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Biphenyl Sulfonylamino Methyl Bisphosphonic Acids as Inhibitors of Matrix Metalloproteinases and Bone Resorption

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A number of matrix metalloproteinases (MMPs), proteins important in the balance of bone remodeling, play a critical role both in cancer metastasis and in bone matrix turnover associated with the presence of cancer cells in bone. Here, we report the synthesis and biological evaluation of a new class of MMP inhibitors characterized by a bisphosphonate function as the zinc binding group. Since the bisphosphonate group is also implicated in osteoclast inhibition and provides a preferential affinity to biological apatite, the new molecules can be regarded as bone-seeking medicinal agents. Docking experiments were performed to clarify the mode of binding of bisphosphonate inhibitors in the active site of MMP-2. The most promising of the studied bisphosphonates showed nanomolar inhibition against MMP-2 and resulted in potent inhibition of osteoclastic bone resorption in vitro.

Introduction

Bone is a dynamic tissue undergoing continuous remodeling. There are two types of bone cells, osteoblastic cells responsible for bone formation by synthesizing the components of the bone matrix and osteoclastic cells that facilitate bone resorption by degrading bone matrix. Under normal physiological conditions of skeletal remodeling, the activities of osteoclasts and osteoblasts are coordinated such that bone resorption is balanced by bone formation. During normal bone turnover, osteoclast-mediated bone resorption requires the solubilization of hydroxyapatite (HAP) and degradation of the type I collagen-containing organic matrix of bone; this is facilitated by the capacity of osteoclasts to secrete specific collagenolytic proteinases, including MMPs, to degrade fibrillar and/or denatured collagen.[1,2] Concerted actions of different MMPs (collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs)) are required to fully degrade all the components of the bone matrix during bone remodeling.[1,3,4] Although normal expression of MMPs and their regulation by bone-remodeling agents are essential for maintaining bone mass,[3] overexpression of MMPs appears to play critical roles in both cancer metastasis and bone matrix turnover associated with the presence of cancer cells in bone. Malignant cells of tumors that metastasize to bone are a rich source of MMPs contributing to bone degradation and tumor migration.[1] In pathological conditions, the imbalance between bone formation and resorption results in bone loss or excessive bone formation.[3] Tumor-induced bone disease is a common clinical feature of certain cancers, including breast, prostate, and lung carcinomas, for which the likelihood to develop bone metastases increases considerably and the incidence is as high as 70%.[5] The presence of metastatic cells in bone leads to a tremendous increase in bone matrix turnover. It has been proposed that stimulation of bone matrix turnover by metastatic cells may be responsible for the tendency of prostate cancer cells to thrive within the bone environment. The hypothesis is that a vicious cycle exists in which tumor cells stimulate matrix turnover and matrix turnover stimulates tumor growth.[4] Nemeth and coworkers[4] suggested that MMP inhibitors may disrupt the cycle of bone matrix turnover and tumor cell growth, thus acting also as potential therapeutic agents for certain bone diseases.[3]

In the last 20 years, a great variety of synthetic, low-molecular-weight MMP inhibitors (MMPIs) have been synthesized and

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tested, and some of them entered phase III clinical trials as anticancer drugs, although none has reached clinical utility. The general structure of an effective MMPI includes a zinc binding group (ZBG) capable to bind the catalytic zinc(II) ion of these proteinases, at least one functional group that provides crucial hydrogen bonding interactions with the enzyme backbone, and one or more side chains giving rise to effective van der Waals interactions with the enzyme subsites. The hydroxamic acid group is the most commonly used ZBG in inhibitor design.[6–8] Hydroxamate has been considered for long time the most potent ZBG that binds the catalytic Zn (II) ion in an ideal bidentate fashion,[9] and also forms effective hydrogen bonds with complementary functional groups in the enzyme active site. Recently, Cohen and co-workers identified new bidentate ZBGs that are more potent than hydroxamic acids,[9–11] some of which have been developed as potent inhibitors of MMPs.[11–13] With only a single coordinate bond to the metal center, inhibitors with monodentate ZBGs (such as carboxylic or phosphonic acids) are generally weaker inhibitors.[14,15]

