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# A novel mercapto-bisphosphonate as an efficient anticalcification agent for bioprosthetic tissues

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**Abstract**

The synthesis of 2-(2-mercaptoethylamino)ethylidene–1,1-bisphosphonic acid (**2**) is reported (overall yield >90%), via nucleophilic addition of cystamine to vinylidene–bisphosphonic acid (**1**) followed by reduction of disulfide bonds with $Me_3P$. Reaction of **1** with cysteamine forms the isomeric 2-(2-aminoethylthio)ethylidene–1,1-bisphosphonate (**3**) in an almost quantitative yield. Thiol groups of **2** in water at pH 7 react with epoxy rings more than 30 times faster compared to the known 2-mercaptoethylidene–1,1-bisphosphonate. Elimination of the thiol group is observed as a side-reaction in the reduction of disulfides with phosphines. Stabilization of bioprosthetic tissues with triglycidylamine in the presence of **2** results in covalent immobilization of **2** via an epoxy-SH reaction; this inhibits the long-term calcification of bioprosthetic heart valve tissues to almost undetectable levels.


*Keywords:* Bisphosphonates; Nucleophilic addition; Phosphines; Thiols; Epoxides

## 1. Introduction

Bisphosphonates remain a focus of interest as candidate drugs for treatment of calcium-related disorders and arthritis [1–3]. 3-Amino-1-hydroxypropylidene–1,1-bisphosphonate (pamidronate) was successfully used to inhibit calcification of glutaraldehyde-treated bioprosthetic tissues [4]. Amino groups of pamidronate were covalently bound to the tissue via residual aldehyde groups.

We have become interested in the use of a similar approach to prevent calcification of heart valve bioprostheses stabilized with polyepoxide cross-linkers (particularly, triglycidylamine) as a promising alternative to glutaraldehyde [5]. In the conditions of tissue stabilization with triglycidylamine (pH near 7), the primary amino group of pamidronate is poorly reactive with the residual epoxy groups. To ensure an efficient covalent binding of bisphosphonate moieties to the residual epoxy groups of triglycidylamine-treated tissues, thiol-containing bisphosphonates should hypothetically be preferable to amino-bisphosphonates. Only a few geminal mercapto-bisphosphonates are reported in the literature. The syntheses of all these compounds are based on the nucleophilic addition to double bonds of either non-esterified vinylidene–bisphosphonic acid (**1**) [6] or **1** tetraethyl ester [7].

In this paper, we report the use of nucleophilic addition to the non-esterified **1** in the synthesis of a novel thiol-containing bisphosphonate, that is highly reactive towards the epoxy-ring opening and capable of efficiently reducing the in vivo calcification of triglycidylamine-treated bioprostheses.

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## 2. Results and discussion

Addition of various nucleophiles to the double bond of non-esterified **1** proved to be a versatile synthetic method for preparation of 2-substituted ethylidene–1,1-bisphosphonic acids. Amines [8], ammonia and hydrazine [9], thiols [10], thiourea [6], amino-acids [11], nitrogen-containing heterocycles [12] and polymers [13] were successfully reacted with **1** in polar media (water or AcOH). Tertiary phosphines, sulfinic acids, hydrosulfite and halogenide ions, aliphatic sulfides and alcohols also can be added to the double bond of non-esterified **1** [14]. Tetraesters of **1** were also used in the reactions of nucleophilic addition [7,15]. However, removal of ester groups was required in this case to prepare free bisphosphonic acids. Moreover, the products of nucleophilic addition to the non-esterified **1** showed much higher stability towards the reverse reaction than the corresponding tetraesters, especially in neutral and alkaline media.

