation agents in textile industry or detergent formulations.

Treatment of calcium metabolism diseases is not the only medical application of bis(phosphonates). Nowadays, geminal bis(phosphonates) are utilized as potential vectors of other compounds to target bone tissues [5–7]. Attachment of bis(phosphonate) group changes significantly biodistribution and/or pharmacokinetics of various pharmaceuticals, and mostly leads to selective delivery of the drugs to bone tissue. This concept is applied not only to

the treatment of bone diseases but also to imaging of bone-related diseases. Radiopharmaceuticals based on ^{99m}Tc-bis(phosphonate) complexes used for SPECT (single-photon emission computed tomography) imaging of bone metastases are clinically important [7, 8]. Similar complexes with ^{186}Re and ^{188}Re have been used for the bone-pain palliation therapy [7]. Specific chelators bearing the bis(phosphonate) group in a side chain have been studied as bone-targeted transporters of other metal ions in magnetic resonance imaging (Gd^{3+}) [9, 10], nuclear medicine methods SPECT (^{99m}Tc, ^{111}In, ^{177}Lu) [7, 11, 12], or positron emission tomography (^{68}Ga, ^{64}Cu) [7, 13–15]. Fluorescent dyes [6] or nanoparticles [16] containing the bis(phosphonate) group have also been used for optical imaging of calcified tissues.

Phosphonic acids are also known to exhibit a high affinity for the surface of many inorganic oxides and other inorganic materials [17–19]. The geminal bis(phosphonate) group has been proved to have much higher affinity to the metal oxide surfaces than the simple phosphonate group [20] and is able to anchor other molecules to the surfaces with long-term stability [21–25].

Diverse spectrum of compounds with unique properties has been generated during studies on bis(phosphonates) over past 40 years [26]. Derivatization of the simplest bis(phosphonates) has been usually employed for preparation of the more complex molecules [27, 28]. However, for a number of applications, the most important matter is just the presence of the bis(phosphonate) group as it is solely responsible for the desired properties as targeting or surface modification. Thus, more easily available bifunctional bis(phosphonates) as starting materials, mainly the ester form, are sought as they could simplify further modification of various molecules. Such bis(phosphonate) "building blocks" containing a reactive functional group in the side chain (e.g., in Fig. 1) might be attachable to a wide range of substrates having the availability of a complementary functional group [9, 10, 28–33]. The general conjugation is illustrated in Fig. 1; the coupling reaction should simplify the introduction of the bis(phosphonate) group, decrease a number of reaction steps, and reduce the time to synthesize the conjugates.

Thus, the useful compound should be easily synthesized in large scale in both ester and acid forms. Therefore, we decided to synthesize and study two bis(phosphonate)-building blocks containing amine or carboxylate groups (compounds **3**–**7**, Fig. 2) fulfilling these requirements. The compounds were, consequently, used to attach the bis(phosphonate) group to fluorescent dye moieties - dansyl group, rhodamine, and fluorescein (Fig. 2). The conjugates with these common dyes were used for HA surface modification.

EXPERIMENTAL

Materials and Methods

The commercially available chemicals had synthetic purity and were used as received. Dry solvents for the synthesis were purchased from Sigma–Aldrich (Prague, Czech Republic). Tetraethyl methylenebis(phosphonate) [34] and cationic piperazine derivative of rhodamine B (*III*) [35] were prepared according to the published procedures. ^{1}H, ^{13}C, and 2D NMR (H,H-COSY, H,C-HSQC, and H,C-HMBC) spectra were recorded on a Bruker Avance III 600 MHz spectrometer (Scientific Instruments, Brno, Czech Republic) equipped with the triple resonance cold probe. Chemical shifts were referenced according to the literature [36]. The ^{31}P NMR spectra were recorded on a Varian NMR system 300 MHz spectrometer (Agilent, Santa Clara, CA, USA) equipped with an AutoSwitchable probe, and 85% H_3PO_4 was used as an external reference ($\delta = 0.00$ ppm). The NMR spectra were recorded at 25°C. Chemical shifts (δ) are given in ppm and the coupling constants (J) in Hz.

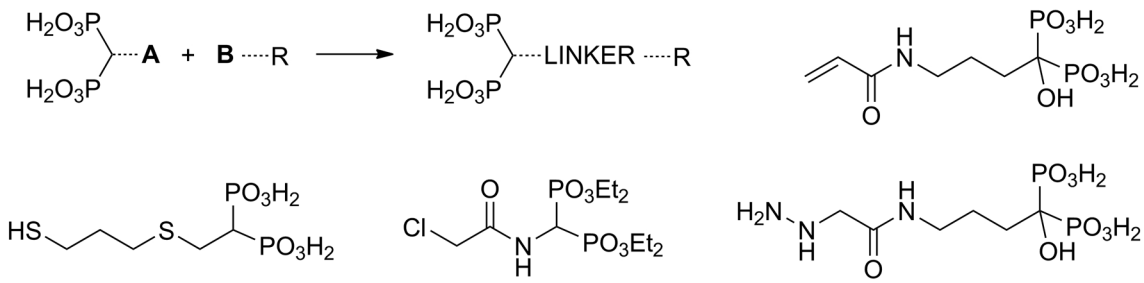


FIGURE 1 Schematic illustration of the conjugation reaction between a bis(phosphonate) building block with a general substrate and examples of such building blocks. **A** is a functional group specifically reacting with a complementary functional group **B**.

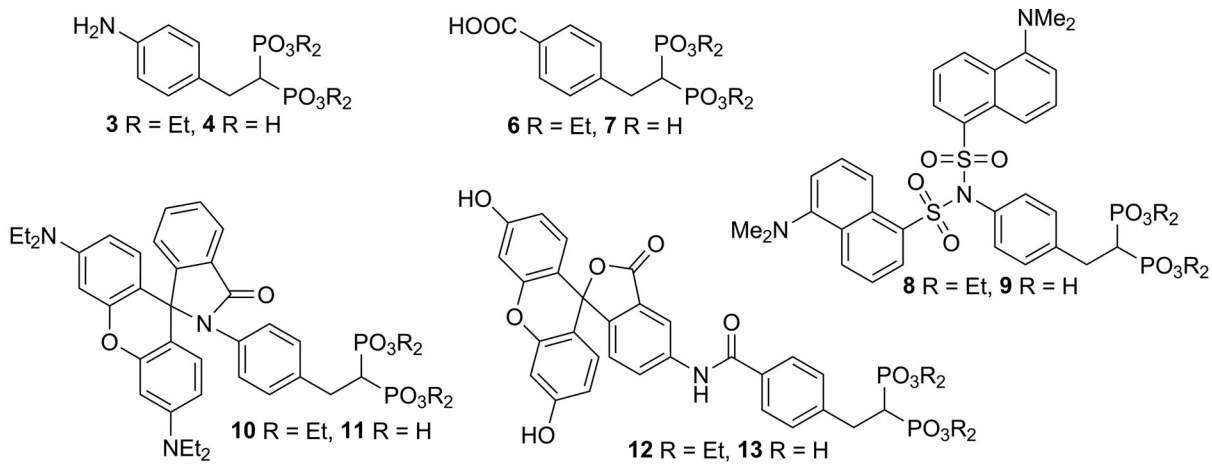


FIGURE 2 Structures of the studied bis(phosphonate)-building blocks (**3, 4, 6, 7**) and the modified dyes (**8**–**13**).

Numbering schemes of bis(phosphonate) dye conjugates can be found in the Supporting Information (Fig. S1). The ESI-MS spectra were recorded on a Bruker Esquire 3000 spectrometer. High-resolution MS spectra were measured on a Bruker APEX-Q Fourier transform mass spectrometer. Fluorescence spectra were recorded on a luminescence thermo spectronic spectrometer AMINCO Bowman series 2 (Thermo Fisher Scientific, Pardubice, Czech Republic). UV–vis spectra were recorded on a Biochrom WPA Lightwave II spectrophotometer (ChromSpec, Prague, Czech Republic). HA was purchased from Fluka (catalogue number 55496; Prague, Czech Republic). The specific surface area, 73 $m^2\ g^{-1}$, was determined by N_2 adsorption by means of a Quantachrome Autosorb-6B apparatus (Quantachrome, Odelzhausen, Germany). Surface analysis showed the Ca:P ratio 5:4, as determined on an Omicron ESCAProbeP apparatus (Omicron NanoTechnology, Taunusstein, Germany). The ζ potential, –18 mV at pH 7.5, was determined on a Brookhaven BI-Zeta PALS apparatus (Brookhaven Instruments Corporation, Holtsville, NY, USA). For thin-layer chromatography, Merck (Prague, Czech Republic) aluminum foils with silica gel 60 F_{254} were used.