A number of broad-spectrum synthetic MMPIs have been tested to evaluate their ability to inhibit bone resorption in a variety of different bone culture systems.[1,4,16] Treatment of tumor-bearing mice with batimastat, a non-selective MMPI, reduced tumor-associated osteolysis and tumor growth. In vitro treatment of these tumor cells with the same drug blocked the ability of the tumor cells to degrade osteoblast-like matrices and reduced the number of resorption pits generated in bovine cortical bone.[17] In further support of the importance of MMPs in the bone metastatic process, treatment of breast cancer-bearing mice with the general MMP inhibitor GM6001 led to a reduction in bone breakdown and tumor growth.[18] Furthermore, the zinc chelator 1,10-phenanthroline or the physiological MMP inhibitors TIMP-1 and TIMP-2 reduced migration of the aggressive breast cancer cells through the bone marrow fibroblast layer.[19]

We have been studying non-hydroxamic MMPIs for a long time,[20–26] with a particular attention towards phosphonic derivatives.[20,22,23,25] As an extension of our phosphonate program, we report herein the synthesis of new analogues containing the bisphosphonic group as a ZBG (Figure 1) and their biological evaluation against various MMPs and against the macrophage cell line J774, a model system to screen the inhibitory effects on osteoclast activity.[27,28] Moreover, we report the activity of the most promising compounds against osteoclastic bone resorption in vitro.

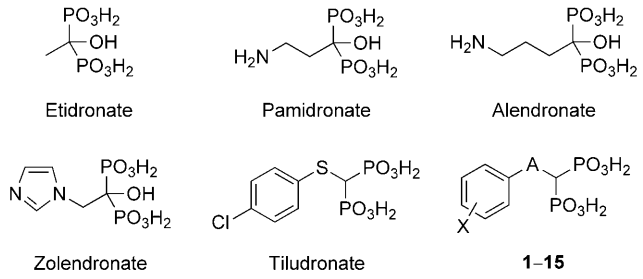


Figure 1. Chemical structures of bisphophonate derivatives. Substituents X and A in **1**–**15** are presented in Table 2.

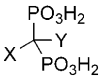
The bisphosphonic moiety is already present in some well-known potent inhibitors (bisphosphonates, BPs) of osteoclast-mediated bone resorption that have demonstrated clinical utility in the palliative treatment of bone metastases.[29] Like inorganic pyrophosphate, BPs are capable of binding divalent cations, such as Ca^{2+}[30] and Zn^{2+} ions,[31] by coordination from the two phosphonate groups. Their ability to chelate Ca^{2+} ions is the basis for the bone-targeting property of BPs. It is now becoming clear that bisphosphonates exhibit direct and indirect antitumor effects in preclinical models, inhibit tumor cell adhesion to mineralized bone, and prevent invasion and proliferation of metastatic tumor cells.[30] Inhibition of tumor cell invasion by BPs seems to occur through two distinct mechanisms: at low concentrations (10^{-8} to 10^{-6} M), BPs inhibit the mevalonate pathway, whereas at higher concentrations (10^{-4} M) they inhibit the activity of MMPs.[4,5,32,33] Reported findings indicate that known BPs are moderate inhibitors of MMPs.[30,32,33] For example, Heikkilä and co-workers[33] reported that clodronate and alendronate, when used at therapeutically attainable non-cytotoxic concentrations, inhibited MMP-1, -2, -3, -8, -9, and -13 in a dose-dependent manner with IC_{50} values in the micromolar range. Boissier and colleagues[32] reported the activities of clodronate, ibandronate, and zolendronate against MMP-2, -9, and -12. The results of both studies suggest that the phosphonate groups of BPs could inhibit the proteolytic activity of MMPs through zinc chelation.