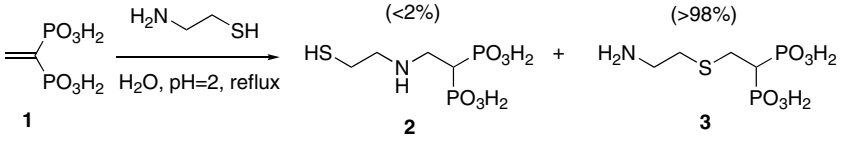
Using the described [6] procedure, we prepared 2-mercaptoethylidene–1,1-bisphosphonic acid and tested it in a model epoxy-ring opening (*tert*-butyl glycidyl ether in water at 20 °C and pH ca. 7). Surprisingly, the thiol group of this compound was found to be poorly reactive towards the epoxides ($k = 0.035$ l/mol h, whereas for cysteine $k = 1.62$ l/mol h). Based on these kinetic estimations, 2-mercaptoethylidene–1,1-bisphosphonate was deemed a suboptimal candidate for covalent binding to the residual epoxy groups of bioprosthetic tissues. We have hypothesized that the presence of an amino function in a β-position to the thiol group (as in cysteine) renders the thiol group especially active towards the epoxy-ring opening under the given conditions.

In a straightforward attempt to obtain a thiol-containing bisphosphonate with a structure resembling this of cysteine, namely 2-(2-mercaptoethylamino)ethylidene–1,1-bisphosphonic acid (**2**), we reacted an excess of cysteamine (a two-centered nucleophile) with **1** (Scheme 1). However according to $^{31}$P NMR analysis of the reaction mixture, the main product of the reaction ($^{31}$P: $\delta = 18.2$ ppm) was the isomeric 2-(2-aminoethylthio)ethylidene–1,1-bisphosphonic acid (**3**), formed in a yield of more than 98%. The acid **3** was easily isolated as an individual crystalline product. No unreacted **1** was found in the reaction mixture, the reaction is virtually irreversible under the given conditions.

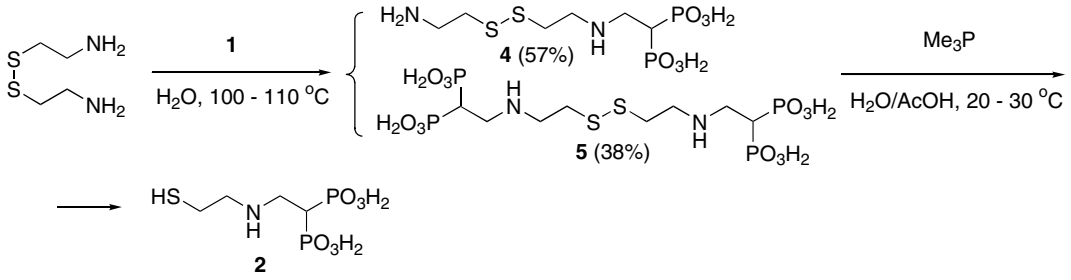
The amount of **2** in the reaction mixture was negligible (two close peaks with chemical shifts characteristic of **2** were observed: at $\delta = 15.57$ ppm, 1% and $\delta = 15.62$ ppm, 0.5%; the latter is most likely due to the tetraphosphonate with both the nucleophilic centers of cysteamine reacted). It became obvious that some kind of chemical protection is necessary to prevent the nucleophilic addition of the cysteamine thiol group (that is much more reactive than the amino group) to **1**. With this aim, we reacted **1** with the equimolar amount of cystamine (Scheme 2).

$^{31}$P NMR Analysis of the reaction mixture indicated that the disulfide bond is inert under the reaction conditions. Two main $^{31}$P signals were observed ($\delta = 16.4$ ppm, 57% and $\delta = 16.2$ ppm, 38%). We ascribed structure **4** to be the major product and the structure **5** to be the minor one. No compounds displayed the $^{31}$P chemical shifts characteristic of 2-alkylthioethylidene–1,1-bisphosphonates (near 18 ppm), and only 0.4% of unreacted **1** ($\delta = 11.7$ ppm) was found.

Tertiary phosphines, especially the water-soluble and odorless tris-2-carboxyethyl-phosphine (TCEP) [16]




Scheme 1. Reaction of **1** with an excess of cysteamine.



