Bioorganic Chemistry

A Dendritic Tetra(bisphosphonic acid) for Improved Targeting of Proteins to Bone**

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Protein-based therapeutic agents are being actively pursued for the treatment of skeletal diseases.[1] As endogenous regulators of cellular activity, proteins have the potential to modulate cellular activity at skeletal sites to obtain a desired tissue response. For example, protein-based cytokines that can stimulate the formation of new bone or inhibit the cell activity that is responsible for bone loss will be beneficial for the treatment of diseases such as osteoporosis and arthritis. Despite the demonstrated efficacy of several proteins in preclinical disease models,[2] the clinical entry of most proteins has been hampered as a result of undesired side effects at extraskeletal sites. As systemically administered proteins are not naturally targeted to skeletal tissue, the desired efficacy is achieved only at exuberant doses of the protein by nonspecific deposition at skeletal tissues. In this case, pharmacological activity at extraskeletal sites becomes unacceptable. If proteins could be engineered to exhibit a strong affinity for bone, then systemic administration would allow delivery of the proteins selectively to the skeletal sites.

Bone affinity can be imparted to a molecule by chemical modification with bisphosphonic acids (BPs), a class of compounds with exceptionally high affinity for the bone mineral hydroxyapatite (HA). Bisphosphonic acids are chemical analogues of endogenous pyrophosphate in which the central, hydrolytically labile P-O-P linkage has been replaced by the hydrolysis-resistant P-C-P bond. Intravenous administration of molecules that contain bisphosphonic acid groups results in significant deposition (20–50 % of injected dose) of the molecules at bone tissue.[3,4] The exceptional affinity of bisphosphonic acid derivatives for minerals has been utilized to deliver radiopharmaceutical and imaging agents to skeletal tissue.[5,6] To target proteins to bone, we devised conjugation schemes by which 1-amino-1,1-diphosphonic acid (aminoBP)[7] and 2-(3-mercapto-propylsulfanyl)-ethyl-1,1-bisphosphonic acid (SH-BP)[8] were conjugated to proteins

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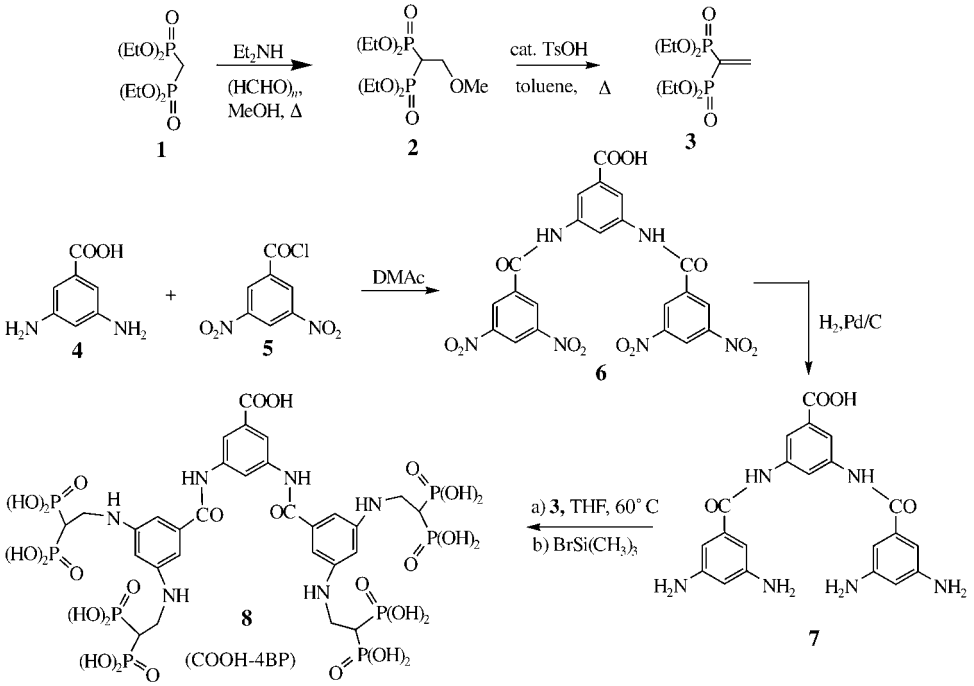
[**] Financial support was provided by the Canadian Institutes of Health Research (CIHR) and the Whitaker Foundation.