Synthesis of Bis(phosphonate)-Building Blocks

*General Procedure for Preparation of 4-Substituted Benzyl-bis(phosphonates) (***1, 5***).* Under an argon atmosphere, a solution of $CH_2(PO_3Et_2)_2$ (9.51 g; 33 mmol) in dry solvent (50 mL; see below) was slowly added, dropwise, to NaH (60% suspension in mineral oil; 1.85 g, 46.3 mmol) suspended in dry solvent (50 mL) and cooled to 0°C. After liberation of all hydrogen gas, the reaction mixture was left to warm up to room temperature (RT). The solution of the substituted benzylbromide (22 mmol) in dry solvent (50 mL) was added to the reaction mixture. The resulting mixture was stirred at RT for additional 12 h. The reaction was quenched by addition of EtOH (20 mL). The mixture was evaporated to dryness. The crude product was dissolved in $CHCl_3$ (150 mL) and extracted with water (3 × 150 mL). The organic layer was evaporated to dryness, and the resulting oil was extracted with water (3 × 25 mL). The crude oily product was purified by column chromatography (SiO_2). Fractions containing pure product were combined, dried with anhydrous $MgSO_4$, and evaporated in vacuum to dryness. The product was obtained as a viscous oil.

*Tetraethyl 2-(4-Nitrophenyl)-ethane-1,1-bis (phosphonate) (***1***).* The reaction was performed with 4-nitrobenzylbromide (4.75 g) in dry toluene with 90% purity after extractions (determined by ^{31}P NMR). The mobile phase MeOH:MeCN:$CHCl_3$ 1:10:10 was used for column chromatography. The product was obtained as a yellow viscous oil. Yield: 5.03 g (72%). NMR ($CDCl_3$) ^1H: δ 1.24 (CH_3, m, 12H); 2.59 (C*H*—P, tt, 1H, $^2J_{HP}$ = 24, $^3J_{HH}$ = 6); 3.27 (CH_2—CH, td, 2H, $^3J_{HP}$ = 16, $^3J_{HH}$ = 6); 4.07 (CH_2—O, m, 8H); 7.39 (C*H*—C—CH_2, d, 2H, $^3J_{HH}$ = 9); 8.08 (C*H*—C—N, d, 2H, $^3J_{HH}$ = 9); $^{13}C\{^1H\}$: δ 16.4 (CH_3, m); 31.4 (CH_2—CH, m); 38.9 (C*H*—P, t, $^1J_{CP}$ = 133); 62.9 (CH_2—O, m); 123.5 (C*H*—C—N, s); 130.0 (C*H*—C—CH_2, s); 146.8 (*C*—N, s); 147.5 (*C*—CH_2, t, $^3J_{CP}$ = 8); $^{31}P\{^1H\}$: δ 24.0 (s). ESI-MS *m/z* (–): 394.0 [M – Et]$^-$; 422.0 [M – H]$^-$; (+): 424.2 [M + H]$^+$; 446.1 [M + Na]$^+$; 462.0 [M + K]$^+$. TLC (SiO_2, MeOH:MeCN:$CHCl_3$ 1:10:10) R_f = 0.6.

*Tetraethyl 2-[4-(Methoxycarbonyl)phenyl]-ethane-1,1-bis(phosphonate) (***5***).* The reaction was performed with methyl 4-(bromomethyl)benzoate

(5.04 g) in dry THF with 87% purity after extractions (determined by ^{31}P NMR). The mobile phase MeCN:$CHCl_3$ 1:1 was used for SiO_2 column chromatography. The product was obtained as a light yellow viscous oil. Yield: 7.1 g (75%). NMR ($CDCl_3$) ^{1}H: δ 1.24 (CH_3—CH_2, m, 12H); 2.62 (C*H*—P, tt, 1H, $^2J_{HP}$ = 24, $^3J_{HH}$ = 7); 3.25 (CH_2—CH, td, 2H, $^3J_{HP}$ = 17, $^3J_{HH}$ = 7); 3.88 (CH_3—O, s, 3H); 4.09 (CH_2—O, m, 8H); 7.32 (C*H*—C—CH_2, d, 2H, $^3J_{HH}$ = 8); 7.93 (C*H*—C—CO, d, 2H, $^3J_{HH}$ = 8); ^{13}C{^{1}H}: δ 16.5 (*C*H_3, m); 31.5 (*C*H_2—CH, s); 39.0 (*C*H—P, t, $^1J_{CP}$ = 130); 52.3 (*C*H_3—O, s) 62.8 (*C*H_2—O, m); 128.6 (*C*—CO, s); 129.1 (*C*H—C—CH_2, s); 129.8 (*C*H—C—CO, s); 145.3 (*C*—CH_2, t, $^3J_{CP}$ = 8); 167.2 (*C*O, s); ^{31}P{^{1}H}: δ 22.6 (s). ESI-MS *m/z* (–): 434.8 [M – H]$^-$; (+): 437.0 [M + H]$^+$; 459.0 [M + Na]$^+$; 473.0 [M + K]$^+$. TLC (SiO_2, MeCN:$CHCl_3$ 1:1) R_f = 0.3.

*Tetraethyl 2-(4-Aminophenyl)-ethane-1,1-bis (phosphonate) (**3**).* Under an argon atmosphere, 10% Pd/C (400 mg) was suspended in dry EtOH (50 mL). The solution of **1** (4.05 g; 9.57 mmol) in dry EtOH (100 mL) was added to the mixture, and H_2 was continuously bubbled through the suspension at RT overnight. The end of the reaction was detected by TLC (a vanishing spot of the parent compound, MeOH:MeCN:$CHCl_3$ 1:10:10). The catalyst was filtered, and the filtrate was evaporated to dryness. The product was obtained as a pale yellow viscous oil. Yield: 3.54 g (94%). NMR ($CDCl_3$) ^{1}H: δ 1.24 (CH_3, m, 12H); 2.54 (C*H*—P, tt, 1H, $^2J_{HP}$ = 24, $^3J_{HH}$ = 6); 3.10 (CH_2—CH, td, 2H, $^3J_{HP}$ = 17, $^3J_{HH}$ = 6); 4.06 (CH_2—O, m, 8H); 6.57 (C*H*—C—N, d, 2H, $^3J_{HH}$ = 9); 7.02 (C*H*—C—CH_2, d, 2H, $^3J_{HH}$ = 9); ^{13}C{^{1}H}: δ 16.3 (*C*H_3, m); 30.4 (*C*H_2—CH, s); 39.3 (C*H*—P, t, $^1J_{CP}$ = 132); 62.5 (*C*H_2—O, m); 115.0 (*C*H—C—N, s); 129.4 (*C*—CH_2, t, $^3J_{CP}$ = 7); 129.7 (*C*H—C—CH_2, s); 145.0 (*C*—N, s); ^{31}P{^{1}H}: δ 23.2 (s). ESI-MS *m/z* (–): 392.1 [M – H]$^-$. 394.2; (+): [M + H]$^+$; 416.2 [M + Na]$^+$. TLC (SiO_2, MeOH:MeCN:$CHCl_3$ 1:10:10) R_f = 0.4.

*Tetraethyl 2-(4-Carboxyphenyl)-ethane-1,1-bis (phosphonate) (**6**).* To a solution of **5** (7.1 g; 16.3 mmol) in MeOH (200 mL), LiOH·H_2O (3.42 g; 81.5 mmol) was added and the resulting suspension was stirred at RT for 12 days. The reaction mixture was evaporated to dryness, the residue was dissolved in water (150 mL), and aqueous HCl (6 M) was slowly added to reach pH 3. The resulting mixture was extracted with $CHCl_3$ (3 × 150 mL). The organic layers were combined and evaporated to dryness. The crude product was purified by column chromatography (SiO_2; CH_2Cl_2:MeOH 10:1). Fractions containing pure product were combined, dried with anhydrous $MgSO_4$, and evaporated to dryness in vacuum. The product was obtained as a light yellow viscous oil. Yield: 4.07 g (59%). NMR ($CDCl_3$) ^{1}H: δ 1.22 (CH_3, m, 12H); 2.63 (C*H*—P, tt, 1H, $^2J_{HP}$ = 24, $^3J_{HH}$ = 6); 3.27 (CH_2—CH, td, 2H, $^3J_{HP}$ = 17, $^3J_{HH}$ = 6); 4.09 (CH_2—O, m, 8H); 7.32 (C*H*—C—CH_2, d, 2H, $^3J_{HH}$ = 8); 7.94 (C*H*—C—CO, d, 2H, $^3J_{HH}$ = 8); ^{13}C{^{1}H}: δ 16.0 (*C*H_3, m); 30.9 (*C*H_2—CH, s); 38.2 (*C*H—P, t, $^1J_{CP}$ = 130); 62.7 (*C*H_2—O, m); 128.3 (*C*—CO, s); 128.7 (*C*H—C—CH_2, s); 129.7 (*C*H—C—CO, s); 144.7 (*C*—CH_2, t, $^3J_{CP}$ = 8); 169.1 (*C*O, s); ^{31}P{^{1}H}: δ 22.6 (s). ESI-MS *m/z* (–): 392.7 [M – Et]$^-$; 420.8 [M – H]$^-$; (+): 422.9 [M + H]$^+$; 445.0 [M + Na]$^+$; 460.9 [M + K]$^+$. TLC (SiO_2, CH_2Cl_2:MeOH 10:1) R_f = 0.3.