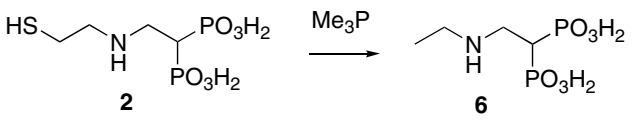

Scheme 2. Reaction of **1** with cystamine and the following reduction of disulfide bonds.

were reported as energetic reducing agents for disulfide bonds. We have preferred less polar trimethylphosphine for reduction of disulfide bonds in the reaction mixture containing **4** and **5**, to simplify the further purification. The reduction smoothly proceeded in water–AcOH under mild conditions. Both the compounds **4** and **5** resulted in the acid **2**, which was isolated as a crystalline compound. The bisphosphonate **2** has much higher water solubility than the isomer **3** (containing no thiol group) and readily decolorizes the solution of $I_2$ in KI. The isomers are clearly distinguishable in $^{31}P$ NMR (for **2**: $\delta = 16.0$ ppm; for **3**: $\delta = 18.2$ ppm) and in $^{1}H$ NMR (chemical shifts of the most characteristic diphosphonoethyl $CH_2$ in $D_2O$ at pD near 7: $\delta = 3.42$ ppm for **2** and $\delta = 3.00$ ppm for **3**).

An unexpected side-reaction was observed in the reduction of **4** and **5** to **2**. When the reaction medium was occasionally made insufficiently acidic (due to failure to add AcOH), a significant part of **2** (up to 15%) was transformed into 2-ethylaminoethylidene–1,1-bisphosphonic acid (**6**), as shown on Scheme 3. The impurity of bisphosphonate **6** (described previously [8], prepared by reaction of **1** with ethylamine) could be easily recognized in the $^{1}H$ and $^{31}P$ spectra of **2** crystallized from the reaction mixture. Purification of **2** from **6** was almost impossible.

Because tertiary phosphines are widely used in biochemical applications for reductions of disulfide bonds (in proteins, etc.), one should be aware about the possible desulfurization in the course of such reductions. We have found that other thiols (cysteine derivatives) also form analogous desulfurization products with other phosphines (TCEP) under similar conditions. Most likely, phosphines attack the already formed thiol groups, since the amounts of desulfurization products continue to increase even when no more disulfides remain in the reaction mixtures. The reaction can proceed as well in non-aqueous media (like MeOH), and basic conditions favor the reaction.

The bisphosphonate **2** was tested in the model epoxy-ring opening as described above. The kinetic estimations showed that the thiol group of **2** opens the epoxy ring more than 30 times faster ($k = 1.12$ l/mol h) compared to 2-mercaptoethylidene–1,1-bisphosphonate ($k = 0.035$ l/mol h) and almost as fast as the thiol group of cysteine ($k = 1.62$ l/mol h). Based on these results, we investigated **2** in the treatment of different heterograft bioprosthetic tissues simultaneously with triglycidylamine as a cross-linking agent. The bioprosthetic tissues stabilized




**2** **6**

Scheme 3. Desulfurization of **2** with an excess of trimethylphosphine.

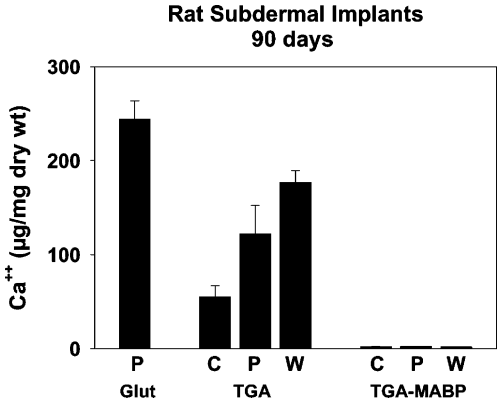



Fig. 1. Long term calcification of bioprosthetic tissues stabilized with different agents. Glut, glutaraldehyde; TGA, triglycidylamine; TGA-MABP, triglycidylamine and **2** P, bovine pericardium; C, porcine aortic cusp; W, bovine aortic wall.

with triglycidylamine in the absence of **2** and with glutaraldehyde served as controls. The tissues were implanted in rats subdermally [4], and the extent of calcification was estimated after 90 days from the calcium content in the explanted tissues (Fig. 1). As can be seen from the results, in the presence of **2** the calcification of bioprosthetic heart valve tissues is almost completely inhibited.

## 3. Experimental

Elemental analyses were carried out by Desert Analytics Laboratory (Tucson, AZ). A Bruker Avance DMX 400 spectrometer was used for recording the $^{1}H$ and $^{31}P$ NMR spectra. Tetrasodium salt of vinylidene–bisphosphonic acid (**1**) was kindly provided by Dr. Gary Woodward (Rhodia, UK). According to the $^{31}P$ NMR analysis, the salt was 93% pure, the main impurities being phosphate and pyrophosphate. Free **1** (>99% pure in $^{31}P$ NMR, close to monohydrate) was prepared from the salt as described previously [17].