  DOI: 10.1002/anie.200500350

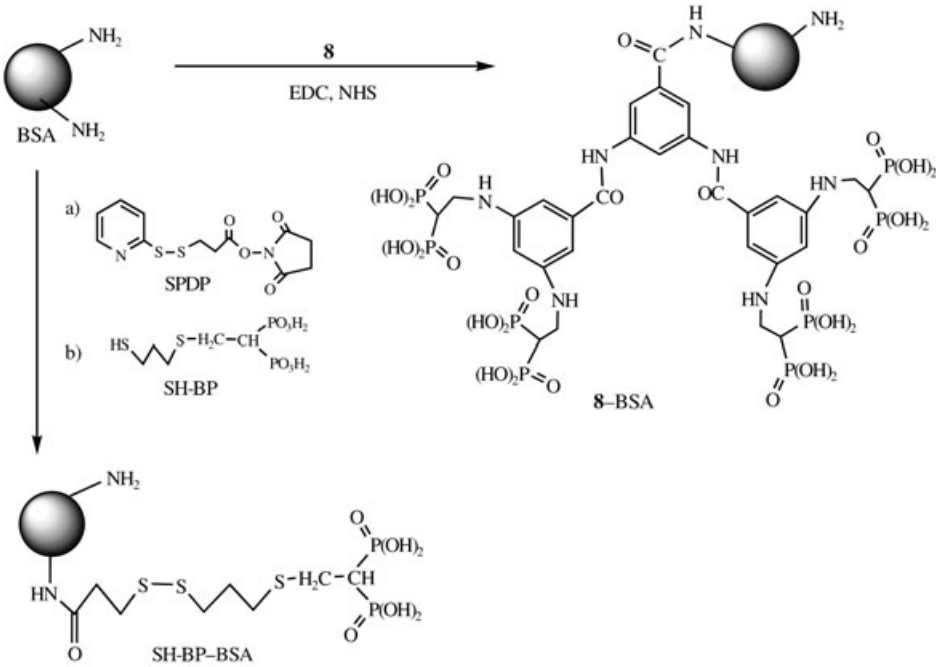
with heterofunctional linkers in aqueous medium.[9–14] The affinity of proteins for hydroxyapatite in vitro was shown to be proportional to the extent of conjugation of the bisphosphonic acid groups.[9,12,14] Intraosseous injection of the aminoBP conjugates confirmed the high affinity in vivo of the conjugates for bone[10] as well as their capability to "seek" bone tissue after systemic injection in rats.[11] The length of the tether between the bisphosphonic acid derivative and the protein was found to be an important factor for the affinity of the conjugates to minerals: conjugates with shorter tethers displayed a higher mineral affinity than those with longer tethers.[13] A higher density of the bisphosphonic acid ligand at the vicinity of proteins was postulated for the beneficial effect of shorter tethers. On the basis of these observations, the ideal approach to target bone would rely on creating a high density of bisphosphonic acids on proteins through short tethers. To this end, we have prepared a new dendritic molecule composed of four bisphosphonic acid moieties linked through a benzene ring (**8**, COOH-4BP; Scheme 1). This compound should provide a higher density of bisphosphonic acid groups on the proteins as four BP units are attached per protein site, unlike the conventional approach that relies on one-to-one linkage (Scheme 2). A carboxylic acid moiety was also incorporated into **8** to allow its conjugation to proteins through a short (zero carbon atom) tether using carbodiimide chemistry (Scheme 2).

The synthesis of COOH-4BP (**8**) is outlined in Scheme 1. Tetraethyl methylenebisphosphonate (**1**; 4 mmol) was converted into tetraethyl ethylidenebisphosphonate (**3**) by reaction, first, with formaldehyde (20 mmol) in diethylamine (0.42 mL) according to the method of Degenhardt and Burdsell to give the intermediate tetraethyl 2-methoxyethylene-bisphosphonate (**2**).[15] Methanol was removed from **2** by adding a catalytic amount of *p*-toluenesulfonic acid monohydrate to a solution of **2** in toluene and then heating the mixture at reflux with a Soxhlet apparatus (CaH_2) to give **3**, which was purified by chromatography on silica gel (60–270 mesh; 4:1 $CHCl_3$/hexane as eluent). Meanwhile, 3,5-bis(3,5-dinitrobenzoylamino)benzoic acid (**6**) was prepared from a mixture of 3,5-diaminobenzoic acid (**4**; 19.7 mmol) and 3,5-dinitrobenzoyl chloride (**5**; 43.4 mmol) in *N*,*N*-dimethylacetamide and was then reduced to 3,5-bis(3,5-diaminobenzoylamino)benzoic acid (**7**) with H_2 and 10% Pd/C.[16] Then compound **3** (1.6 mmol) was coupled to the amine moieties of **7** (0.4 mmol) by an anti-Markovnikov reaction in THF (15 mL) at 60 °C for 5 hours to give 3,5-bis[3,5-di(ethylamino-2,2-tetraethylbisphosphonate)benzoylamino]benzoic acid. After removal of the solvent, the residue was dissolved in CH_2Cl_2 and the phosphonate esters were hydrolyzed with bromotrimethylsilane ($BrSi(CH_3)_3$)[15] for 48 hours to afford the dendritic tetra(bisphosphonic acid) **8** (Scheme 1).[17]