*General Procedure for Ester Hydrolysis (**2, 4, 7**).* A solution of particular bis(phosphonate) was dissolved in aqueous HCl (6 M; 15 mL) and heated at 90°C (for reaction time see below). After cooling to RT, the mixture was evaporated to dryness and three times coevaporated with H_2O (20 mL) and isolated as described below.

*2-(4-Nitrophenyl)-ethane-1,1-bis(phosphonic acid) (**2**).* The solution of **1** (1.00 g; 2.36 mmol) was heated for 24 h. After coevaporation with H_2O, the product was obtained as a light yellow viscous oil. Slow crystallization from aqueous solution afforded single crystals suitable for X-ray diffraction analysis. Yield: 0.70 g (95%). Elemental analysis Calcd for $C_8H_{11}NO_8P_2{\cdot}H_2O$ (M_r = 329.1): C 29.5; H 3.7; N 4.3; found: C 29.2; H 4.0; N 4.3 (%). NMR (DMSO-d_6) ^{1}H: δ 2.41 (C*H*—P, tt, 1H, $^2J_{PH}$ = 23, $^3J_{HH}$ = 6); 3.17 (CH_2, dt, 2H, $^3J_{PH}$ = 16, $^3J_{HH}$ = 6); 7.53 (C*H*—C—CH_2, d, 2H, $^3J_{HH}$ = 9); 8.11 (C*H*—C—N, d, 2H, $^3J_{HH}$ = 9); ^{13}C{^{1}H}: δ 30.8 (*C*H_2, m); 39.8 (*C*H—P, t, $^1J_{PC}$ = 126); 123.1 (*C*H—C—N, s); 130.1 (*C*H—C—CH_2, s); 145.9 (*C*—N, s); 149.1 (*C*—CH_2, t, $^3J_{PC}$ = 7); ^{31}P: δ 22.1 (dt, $^2J_{PH}$ = 23, $^3J_{PH}$ = 16). ESI-MS *m/z* (–): 291.9 [M – (H_3O)]$^-$; 309.9 [M – H]$^-$; (+): 334.1 [M + Na]$^+$; 356.1 [M + 2Na – H]$^+$; 377.1 [M + 3Na – 2H]$^+$. HRMS *m/z* (–): 309.989 [M – H]$^-$; 620.985 [2M – H]$^-$; (+): 312.003 [M + H]$^+$; 333.985 [M + Na]$^+$; 349.959 [M + K]$^+$; 622.999 [2M + H]$^+$; 644.981 [2M + Na]$^+$.

*2-(4-Aminophenyl)-ethane-1,1-bis(phosphonic acid) (**4**).* The solution of **3** (715 mg; 1.81 mmol) was heated for 24 h. After coevaporation with H_2O, the residue was redissolved in H_2O (10 mL) and excess of *i*-PrOH was added to cloudiness. The white precipitate formed was collected on glass frit and dried over P_2O_5 under vacuum to give hydrochloride of the

product as an off-white powder. Crystallization from 10% aqueous acetic acid afforded single crystals suitable for X-ray diffraction Yield: 401 mg (69%). Elemental analysis Calcd for $C_8H_{13}NO_6P_2 \cdot HCl$ (M_R = 317.6): C, 30.3; H, 4.4; N, 4.4. Found: C, 30.3; H, 4.6; N, 4.4. NMR (D_2O/NaOD, pD = 10) ^{1}H: δ 2.28 (C*H*—P, tt, 1H, $^2J_{PH}$ = 21, $^3J_{HH}$ = 6); 3.16 (CH_2, dt, 2H, $^3J_{HH}$ = 6, $^3J_{PH}$ = 16); 6.93 (C*H*—C—N, d, 2H, $^3J_{HH}$ = 8); 7.37 (C*H*—C—CH_2, d, 2H, $^3J_{HH}$ = 8); ^{13}C{^{1}H}: δ 30.8 (*C*H$_2$, m); 41.8 (*C*H—P, t, $^1J_{PC}$ = 111); 116.3 (*C*H—C—N, s); 129.9 (*C*H—C—CH_2, s); 133.8 (*C*—CH_2, m); 143.6 (*C*—N, s); ^{31}P: δ 22.1 (dt, $^2J_{PH}$ = 21, $^3J_{PH}$ = 16). ESI-MS *m/z* (–): 261.9 [M – (H_3O)]$^-$; 279.9 [M – H]$^-$; (+): 304.1 [M + Na]$^+$; 226.1 [M + 2Na – H]$^+$; 348.1 [M + 3Na – 2H]$^+$. HRMS *m/z* (–): 280.015 [M – H]$^-$; (+): 282.029 [M + H]$^+$; 304.011 [M + Na]$^+$; 319.985 [M + K]$^+$; 563.051 [2M + H]$^+$; 585.033 [2M + Na]$^+$.

*2-(4-Carboxyphenyl)-ethane-1,1-bis(phosphonic acid) (**7**).* The solution of **5** (671 mg; 1.54 mmol) was heated for 3 days. After co-evaporation with H_2O, product was re-dissolved in H_2O (20 mL) and filtered with charcoal. The filtrate was then evaporated to dryness in vacuum and three times coevaporated with H_2O (20 mL). The solid product was dried over P_2O_5 under vacuum to give a white powder. Yield: 458 mg (96%). Elemental analysis Calcd for $C_9H_{12}O_8P_2 \cdot H_2O$ (M_r = 328.2): C, 33.0; H, 4.3. Found: C, 33.0; H, 4.0. NMR (DMSO-d_6) ^{1}H: δ 2.33 (C*H*—P, tt, 1H, $^2J_{HP}$ = 24, $^3J_{HH}$ = 7); 3.11 (CH_2, td, 2H, $^3J_{HP}$ = 17, $^3J_{HH}$ = 7); 7.37 (C*H*—C—CH_2, d, 2H, $^3J_{HH}$ = 8); 7.83 (C*H*—C—CO, d, 2H, $^3J_{HH}$ = 8); ^{13}C{^{1}H}: δ 31.1 (*C*H$_2$, s); 128.9 (*C*—CO, s); 129.3 (*C*H—C—CH_2, s); 129.6 (*C*H—C—CO, s); 146.6 (*C*—CH_2, t, $^3J_{CP}$ = 8); 167.8 (*C*O, s); ^{31}P: δ 20.3 (dt, $^2J_{PH}$ = 24; $^3J_{PH}$ = 17). ESI-MS *m/z* (–): 290.5 [M – (H_3O)]$^-$; 308.5 [M – H]$^-$. HRMS *m/z* (–): 308.994 [M – H]$^-$; 618.993 [2M – H]$^-$; (+): 311.008 [M + H]$^+$; 332.990 [M + Na]$^+$; 348.964 [M + K]$^+$; 621.009 [2M + H]$^+$; 642.991 [2M + Na]$^+$; 658.965 [2M + K]$^+$.