### 3.1. 2-Mercaptoethylidene–1,1-bisphosphonic acid

2-Mercaptoethylidene–1,1-bisphosphonic acid was prepared according to the procedure described previously [17] from 2-(S-isothiuronio)ethylidene–1,1-bisphosphonic acid betaine [6]. Omitting the neutralization with tetrabutylammonium hydroxide, the eluate was concentrated in vacuo to a syrup. Attempts to crystallize the acid were unsuccessful, although the purity in $^{31}P$ NMR exceeded 99%. Characterization data: $^{1}H$ NMR ($D_2O$): $\delta = 2.60$(tt, 1H, $J = 6$ Hz, $J = 23$ Hz), 3.05(td, 2H, $J_d = 6$ Hz, $J_t = 17$ Hz). $^{31}P$ NMR ($D_2O$): $\delta = 20.3$(dt, $J_t = 17$ Hz, $J_d = 23$ Hz, $^{1}H$ decoupled: s).

### 3.2. Cystamine free base

Cystamine dihydrochloride (60.8 g, 0.27 mol) was dissolved in water (80 ml), then $Et_2O$ (300 ml) and THF (120 ml) were added. The mixture was cooled in an ice bath, and a 40% aqueous NaOH solution (400 g, 4 mol) was slowly added at the temperature not exceeding 15 °C. The organic layer was separated, the residue was additionally extracted with a mixture of $Et_2O$ (250 ml) and THF (90 ml). The combined organic layers were dried at 4 °C over NaOH, the desiccant was filtered off and washed with a mixture of $Et_2O$ (250 ml) and THF (50 ml), and the filtrate was dried in vacuo to give 40.8 g of residue. Vacuum distillation gave 34.7 g (85%) of pure cystamine base; b.p. = 85–87 °C at 0.1 mm Hg. The product (viscous colorless syrup) slowly decomposes at room temperature and should be stored at −20 °C. Characterization data: $^1$H NMR ($CDCl_3$): $\delta$ = 1.27(br, 4H), 2.70(m, 4H), 2.95(m, 4H).

### 3.3. 2-(2-Mercaptoethylamino)ethylidene–1,1-bisphosphonic acid (**2**)

To a solution of **1** hydrate (30.0 g, ca. 0.15 mol) in water (25 ml), cystamine base (23.95 g, 0.157 mol) was added portionwise. The mixture was dried on a steam bath at 100–200 mm Hg to a viscous homogeneous syrup (63.3 g) and further heated at 100–110 °C for 5 h. After cooling, the mixture was dissolved in water (200 ml), acidified with AcOH (17 ml, 0.30 mol), protected with the argon flow and cooled to 16 °C. Trimethylphosphine (27 ml, 0.26 mol) was added in two equal portions with a 5-min interval between them, while the temperature was not allowed to exceed 29 °C. The mixture was stirred at 20–22 °C for 1.5 h, filtered, diluted with water (60 ml) and briefly vacuum-concentrated at 30 °C, to partly remove the unreacted phosphine (about 40 g of water was distilled off). The residue was diluted with water to 560 ml, passed through Dowex 50WX8-40 in H-form, and eluted with water until neutrality. The eluate (ca. 1900 ml) was vacuum-concentrated at 30–45 °C to 267 g, and MeOH (240 ml) was added. The mixture (remaining homogeneous) was warmed to 35 °C, and crystallization of **2** was initiated by rubbing or seeding. An additional portion of MeOH (175 ml) was added after the end of crystallization, the suspension was protected with argon and left overnight at 4 °C. The crystals of **2** were filtered off, washed with MeOH–water (2:1 by volume), with MeOH, and dried in vacuo at room temperature, to afford crystalline **2** (36.84 g, 93%). The product was further purified by reprecipitation from water with MeOH. Characterization data: Anal. (%) Calc. for $C_4H_{13}NO_6P_2S$ (found): C, 18.12(18.24); H, 4.94(4.93) and P, 23.36(23.36); m.p. 205–210 °C (dec.). $^1$H NMR ($D_2O$): $\delta$ = 2.58(tt, 1H, $J$ = 7 Hz, $J$ = 21 Hz), 2.89(t, 2H, $J$ = 6 Hz), 3.33(t, 2H, $J$ = 6 Hz), 3.50(td, 2H, $J_d$ = 7 Hz, $J_t$ = 14 Hz). $^1$H NMR ($D_2O$ + $NaHCO_3$, pD 7): $\delta$ = 2.20(tt, 1H, $J$ = 6 Hz, $J$ = 20 Hz), 2.86(t, 2H, $J$ = 6 Hz), 3.26(t, 2H, $J$ = 6 Hz), 3.42(td, 2H, $J_d$ = 6 Hz, $J_t$ = 15 Hz). $^{31}$P NMR ($D_2O$): $\delta$ = 16.0(dt, $J_t$ = 14 Hz, $J_d$ = 21 Hz, $^1$H decoupled: s).