Bovine serum albumin (BSA) served as the model protein for conjugation of the bisphosphonic acid derivative. Acid **8** was activated by treating equimolar concentrations (0–20 mM) of NHS and EDC in MES (morpholinoethanesulfonic acid) buffer (0.1 M, pH 4.5) for 45 minutes. The activated acid **8** (0.15–1.25 mM) was then incubated with BSA (5 mg mL^{-1}) in MES buffer for 3 hours, after which time the unreacted components were removed by dialysis against 0.2 M carbonate buffer (pH 10; ×4) and deionized water (×2). The dialyzed samples were further purified by gel permeation chromatography (Bio-Rad P2 gel-fine; 18 × 1.2 cm^2 column) with deionized water as eluent. Samples of BSA mixed with **8** in the absence of EDC/NHS as well as BSA conjugated to SH-BP[14] served as controls. These control samples were purified by dialysis only. All conjugates were analyzed for an average



Scheme 1. Synthesis of tetra(bisphosphonic acid) **8**. TsOH = *p*-toluenesulfonic acid, DMAc = *N*,*N*-dimethylacetamide.



Scheme 2. Conjugation of **8** (COOH-4BP) and SH-BP to model protein BSA. EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, NHS = *N*-hydroxysuccinimide, SPDP = *N*-succinimidyl 3-(2-pyridyldithio)propionate.

number of bisphosphonic acid substituents (i.e. the number of BP groups attached per BSA) by using the Bradford assay for protein concentrations[18] and the Ames assay[19] for phosphate concentrations.

SDS-PAGE (sodium dodecylsulfate–polyacrylamide-gel electrophoresis) analysis of the obtained proteins indicated the migration of protein as single bands, which confirmed a lack of protein–protein linking during the conjugation reactions (data not shown). The number of bisphosphonic acid substituents for a representative set of **8**–BSA conjugates ([EDC] = [NHS] = 0–20 mM; [**8**] = 1.25 mM) are summarized in Figure 1 a. The control sample of BSA ([EDC] = [NHS] = 0 mM) revealed 0.8 molecules of **8** per BSA protein which presumably represents the extent of free COOH-4BP (**8**) not removed by dialysis (or gel chromatography). The conjugates

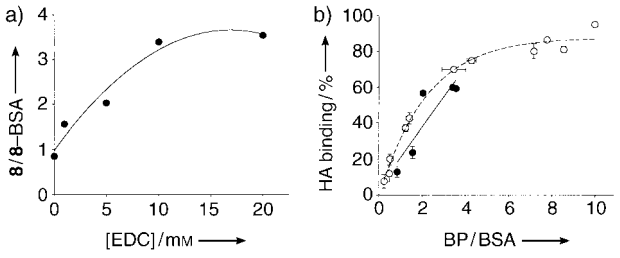


Figure 1. a) Number of tetra(bisphosphonic acid) substituents **8** per BSA conjugate upon coupling **8** (1.25 mM) to BSA in the presence of equimolar quantities of EDC and NHS ([EDC] = [NHS] = 0–20 mM). b) Correlation between the number of bisphosphonic acid (BP) substituents per BSA protein conjugate (mean ± SD) and the percentage binding to hydroxyapatite (HA; mean ± SD) in phosphate buffer (100 mM). Note the linear increase in the percentage binding of the conjugates at low numbers of BP groups for both SH-BP–BSA (○) and **8**–BSA (●). See Ref. [9] for details about the methods.