Synthesis of Bis(phosphonate) Dyes

*Tetraethylester of Dansyl–Bis(phosphonate) Conjugate (**8**).* To the stirred solution of **3** (1.08 g; 2.75 mmol) in dry MeCN (100 mL), dried K_2CO_3 (1.85 g; 13.4 mmol) was added. After 10 min, dansyl chloride (1.48 g; 5.50 mmol) was added to the mixture. The resulting suspension was stirred at RT for 5 days. Solids were filtered, and the filtrate was evaporated to dryness. The crude product was purified by column chromatography (SiO_2; MeOH:MeCN:$CHCl_3$ 1:10:10). The fractions containing pure product were combined, dried with anhydrous $MgSO_4$ and evaporated to dryness in vacuum. The resulting oil was dissolved in benzene and lyophilized to get the product as a dark yellow powder (1.16 g). Yield: 68%. Elemental analysis Calcd for $C_{40}H_{51}N_3P_2O_{10}S_2$ (M_r = 859.9): C, 55.8; H, 6.0; N, 4.9. Found: C, 55.1; H, 5.9; N, 4.8. NMR ($CDCl_3$) ^{1}H: δ 1.26 (CH_3—CH_2, m, 12H); 2.54 (C*H*—P, tt, 1H, $^2J_{HP}$ = 24, $^3J_{HH}$ = 6); 2.85 (CH_3—N; s, 12H); 3.18 (CH_2—CH, td, 2H, $^3J_{HP}$ = 16, $^3J_{HH}$ = 6); 4.07 (CH_2—O, m, 8H); 7.01 (*2*, d, 2H, $^3J_{HH}$ = 8); 7.08 (*H6*, m, 2H); 7.10 (*H7*, m, 2H); 7.12 (*H3*, m, 2H); 7,45 (*H12*, m, 2H); 7.75 (*H8*, d, 2H, $^3J_{HH}$ = 8); 8.26 (*H11*, d, 2H, $^3J_{HH}$ = 8); 8.56 (*H13*, d, 2H, $^3J_{HH}$ = 8); ^{13}C{^{1}H}: δ 16.3 (*C*H$_3$—CH$_2$, m); 30.8 (*C*H$_2$—CH, s); 38.9 (*C*H—P, t, $^1J_{CP}$ = 133); 45.6 (*C*H$_3$—N, s); 62.8 (*C*H$_2$—O, m); 115.3 (*C6*, s); 119.1 (*C8*, s); 122.9 (*C12*, s); 127.9 (*C7*, s); 129.4 (*C3*, s); 129.5 (*C14*, s); 129.9 (*C9*, s); 131.6 (*C1*, s); 131.9 (*C13*, s); 132.1 (*C2*, s); 132.7 (*C11*, s); 133.9 (*C10*, s); 142.0 (*C4*, t, $^3J_{CP}$ = 7); 151.6 (*C5*, s); ^{31}P{^{1}H}: δ 22.7 (s). ESI-MS *m/z* (+): 860.4 [M + H]$^+$; 882.4 [M + Na]$^+$; 898.3 [M + K]$^+$. TLC (SiO_2, MeOH:MeCN:$CHCl_3$ 1:10:10) R_f = 0.8.

*Tetraethylester of Rhodamine–Bis(phosphonate) Conjugate (**10**).* Under an argon atmosphere, O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate (TBTU) (740 mg; 2.30 mmol) was added to a solution of rhodamine B hydrochloride (1.10 g; 2.30 mmol) and triethylamine (640 μL; 4.60 mmol) in dry MeCN (25 mL). The mixture was stirred at RT for 15 min. The solution of **3** (594 mg; 1.51 mmol) and triethylamine (320 μL; 2.30 mmol) in dry MeCN (5 mL) was added to the rhodamine derivative solution. The resulting mixture was stirred at RT for 5 h. The mixture was evaporated to dryness; the residue was dissolved in $CHCl_3$ (50 mL) and extracted with water (5×50 mL). The organic layer was evaporated to dryness. The crude product was purified by column chromatography (SiO_2; MeOH:MeCN:$CHCl_3$ 1:10:10). The fractions containing pure product were combined and evaporated to dryness in vacuum to get the product as a purple viscous oil (807 mg). Yield: 65%. NMR (CD_3CN) ^{1}H: δ 1.10 (CH_3—CH_2—N, t, 12H, $^3J_{HH}$ = 7); 1.16 (CH_3—CH_2—O, m, 12H); 2.60 (C*H*—P, tt, 1H, $^2J_{HP}$ = 24, $^3J_{HH}$ = 7); 3.04 (CH_2—CH, td, 2H, $^3J_{HP}$ = 16, $^3J_{HH}$ = 7); 3.33 (CH_2—N, q, 8H, $^3J_{HH}$ = 7); 3.94 (CH_2—O, m, 8H); 6.26 (*H6*, d, 2H, $^4J_{HH}$ = 2); 6.39 (*H10*, dd, 2H, $^3J_{HH}$ = 9, $^4J_{HH}$ = 2); 6.63 (*H9*, d, 2H, $^3J_{HH}$ = 9); 6.76 (*H2*, d, 2H, $^3J_{HH}$ = 9); 7.02 (*H17*, d, 1H, $^3J_{HH}$ = 7); 7.05 (*H3*, d, 2H, $^3J_{HH}$ = 9); 7.52 (*H15*, m, 1H); 7.54 (*H16*, m, 1H); 7.88 (*H14*, d, 1H, $^3J_{HH}$ = 7); ^{13}C{^{1}H}: δ 12.7 (*C*H$_3$—CH$_2$—N, s); 16.6 (*C*H$_3$—CH$_2$—O, m); 31.5 (*C*H$_2$—CH, t, $^2J_{CP}$ = 5); 38,9 (*C*H—P, t, $^1J_{PC}$ = 132); 44.9 (*C*H$_2$—N, s);

63.1 (CH_2—O, m); 66.7 (*C11*, s); 98.2 (*C6*, s); 106.8 (*C8*, s); 109.1 (*C10*, s); 123.7 (*C14*, s); 124.5 (*C17*, s); 127.7 (*C2*, s); 129.2 (*C15*, s); 129.8 (*C9*, s); 130.0 (*C3*, s); 131.3 (*C13*, s); 134.0 (*C16*, s); 136.4 (*C1*, s); 138.9 (*C4*, t, $^3J_{CP} = 7$); 149.7 (*C5*, s); 153.7 (*C7*, s); 154.9 (*C12*, s); 168.3 (*C*O, s); $^{31}P\{^1H\}$: δ 23.2 (s). ESI-MS *m/z* (–): 788.2 $[M - Et]^-$; 816.3 $[M - H]^-$; (+): 818.4 $[M + H]^+$; 840.4 $[M + Na]^+$; 856.4 $[M + K]^+$. TLC (SiO_2, MeOH:MeCN:$CHCl_3$ 1:10:10) $R_f = 0.6$.

*Tetraethylester of Fluorescein–Bis(phosphonate) Conjugate (***12***).* Under an argon atmosphere, TBTU (652 mg; 2.03 mmol) was added to a solution of **6** (660 mg; 1.56 mmol) and triethylamine (1.10 mL; 7.90 mmol) in dry MeCN (100 mL). The mixture was stirred at RT for 15 min. The solution of 5-aminofluorescein (705 mg; 2.03 mmol) in MeCN:THF (5:1; 120 mL) was then added to the reaction mixture. The resulting solution was stirred at RT for 12 h. The mixture was evaporated to dryness, the residue was dissolved in $CHCl_3$ (100 mL), and the solution was extracted with water (5×100 mL). The organic layer was evaporated to dryness. The crude product was purified by column chromatography (SiO_2; acetone:toluene 8:1; R_f(**12**) = 0.7). The fractions containing pure product were combined and evaporated to dryness in vacuum to get the product as a dark yellow viscous oil (880 mg). Yield: 75%. NMR ($CDCl_3$) ^1H: δ 1.27 (CH_3, m, 12H); 2.74 (C*H*—P, tt, 1H, $^2J_{HP} = 19$, $^3J_{HH} = 5$); 3.26 (CH_2, td, 2H, $^3J_{HP} = 16$, $^3J_{HH} = 5$); 4.12 (CH_2—O, m, 8H); 6.55 (*H10*, d, 1H, $^3J_{HH} = 8$); 6.63 (*H9*, d, 1H, $^3J_{HH} = 8$); 6.73 (*H6*, s, 1H); 6.81 (*H22* + *H23*, m, 2H); 6.89 (*H17*, d, 1H, $^3J_{HH} = 8$); 7.04 (*H16*, d, 1H, $^3J_{HH} = 8$); 7.09 (*H19*, s, 1H); 7.29 (*H14*, d, 1H, $^3J_{HH} = 8$); 7.41 (*H3*, d, 2H, $^3J_{HH} = 8$); 8.06 (*H2*, d, 2H, $^3J_{HH} = 8$); $^{13}C\{^1H\}$: δ 11.5 (*C*H_3, d, $^3J_{CP} = 6$); 26.6 (*C*H_2—CH, t, $^2J_{CP} = 5$); 33.8 (*C*H—P, t, $^1J_{PC} = 132$); 58.4 (*C*H_2—O, m); 83.3 (*C11*, s); 102.8 (*C6*, s); 109.9 (*C14*, s); 110.2 (*C8* + *C19*, s); 112.8 (*C10*, s); 117.2 (*C21*, s); 117.3 (*C23*, s); 123.4 (*C16*, s); 124.8 (*C17*, s); 127.4 (*C1*, s); 128.0 (*C15*, s); 129.0 (*C22*, s); 129.1 (*C9*, s); 129.2 (*C3*, s); 130.3 (*C2*, s); 143.6 (*C12*, s); 145.7 (*C4*, t, $^3J_{CP} = 6$); 146.6 (*C13*, s); 151.9 (*C20*, s); 152.0 (*C18*, s); 152.3 (*C7*, s); 159.4 (*C*5, s); 164.4 (*C*O—N, s); 169.7 (*C*O—O, s); $^{31}P\{^1H\}$: δ 22.3 (s). ESI-MS *m/z* (–): 750.1 $[M - H]^-$; (+): 752.2 $[M + H]^+$; 774.2 $[M + Na]^+$; 790.1 $[M + K]^+$. TLC (SiO_2, acetone:toluene 8:1) $R_f = 0.7$.