### 3.4. 2-(2-Aminoethylthio)ethylidene–1,1-bisphosphonic acid (**3**)

A solution of **1** hydrate (1.16 g, ca. 5.8 mmol) and cysteamine hydrochloride (1.59 g, 14.0 mmol) in water (1.0 ml) was partly neutralized with $Et_3N$ (0.84 ml, 6.0 mmol) to pH ca. 2 and heated at reflux for 3 h. The resulting thick crystalline suspension was diluted with water (6 ml), acidified with 12.1 M HCl (0.7 ml, 8.5 mmol), and MeOH (15 ml) was added. The crystals were filtered off, washed with MeOH, air-dried, and the crude 3 (1.60 g) was dissolved in water in the presence of $Et_3N$ and precipitated by acidification with an excess of HCl. Yield 1.50 g (97%). Characterization data: Anal. (%) Calc. for $C_4H_{13}NO_6P_2S$ (found): C, 18.12(18.01); H, 4.94(5.24) and P, 23.36(22.67); m.p. 280–285 °C (dec.). $^1$H NMR ($D_2O$ + $KHCO_3$, pD 6.5): $\delta$ = 2.14(tt, 1H, $J$ = 7 Hz, $J$ = 21 Hz), 2.90(t, 2H, $J$ = 6 Hz), 3.00(td, 2H, $J_d$ = 7 Hz, $J_t$ = 14 Hz), 3.25(t, 2H, $J$ = 6 Hz). $^{31}$P NMR ($D_2O$ + $KHCO_3$, pD 6.5): $\delta$ = 18.2(dt, $J_t$ = 14 Hz, $J_d$ = 21 Hz, $^1$H decoupled: s).

### 3.5. Desulfurization of **2** with trimethylphosphine

A solution of **1** hydrate (3.00 g, ca. 15 mol) and cystamine base (2.40 g, 15.7 mmol) in water was concentrated to a syrup (6.3 g) and reacted at 100–110 °C as described above in the preparation of **2**. The residue was dissolved in water (20 ml), and trimethylphosphine (4.5 ml, 42 mmol) was introduced without AcOH addition. The mixture was reacted at 20–22 °C for 2.5 h and treated as described above to generate 3.13 g of crystalline product. Reprecipitation (as above) gave 2.75 g of a crystalline mixture consisted of **2** (86% molar) and **6** (14% molar). $^1$H NMR of this mixture ($D_2O$) displayed signals typical for Et: $\delta$ = 1.30(t, $J$ = 7 Hz), 3.16(q, $J$ = 7 Hz). $^{31}$P NMR ($D_2O$, $^1$H decoupled): $\delta$ = 16.1(**2**), 16.2(**6**).

### 3.6. Kinetic estimations of epoxy-ring opening with thiols

Kinetic estimations were performed at 20 °C in aqueous solutions containing 0.1–0.2 M of substrates and 0.06–0.1 M of freshly distilled *tert*-butyl glycidyl ether under protection with argon. The solutions were buffered to pH 7 (phosphate buffer for cysteine and no additional buffer for bisphosphonates). After the definite reaction time, probes of the mixture were extracted with toluene, dried in vacuo below 20 °C, dissolved in $D_2O$, and subjected to the $^1$H NMR spectral analysis.

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