8–BSA showed an increasing efficiency of conjugation (up to 3.6 molecules of **8** per BSA) as the concentration of EDC and NHS was increased (Figure 1 a). The binding of the conjugates to hydroxyapatite was assessed in phosphate buffer (100 mM, pH 7.4), which was previously optimized for investigating such protein–HA binding.[7] Figure 1 b summarizes the relationship between the binding of the conjugates to hydroxyapatite and the conjugation efficiency (that is, the number of bisphosphonic acid groups per BSA) for SH-BP–BSA and **8**–BSA. Both conjugates exhibited an increased level of binding to hydroxyapatite as a function of conjugation efficiency. Whereas a plateau in the percentage binding to hydroxyapatite was evident with SH-BP–BSA, no such plateau was seen for **8**–BSA, partly as a result of our inability to obtain more than 3.6 bisphosphonic acid groups per BSA (two other independent reactions did not yield higher numbers of bisphosphonic acid substituents). This excellent correlation between the conjugation efficiency and the affinity for hydroxyapatite was in accord with the results obtained previously for aminoBP and SH-BP conjugations with hydroxyapatite.[9,14]

The conjugates were then analyzed for binding to hydroxyapatite and bone in the presence of serum proteins. Fresh bone matrix was obtained from Sprague–Dawley rats,[9] and the serum proteins originated from tissue-culture grade adult bovine serum. The use of bone matrix and serum proteins in the binding medium provides more stringent conditions for an assessment of binding and better represents the situation in vivo. The BSA conjugates for this analysis had similar levels of substitution by bisphosphonic acids: 3.4 and 3.6 for SH-BP–BSA and **8**–BSA, respectively. Owing to the presence of serum, ^{125}I-labeled conjugates were used for this analysis and were added to unlabeled (cold) proteins to give

approximately 10^6 cpm (counts per minute) at a concentration of 0.1 mg mL^{-1} protein (hot/cold = 1:100).[9] In 30 and 60% serum media, both conjugates exhibited a higher level of binding to hydroxyapatite than unmodified BSA (Figure 2a).

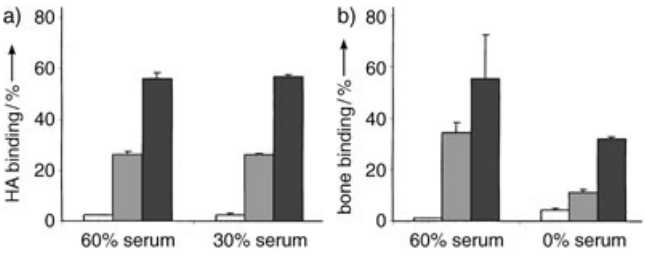


Figure 2. Percentage binding of BSA protein (□) and its conjugates (SH-BP–BSA ■, **8**–BSA ■) to a) HA and b) bone in serum-containing media (200 mM phosphate buffer). For both binding matrices, **8**–BSA gave the highest extent of binding, followed by SH-BP—BSA, while only a low level of binding was observed with the control (BSA protein) under all conditions. See Ref. [9] for details about the methods.

Conjugates prepared with **8** exhibited a higher level of binding to hydroxyapatite relative to SH-BP–BSA, despite the same number of bisphosphonic acid substituents. A similar result was obtained when hydroxyapatite was replaced by bone as the binding matrix (Figure 2b), with the **8**–BSA conjugate exhibiting superior levels of binding than the SH-BP–BSA conjugate.

Finally, the ability of these two conjugates to target bone was evaluated in rats. The ^{125}I-labeled conjugates (as above) were injected intravenously through the tail vein and bone (femora and tibiae), and deposition of the proteins was assessed by explanting the bones and determining the counts in these tissues. The depositions of the control BSA and the SH-BP–BSA conjugate at the bone were similar at both femora and tibia, which indicates a lack of targeting by the SH-BP–BSA conjugate (Figure 3). The presence of approximately 3.4 bisphosphonic acid substituents per BSA protein was not expected to impart a strong affinity to bone, as our previous studies required around 10 bisphosphonic acid groups per BSA for successful bone-targeting.[11] However,