*General Procedure for Transesterification and Subsequent Hydrolysis (***9, 11, 13***).* To a stirred solution of particular bis(phosphonate) conjugate in dry solvent, Bromotrimethylsilane (TMSBr) was added (see below). The reaction mixture was stirred in dark at RT for 24 h. Volatiles were then evaporated in vacuum, and the residue was worked up as described below.

*Dansyl–Bis(phosphonate) Conjugate, Free Acid (***9***).* TMSBr (1.20 mL; 9.1 mmol) was added to a solution of **8** (400 mg; 465 μmol) in dry MeCN (40 mL). The residue after evaporation was dissolved in dry MeCN (20 mL), and the resulting solution was quickly poured into a beaker with H_2O (200 mL). The formed precipitate was collected on glass frit, washed with MeCN and H_2O, and dried over P_2O_5 under vacuum. The product was obtained as a yellow powder. Yield: 347 mg (92%). Elemental analysis Calcd for $C_{32}H_{35}N_3P_2O_{10}S_2$ (M_r = 747.7): C, 51.4; H, 4.7; N, 5.6. Found: C, 51.0; H, 4.7; N, 5.2. NMR (DMSO-d_6): ^1H: δ 2.29 (C*H*—P, tt, 1H, $^2J_{HP} = 22$, $^3J_{HH} = 5$); 2.82 (CH_3—N, s, 12H); 3.13 (CH_2—CH, td, 2H, $^3J_{HP} = 16$, $^3J_{HH} = 5$); 6.89 (*H2*, d, 2H, $^3J_{HH} = 8$); 7.11 (*H7*, m, 2H); 7.18 (*H6*, d, 2H, $^3J_{HH} = 7$); 7.23 (*H3*, d, 2H, $^3J_{HH} = 8$); 7.53 (*H8*, d, 2H, $^3J_{HH} = 8$); 7.57 (*H12*, m, 2H); 8.07 (*H11*, d, 2H, $^3J_{HH} = 7$); 8.54 (*H13*, d, 2H, $^3J_{HH} = 9$); $^{13}C\{^1H\}$: δ 30.8 (*C*H_2—CH, m); 39.1 (*C*H—P, t, $^1J_{CP} = 114$); 45.1 (*C*H_3—N, s); 115.5 (*C6*, s); 118.2 (*C8*, s); 123.5 (*C12*, s); 128.2 (*C7*, s); 128.5 (*C14*, s); 129.0 (*C9*, s); 129.4 (*C3*, s); 130.2 (*C1*, s); 131.3 (*C2*, s); 131.9 (*C13*, s); 132.6 (*C11*, s); 132.7 (*C10*, s); 143.6 (*C4*, t, $^3J_{CP} = 6$); 151.0 (*C5*, s); ^{31}P: δ 21.3 (dt, $^2J_{PH} = 22$, $^3J_{PH} = 16$). ESI-MS *m/z* (–): 728.0 $[M - (H_3O)]^-$; 746.0 $[M - H]^-$; (+): 792.1 $[M + 2Na - H]^+$; 814.1 $[M + 3Na - 2H]^+$. HRMS *m/z* (–): 728.106 $[M - (H_3O)]^-$; 746.117 $[M - H]^-$; (+): 814.078 $[M - 2H + 3Na]^+$.

*Rhodamine–Bis(phosphonate) Conjugate, Free Acid (***11***).* TMSBr (1.90 mL; 14.6 mmol) was added to a solution of **10** (597 mg; 730 μmol) in dry MeCN (20 mL). The residue after evaporation was dissolved in dry MeCN (10 mL), and MeOH (10 mL) was then added. The resulting solution was evaporated to dryness and three times coevaporated with MeOH. The crude product was purified by column chromatography (Amberlite CG50; H^+-form; gradient elution with H_2O:MeOH from 100:0 to 0:100). The fractions containing pure product were combined, evaporated to dryness in vacuum, and coevaporated several times with MeOH. The resulting solid was dried over P_2O_5 under vacuum to give the product as a dark purple powder. Yield: 476 mg (71%). Elemental analysis Calcd for $C_{36}H_{41}N_3O_8P_2 \cdot 2H_2O$ (M_r = 741.7): C, 58.3; H, 6.1; N, 5.7. Found: C, 58.3; H, 5.8; N, 5.7. NMR (DMSO-d_6) ^1H: δ 1.09 (CH_3, t, 12H, $^3J_{HH} = 7$); 2.12 (C*H*—P, tt, 1H, $^2J_{HP} = 23$, $^3J_{HH} = 5$); 2.95 (CH_2—CH, tt, 2H, $^3J_{HP} = 17$; $^3J_{HH} = 5$); 3.31 (CH_2—N, q, 8H, $^3J_{HH} = 7$); 6.29 (*6*, d, 2H, $^4J_{HH} = 2$); 6.38

(*10*, dd, 2H, $^3J_{HH} = 9$, $^4J_{HH} = 2$); 6.55 (*H9*, d, 2H, $^3J_{HH} = 9$); 6.74 (*H2*, d, 2H, $^3J_{HH} = 9$); 7.03 (*H17*, d, 1H, $^3J_{HH} = 8$); 7.05 (*H3*, d, 2H, $^3J_{HH} = 9$); 7.52 (*H15*, m, 1H); 7.54 (*H16*, m, 1H); 7.86 (*H14*, d, 1H, $^3J_{HH} = 7$); $^{13}C\{^1H\}$: δ 12.1 (CH_3, s); 30.0 (CH_2—CH, s); 48.3 (CH_2—N, s); 66.2 (*C11*, s); 97.3 (*C6*, s); 105.6 (*C8*, s); 108.1 (*C10*, s); 122.7 (*C14*, s); 123.6 (*C17*, s); 125.4 (*C2*, s); 128.3 (*C15*, s); 128.4 (*C9*, s); 128.5 (*C3*, s); 129.4 (*C13*, s); 133.2 (*C16*, s); 134.6 (*C1*, s); 139.1 (*C4*, t, $^3J_{CP} = 6$); 148.2 (*C5*, s); 152.1 (*C7*, s); 153.6 (*C12*, s); 166.7 (*C*O, s); ^{31}P: δ 20.3 (dt, $^2J_{PH} = 20$, $^3J_{PH} = 16$). ESI-MS *m/z* (–): 676.1 $[M - Et]^-$; 686.1 $[M - (H_3O)]^-$; 704.1 $[M - H]^-$; (+): 706.3 $[M + H]^+$; 728.3 $[M + Na]^+$; 750.2 $[M - H + 2Na]^+$. HRMS *m/z* (–): 648.167 $[M + H - 2Et]^-$; 676.198 $[M - Et]^-$; 686.219 $[M - (H_3O)]^-$; 704.230 $[M - H]^-$; (+): 706.244 $M + H]^+$; 728.226 $[M + Na]^+$; 750.208 $[M - H + 2Na]^+$.

*Fluorescein–Bis(phosphonate) Conjugate, Free Acid (***13***).* TMSBr (4 mL; 30.3 mmol) was added to a solution of **12** (780 mg; 1.03 mmol) in a mixture of dry MeCN (40 mL) and dry DMF (1 mL). The residue after evaporation was dissolved in dry MeCN (20 mL), and the resulting solution was quickly poured into a beaker with water (200 mL). The precipitate formed was collected on glass frit, washed with MeCN and H_2O, and redissolved in MeOH. The resulting solution was evaporated to dryness and three times coevaporated with MeOH. The solid residue was dried over P_2O_5 under vacuum to give a product as a dark brown powder. Yield: 502 mg (78%). Elemental analysis Calcd for $C_{29}H_{23}NO_{12}P_2 \cdot 2H_2O$ ($M_r = 675.5$): C, 51.6; H, 4.0; N, 2.1. Found: C, 51.8; H, 3.4; N, 2.3. NMR (DMSO-d_6) ^1H: δ 2.36 (C*H*—P, tt, 1H, $^2J_{HP} = 23$, $^3J_{HH} = 6$); 3.17 (CH_2, td, 2H, $^3J_{HP} = 16$, $^3J_{HH} = 6$); 6.60 (*H10*, dd, 1H, $^3J_{HH} = 8$, $^4J_{HH} = 2$); 6.66 (*H9*, d, 1H, $^3J_{HH} = 8$); 6.70 (*H6*, d, 1H, $^4J_{HH} = 2$); 6.90 (*H22*, d, 1H, $^3J_{HH} = 8$); 6.99 (*H17*, d, 1H, $^3J_{HH} = 8$); 7.06 (*H16* + *H23*, m, 2H); 7.12 (*H14*, d, 1H, $^4J_{HH} = 1$); 7.35 (*H19*, d, 1H, $^4J_{HH} = 2$); 7.50 (*H3*, d, 2H, $^3J_{HH} = 8$); 8.02 (*H2*, d, 2H, $^3J_{HH} = 8$); $^{13}C\{^1H\}$: δ 30.8 (CH_2, t, $^2J_{CP} = 6$); 40.0 (*C*H—P, t, $^1J_{PC} = 132$); 81.7 (*C11*, s); 102.1 (*C6*, s); 107.5 (*C14*, s); 109.9 (*C8*, s); 110.3 (*C19*, s); 112.9 (*C10*, s); 117.6 (*C21*, s); 118.0 (*C23*, s); 122.7 (*C16*, s); 124.3 (*C17*, s); 126.2 (*C1*, s); 127.1 (*C15*, s); 129.0 (*C22*, s); 129.1 (*C9*, s); 129.3 (*C3*, s); 129.7 (*C2*, s); 140.7 (*C12*, s); 147.1 (*C4*, t, $^3J_{CP} = 6$); 148.9 (*C13*, s); 151.2 (*C20*, s); 151.6 (*C18*, s); 151.7 (*C7*, s); 159.4 (*C5*, s); 164.2 (*C*O—N, s); 169.0 (*C*O—O, s); ^{31}P: δ 20.7 (dt, $^2J_{PH} = 23$; $^3J_{PH} = 16$). ESI-MS *m/z* (–): 637.9 $[M - H]^-$; (+): 640.1 $[M + H]^+$; 662.1 $[M + Na]^+$; 678.1 $[M + K]^+$. HRMS *m/z* (–): 638.062 $[M + H]^-$.

Adsorption Experiments

*Tetraethylester of Dansyl–Bis(phosphonate) Conjugate (***8***).* In a 25-mL volumetric flask, bis(phosphonate) (50 μmol) was dissolved in a HEPES buffer solution (0.1 M; 25 mL; pH 7.5) to a final bis(phosphonate) concentration of 2 mM. All stock solutions were stored in dark at 8°C.

Adsorption Experiments with Bis(phosphonate)-Building Blocks. In ten 4-mL glass vials, HA (10 mg) was suspended in the HEPES buffer solution (0.1 M; pH 7.5) and the stock solution of the compound under study (**2**, **4**, and **7**) was added to get a final volume of 3.0 mL (the final total concentrations of the studied compounds in the samples were 40–600 μM). The suspensions were gently shaken at 25°C for 72 h and then filtered through a Millipore filter (Rotilabo®-syringe filter; PVDF; 0.22 μm). The amount of the bis(phosphonate) remaining in the supernatant was determined by UV–vis spectroscopy (λ_{max} **2** = 295 nm, λ_{max} **4** = 232 nm, and λ_{max} **7** = 239 nm). The experimental data were treated by a least-squares fitting procedure using the Micromath Scientist program, version 2.0 (Salt Lake City, Utah).

Adsorption Experiments with Bis(phosphonate) Dyes. In five 4-mL glass vials, HA (5–50 mg) was suspended in the HEPES buffer solution (0.1 M; pH 7.5) and the stock solution of the compound under study (**9**, **11**, and **13**) was added to get a final volume of 3.0 mL (the final total concentrations of the studied compounds in the samples were 1.67 M). The suspensions were gently shaken in dark at 25°C for 3 h and then filtered through a Millipore filter (Rotilabo®-syringe filter; PVDF; 0.22 μm). The amount of the bis(phosphonate) remaining in the supernatant was determined by UV–vis spectroscopy (λ **9** = 315, 325, and 335 nm; λ **11** = 230, 265, and 310 nm; and λ **13** = 235, 370, and 485 nm).

X-Ray Diffraction

Single crystals of **2**·H_2O and **4**·H_2O were obtained as described above. The diffraction data were collected at 150 K (Cryostream Cooler, Oxford Cryosystem) using a Nonius Kappa CCD diffractometer and Mo Kα radiation ($\lambda = 0.71073$ Å) and analyzed using the HKL DENZO program package [37]. The structures were solved by direct methods (SIR92) [38], and refined by full-matrix least-squares techniques (SHELXL97) [39]. All nonhydrogen atoms were refined anisotropically. All hydrogen atoms were located in a difference map of electron density; however, they were treated in theoretical (C—H) or original (O—H, N—H) positions with thermal parameters

TABLE 1 Experimental Crystallographic Parameters of the Studied Compounds

	Compound	
Parameter	*2·H_2O*	*4·H_2O*
Formula	$C_8H_{13}NO_9P_2$	$C_8H_{15}NO_7P_2$
M_r	329.13	299.15
Color, habit	Colorless, prism	Colorless, rod
Crystal system	Monoclinic	Monoclinic
Space group	$P2_1/n$	$P2_1/c$
a (Å)	12.9704(2)	8.6409(2)
b (Å)	6.8883(1)	23.2676(7)
c (Å)	15.6311(3)	11.9193(3)
β (°)	113.5613(9)	95.1284(18)
U, Å^3	1280.12(4)	2386.82(11)
Z	4	8
D_{calc}, g·cm^{-3}	1.708	1.665
μ, mm^{-1}	0.385	0.392
Total reflections	2928	4704
Observed reflections ($I > 2\sigma(I)$)	2793	3336
R; $R'(I > 2\sigma(I))$	0.0271; 0.0285	0.0422; 0.0733
wR; $wR'(I > 2\sigma(I))$	0.0732; 0.0743	0.0936; 0.1079

$U_{eq}(H) = 1.2\ U_{eq}(X)$ as their free refinement led to some unrealistic bond lengths. Crystallographic data for the structures of **2**·H_2O and **4**·H_2O have been deposited at the Cambridge Crystallographic Data Centre, deposit number is CCDC-896879 for **2**·H_2O and CCDC-896878 for **4**·H_2O. Table 1 presents selected experimental crystallographic data.

RESULTS AND DISCUSSION

The formation of an amide bond is the most common coupling reaction to form conjugates and various amide-coupling reagents have been developed over the years [40]. However, coupling of simple aminoalkyl-bis(phosphonates), for example, alendronate, brings some problems. Reactions of free zwitterions must be done in water at pH > 9 and

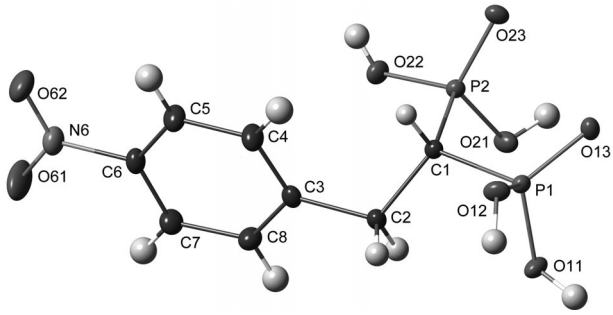
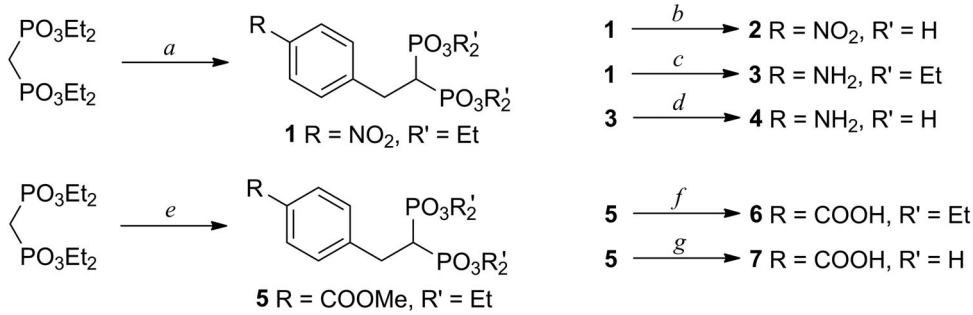


FIGURE 3 Molecular structure of **2** found in the crystal structure of **2**·H_2O.

are, generally, low yielding [27, 32, 41]. Their esters, as it is common for 1-hydroxo-1,1-bis(phosphonate esters), are prone to phosphonate–phosphate rearrangement [42]; however, coupling with the esters can be done in organic solvents. As both carboxylic acid and amine groups are the most frequently utilized functional groups for further modification, we decided to prepare suitable methylene-bis(phosphonate)-building blocks, which can be easily prepared in a gram scale, can be conjugated to other molecules and, thus, enable facile bone targeting of different substrates.