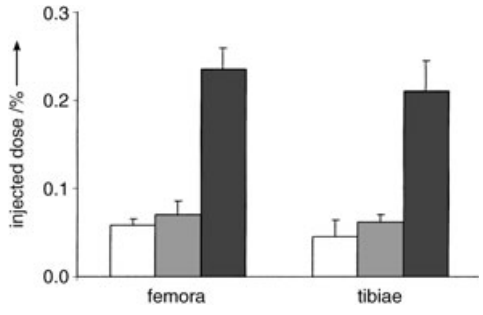


Figure 3. Delivery of BSA (control; □), SH-BP–BSA (■), and **8**–BSA (■) to femora and tibiae after intravenous injection in Sprague–Dawley rats ($n = 3$ per group). Bone-targeting was assessed one day after injection. Note the lack of bone-targeting by the SH-BP–BSA conjugate (i.e. similar to BSA) and the significantly improved bone-targeting by **8**–BSA. See Ref. [11] for details about the methods.

conjugate **8**–BSA, with a similar extent of bisphosphonic acid substituents, gave 4.1- and 4.7-fold higher delivery (relative to control BSA) at the femora and tibiae, respectively (Figure 3). A similar result was obtained in a repeat study in which the control BSA and **8**–BSA were injected subcutaneously: delivery increased by 3.7- (femora) and 3.4-fold (tibiae) as a result of conjugation of **8** (data not shown).

In conclusion, we have reported the synthesis of a novel, dendritic tetra(bisphosphonic acid) **8**. The synthesized molecule is introduced through a minimal tether length at the attachment site and provides a high density of bisphosphonic acid groups per protein site modified. Dendritic **8** gave higher numbers of total bisphosphonic acid groups attached per protein relative to SH-BP conjugates and exhibited its intended effect, namely bone-targeting, at a lower extent of protein modification which is an important consideration for maintaining the pharmacological activity of proteins when they are derivatized with bone-seeking ligands. The studies reported here should further stimulate the efforts to design "bone-seeking" proteins with minimal degree of modification.

Received: January 29, 2005
Published online: May 6, 2005

Keywords: bioorganic chemistry · bone-targeting · drug delivery · protein engineering

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[17] **2**: ^{1}H NMR ($CDCl_3$, J [Hz]): $\delta = 4.19$ (m, 8H), 3.90 (td, 2H, $J =$ 5.4, 16.2), 3.36 (s, 3H), 2.68 (tt, 1H, $J = 10.8$, 24), 1.31 ppm (t, 12H, $J = 9.3$); The hydrogen atom on the carbon atom in P-C_A-P resonates as a triplet of triplets, through coupling to two phosphorus and to two hydrogen atoms on the adjacent C_B atom, whereas the two hydrogen atoms on C_B couple with two

phosphorus nuclei to give a triplet that splits into a doublet by the hydrogen atom on C_A. **3**: ^{1}H NMR ($CDCl_3$, J [Hz]): $\delta = 7.00$ (dd, 2H, $J = 34.2, 33.9$), 4.15 (m, 8H), 1.32 ppm (t, 12H, $J = 6.9$); ^{13}C NMR ($CDCl_3$): $\delta = 149.01$, 132.03, 63.12, 16.2 ppm.[15] **6**: ^{1}H NMR (DMSO): $\delta = 11.08$ (s, 2H), 9.23 (s, 4H), 9.02 (s, 2H), 8.76 (s, 1H), 8.19 ppm (s, 2H); MS: $m/z = 539$ $[M-H]^-$. **7**: ^{1}H NMR (DMSO): $\delta = 10.11$ (s, 2H), 8.42 (s, 1H), 8.04 (s, 2H), 6.31 (s, 4H), 5.99 ppm (s, 2H), in agreement with those reported.[17] The addition of four ethylidenebisphosphonic acid groups to the four amine (NH_2) groups of **7** was confirmed by ^{1}H NMR spectroscopy and mass spectrometry. **8**: ^{1}H NMR (D_2O and NaOD, J [Hz]): $\delta = 7.94$ (m, 1H), 7.81 (m, 2H), 6.74 (m, 4H), 6.52 (m, 2H), 3.45 (m, 8H), 2.05 ppm (tt, 4H, $J = 3.3, 7.5$); ^{13}C NMR: $\delta = 147.52$, 138.96, 121.31, 88.44, 47.66, 40.51 ppm; ^{31}P NMR: $\delta = 18.1$ ppm; MS: $m/z = 1171$ $[M-H]^-$, 1173 $[M+H]^+$; IR: $\tilde{\nu} = 1202$ cm^{-1} (P=O).

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