Synthesis of the Building Blocks

Two building blocks, containing bis(phosphonic acid) group and primary amine (**4**) or carboxylic acid functional groups (**7**), were prepared according to Scheme 1, and the identities of nitroprecursor **2** as well as that of final building block **4** were unambiguously determined by single-crystal X-ray diffraction analysis (Figs. 3 and 4). The amine derivative was described previously by Benedict [43]; however, the yield of the published synthesis was only 8%. A tetrakis(*P*-methyl ester) analogue of **1** and



SCHEME 1 Reagents and conditions: (*a*) NaH, toluene, RT, 2 h, followed by 4-NO_2-C_6H_4-CH_2Br, toluene, RT, 4 h, 72%; (*b*) aqueous HCl, 90°C, 24 h, 95%; (*c*) H_2, 10% Pd/C, atmospheric pressure, EtOH, RT, 94%; (*d*) aqueous HCl, 90°C, 24 h, 69%; (*e*) NaH, THF, RT, 2 h, followed by 4-BrCH_2-C_6H_4-CO_2Me, toluene, RT, 16 h, 75%; (*f*) LiOH, MeOH, RT, 12 days, followed by aqueous HCl, 59%; (*g*) aqueous HCl, 90°C, 3 days, 97%.

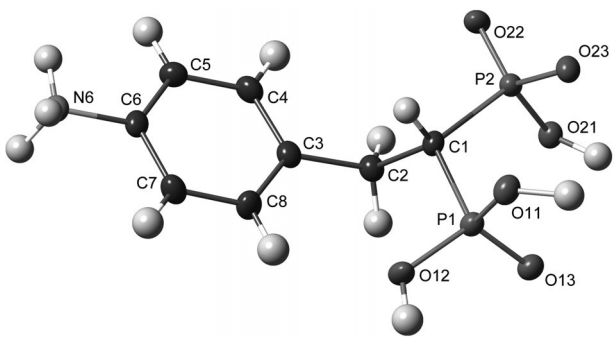
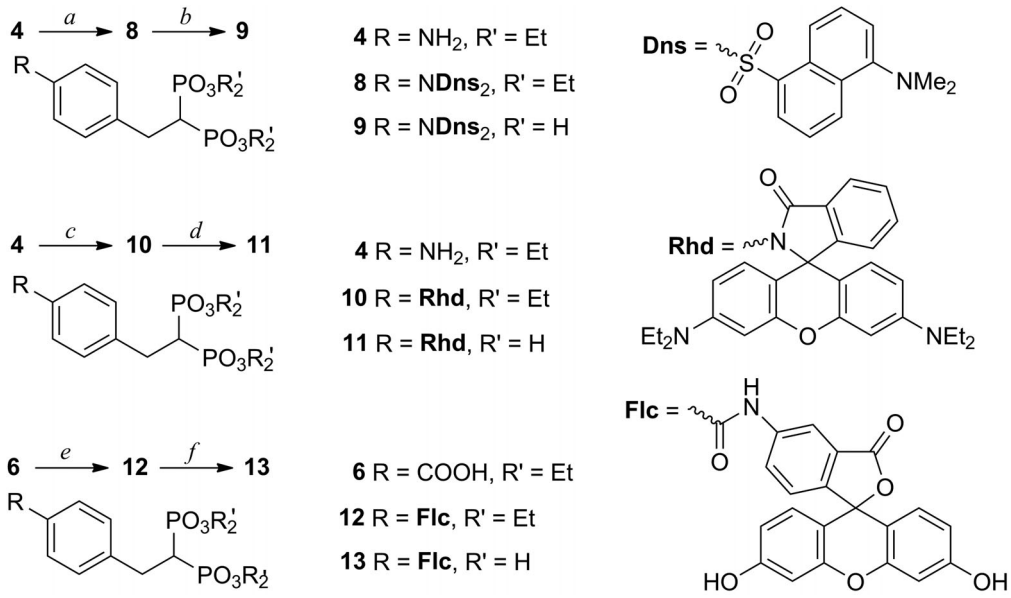


FIGURE 4 Molecular structure of **4** found in the crystal structure of **4**·H_2O.

3 as well as tetrakis(*P*-benzyl ester) analogue of **5** has also been prepared but in only moderate yields [29]. Utilization of compounds **4** and **7** could be advantageous as they are present in a form of the free acids and thus they do not require consequent deesterification. Both compounds are very well soluble in water (except weakly acidic pH in the case of compound **4**) and in DMSO. On the other hand, ethyl esters of these compounds (**3** and **6**) have the true potential of being useful starting material for further modification. They can be prepared in two steps from easily available tetraethyl methylene-bis(phosphonate) with moderate overall yields (68% and 44% for **3** and **6**, respectively). Their synthesis is entirely scalable, and these compounds can be obtained in approximately 90% purity without need of chromatography, which is of sufficient purity for further couplings. For their full characterization, they were purified by column chromatography; it leads to the lower overall yields mentioned in the Experimental. Generally, the phosphonate esters are cleaved by concentrated aqueous acids or bases at nearly reflux temperature or the esters can be conveniently removed by selective transesterification with trimethylbromosilane under nonaqueous conditions [44], followed by cleavage of the silylesters in water or alcohols.

Synthesis of Dyes

As bis(phosphonate)-containing dyes are studied as fluorescent probes for imaging of calcified tissues and microcalcifications [6], conjugates with very common fluorescent moieties were prepared. Three conjugates were prepared from the building blocks and commercially available dye precursors–dansyl chloride, rhodamine B and 5-aminofluorescein (Scheme 2). The reaction of amine-building block **4** with excess of dansyl-chloride in the presence of anhydrous potassium carbonate afforded conjugate **8**. Surprisingly, two bulky dansyl groups can be easily attached to one nitrogen atom by this procedure. Hydrolysis in hydrochloric acid was not suitable for preparation of free bis(phosphonate) due to the instability of the sulfamide bond under acidic conditions. Instead, transesterification with trimethylsilylbromide in dry acetonitrile, followed by aqueous hydrolysis of the silyl groups was utilized. Rhodamine



SCHEME 2 Reagents and conditions: (*a*) dansyl chloride, K_2CO_3, MeCN, RT, 5 days, 68%; (*b*) TMSBr, MeCN, RT, 24 h, followed by H_2O/MeCN, 92%; (*c*) rhodamine B hydrochloride, TBTU, TEA, MeCN, RT, 5 h, 65%; (*d*) TMSBr, MeCN, RT, 24 h, followed by MeOH/MeCN, 71%; (*e*) 5-amino-fluorescein, TBTU, TEA, MeCN, THF, RT, 12 h, 75%; (*f*) TMSBr, MeCN, DMF, RT, 24 h, followed by H_2O/MeCN, 78%.

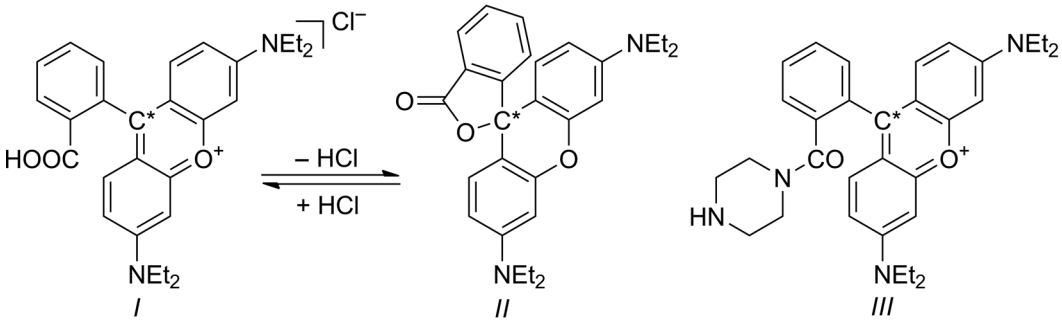


FIGURE 5 Equilibrium between acidic (*I*) and alkaline (*II*) forms of rhodamine B and the structure of the cationic piperazine derivative of rhodamine B (*III*) not undergoing the ring closure. The tautomeric states of the studied compounds were determined according to chemical shifts of the bridging carbon atoms (denoted C*).

derivative **11** was prepared by conjugation of building block **4** with rhodamine B hydrochloride using TBTU followed by the above-mentioned transesterification of resulting esterified product **10**. Similarly, fluorescein derivative **13** was prepared from building block **6** and 5-amino-fluorescein.

Both rhodamine and fluorescein dyes are sensitive toward ring-closing tautomerism generating less fluorescent internal lactones or lactams, respectively [45]. The equilibrium between the open form of rhodamine B (*I*, acid form) and the closed form of rhodamine B (*II*, alkaline form) is shown in Fig. 5. Since common 1D NMR spectroscopy could not distinguish between these two forms, in-depth 2D NMR measurements were performed to figure out the relationship between NMR parameters and the solution structure of the prepared conjugates. We found that chemical shift of one carbon atom (marked C* in Fig. 5) is very sensitive to the tautomerism. Similar analysis was then performed with compounds **10**–**13** and also with compound *III*, whose secondary amido group cannot undergo the ring-closing reaction (Fig. 5). Results are summarized in Table 2. It is clear that higher chemical shifts of C* atoms in the open structures correspond to the aromatic sp^2-hybridized carbon atom. Likewise, lower chemical shifts of C* carbons in the lactones and lactames reflect the aliphatic sp^3-hybridized carbon atom. It is worth to notice that the nature of solvent does not affect the chemical shift significantly. Thus, bis(phosphonated) dyes **10**–**13** were prepared in the closed form.

TABLE 2 Chemical Shifts of the C*-Atoms in the Studied Compounds

Compound	*Dye*	*Solvent*	δ ^{13}*C* (ppm)*	*Tautomer*
I[a]	Rhodamine	DMSO-d_6	159.1	Open
II[a]	Rhodamine	DMSO-d_6	85.3	Lactone
III	Rhodamine	$CDCl_3$	155.8	Open
10	Rhodamine	CD_3CN	66.7	Lactame
11	Rhodamine	DMSO-d_6	66.4	Lactame
12	Fluorescein	$CDCl_3$	84.3	Lactone
13	Fluorescein	DMSO-d_6	81.8	Lactone

[a]Commercially available.

TABLE 3 Excitation and Emission Maxima of the Synthesized Bis(phosphonated) Dyes (0.1 m HEPES buffer, pH 7.5)

Compound	*Dye moiety*	λ_{max}*(ex), nm*	λ_{max}*(em), nm*
9	Dansyl	299	495
11	Rhodamine	523	541
13	Fluorescein	558	586

Florescence spectra of compounds **9**, **11**, and **13** were measured. The maxima of excitation and emission wavelengths are summarized in Table 3. As compounds **11** and **13** are present in the solution in the lactam and lactone forms, respectively; their fluorescence has approximately one order of magnitude lower intensity than that observed for reference materials (the acidic form of rhodamine B and fluorescein, respectively).

Adsorption Behavior of the Studied Compounds

There are several models characterizing adsorptions onto surfaces [46]. The most common ones are the Langmuir and Langmuir–Freudlich isotherms

$$\frac{X}{X_m} = \frac{(Kc)^n}{1 + (Kc)^n}$$

where K is the affinity constant (dm^3 mol^{-1}), X_m the maximum adsorption capacity (mol m^{-2}), X the specific adsorbed amount (mol m^{-2}), c the equilibrium concentration in the solution (mol dm^{-3}), and n is a coefficient describing the adsorption energy distribution (Langmuir, $n = 1$; Langmuir–Freundlich, $0 < n < 1$). The simpler Langmuir model does not take into account the interaction between the adsorbed

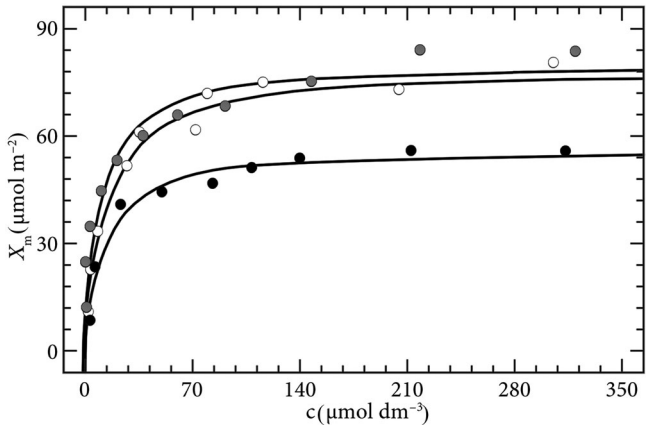


FIGURE 6 Adsorption isotherms of the prepared bis(phosphonate)-building blocks **2** (NO_2 ○), **4** (NH_2 ●), and **7** (COOH •) on HA (pH 7.4, 25°C). The curves correspond to the best fits obtained according to the Langmuir model.

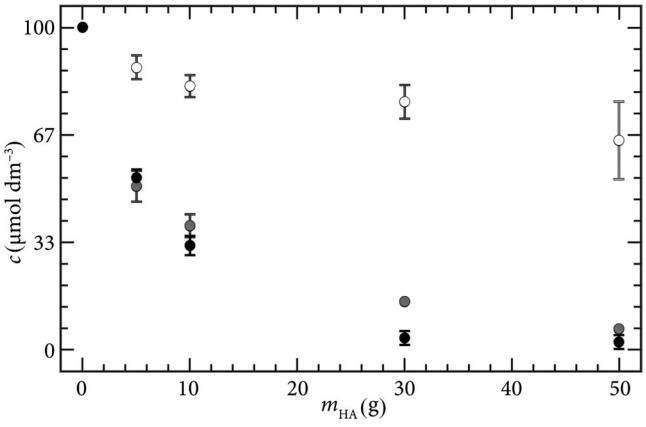


FIGURE 7 Decrease of the supernatant concentration of the dyes **9** (Dns ●), **11** (Rhd •), and **13** (Flc ○), with increasing amount of HA in the suspension.

molecules, but results obtained with the fitting according to the isotherm are usually satisfactory.

To characterize the adsorption behavior of the bis(phosphonate)-building blocks, HA suspension in water was utilized as a model of bone tissue. The vials with various total concentrations of the studied compounds in solutions were incubated with the constant amounts of HA. Adsorbed fractions of the bis(phosphonates) were obtained indirectly as the difference between their starting amount and the amount remaining in solution (as determined by UV–vis spectroscopy). The adsorption isotherms of the bis(phosphonate)-building blocks bearing nitro (**2**), amine (**4**), and carboxylic acid (**7**) groups are depicted on Fig. 6.

The affinity constants, K, and maximum adsorption capacities, X_m, obtained from the fitting are summarized in Table 4. The results prove strong adsorption of all studied compounds onto HA. Comparison of the adsorption parameters with bis(phosphonates) of the similar size (Table 4) supports the widely accepted fact that adsorption properties are determined mainly by the size of the molecule. Similar values of both parameters found for all three studied compounds indicate that the nature of the substituent on phenyl ring does not affect adsorption properties significantly.

The bis(phosphonate) dyes showed a slight shift of the absorption maximum with the increasing concentration. Furthermore, substantial changes in the UV–vis spectra over times were observed, apparently due to photobleaching of the studied compounds. This did not allow the precise quantification and determination of the adsorption parameters and, thus, sorption abilities of the title compounds were demonstrated in a different manner. The vials with the equal total concentration of the dyes were incubated with various amounts of HA. A decrease in the dye solution concentration with increasing amount of HA is depicted in Fig. 7. The results indicate that all compounds are adsorbed onto HA. Significant difference in the adsorption of the fluorescein derivative **13** in comparison with other two derivatives is probably the result of negatively charged phenolic groups leading to a repulsion between molecules on the surface. This is not the case of derivatives **9** and **11** containing tertiary amine groups, which could be uncharged under conditions of the adsorption experiments. Owing to the above-mentioned photobleaching, the adsorption time was reduced to 3 h only. Thus, the difference can also be ascribed to different adsorption kinetics of the studied compounds.

TABLE 4 Adsorption Parameters for the Prepared Bis(phosphonate)-Building Blocks and Other Bis(phosphonates) of the Similar Size on HA (pH 7.4, 25°C)

Compound	*2*	*4*	*7*	*PAM*[a]	*HEDP*[a]
M_r, g·mol^{-1}	311.0	281.1	310.1	235.1	206.0
K ($\times 10^4$, dm^3·mol^{-1})	11.6 ± 2.9	8.6 ± 1.7	9.1 ± 1.9	4.4	5.3
X_m ($\times 10^{-5}$, mol·g^{-1})	8.0 ± 0.4	7.8 ± 0.4	5.7 ± 0.2	11.5	9.9

[a]PAM = 3-aminopropyl-1,1-bis(phosphonic acid), HEDP = 1-hydroxyethyl-1,1-bis(phosphonic acid). Data are taken from the literature [47].

CONCLUSIONS

Two bifunctional bis(phosphonates), containing primary amine or carboxylic acid groups, were synthesized in high overall yields. Both compounds were prepared in the form of bis(phosphonate) ethylesters as well as in the form of free acids. Owing to the common availability of primary amine or carboxylic acid functional groups in various substrates, the synthesized building blocks can be easily attached to various substrates via amide-coupling reactions. Such derivatization was documented in the synthesis of three fluorescent bis(phosphonate) dyes. The conjugates were prepared by one-pot synthesis from tetraethylester of the bis(phosphonates) under nonaqueous conditions. In situ transesterification with trimethylsilylbromide followed by aqueous hydrolysis afforded corresponding free acids in high yields. NMR studies showed that the prepared fluorescein and rhodamine bis(phosphonate) derivatives are present in the lactone and lactame forms, respectively. It results in less intensive fluorescence compared with the starting dyes. All studied compounds show significant adsorption on HA; their affinity to HA is slightly higher than that of common bis(phosphonates) as pamindronate. The results show that the presented bis(phosphonate) derivatives could be considered as promising building blocks for modification of various substrates.

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