Results and Discussion

In order to better explore the ability of the bisphosphonic moiety to inhibit the hydrolytic activity of MMPs, we decided to synthesize the simplest bisphosphonic acid, methylene bisphosphonate (MBP), and to compare its activity against MMP-2 with that of acetohydroxamic acid (AHA), which is commonly used as the representative chelator for the majority of current MMPIs.[34,35] MBP was about 200-fold more potent on MMP-2 than AHA ($IC_{50} = 49 \pm 10$ μM and 12 ± 1.7 mM, respectively), thus suggesting that inhibitors containing bisphosphonic moieties have the potential for even greater binding affinity than those containing a simple hydroxamic acid group.

We then decided to evaluate the inhibitory activities of some clinically relevant bisphosphonates on MMPs (Figure 1); for this purpose, we synthesized two non-nitrogen-containing BPs (etidronate and tiludronate), two amino-alkyl BPs (alendronate and pamidronate), and one heterocyclic amino BP (zolendronate) following previously reported procedures,[36,37] and determined their activity against MMP-2, -8, -9, and -14 (Table 1). These MMPs were selected due to their critical role in mediating skeletal development and remodeling. Etidronate, despite its very simple structure and presumed lack of interaction within the S_1' pocket of MMPs, showed a relatively good activity against MMP-2, -8, and -14. This activity, like that of MBP, seems to be caused only by the bisphosphonic moiety and confirms the validity of this moiety in the design of MMPIs. The introduction of an amino-alkyl substituent on the

Table 1. Inhibition data for commercial bisphosphonates against MMPs.



Compd	X	Y	IC_{50} [μM] MMP-2	MMP-8	MMP-9	MMP-14
Etidronate	CH_3	OH	27.7 ± 3.2	15.4 ± 5.7	80 ± 13	6.6 ± 0.6
Pamidronate[a]	$NH_2CH_2CH_2$	OH	> 100	> 100	> 100	28 ± 3
Alendronate[a]	$NH_2CH_2CH_2CH_2$	OH	> 100	> 100	> 100	29 ± 5
Zolendronate	1-Imidazole-CH_2	OH	7.0 ± 1.3	17.6 ± 4.6	52 ± 6	12.6 ± 0.1
Tiludronate	4-Cl-C_6H_4-S	H	7.2 ± 0.5	32 ± 3	> 100	30.5 ± 1.6

[a] sodium salts.

side chain (alendronate and pamidronate) led to a decrease in inhibitory activity on all tested MMPs in comparison with that of etidronate. Zolendronate, at present the most potent BP in the treatment of bone disease, showed an activity in the range of 7–52 μM against MMP-2, -8, -9, and -14.

It is now widely accepted that a hydroxy group in the α position gives an additional contribution to the HAP binding of the bisphosphonic moiety. In order to evaluate whether the hydroxy group is also essential for the inhibition of MMP activity, we decided to synthesize and evaluate tiludronate against the same enzymes. Despite the lack of the hydroxy group, its activity against MMP-2 was similar to that of zolendronate; interestingly, tiludronate displayed a higher MMP-2/MMP-9 selectivity.

To rationalize the observed activity data, all synthesized compounds were docked into the active site of all tested MMPs using the software Glide[38] and applying the XP (extra precision) mode that, with an extensive sampling and advanced scoring, provides a more accurate binding mode prediction.[39] As expected, MBP is able to bind the catalytic zinc ion of MMPs in a bidentate fashion (Figure 2a), forming two hydrogen bonds with the carboxylic oxygen atoms of Glu202 (MMP-2 numbering is used as a reference throughout; similar interactions occur with the corresponding amino acids of the other MMPs, unless indicated otherwise). Zinc chelation is observed for MBP only; by contrast, none of the other investigated ligands show the same binding mode. The introduction of a substituent on the methylene carbon induces monodentate binding of the zinc ion, as seen with other previously reported phosphonic acids.[25] The correctness of the zinc binding mode of the phosphonate moiety was assessed by comparing the predicted docked poses of the ligands with the bind conformation observed for the phosphonic ligand in the crystallographic complex with MMP-8 (PDB code: 1ZVX), since docking software usually perform poorly in the prediction of the zinc coordination geometry.

For the commercially available BPs (Table 1), the second phosphonic group is hydrogen bonded with the backbone NH of Leu164 and the backbone NH and CO of Ala165 of MMPs.

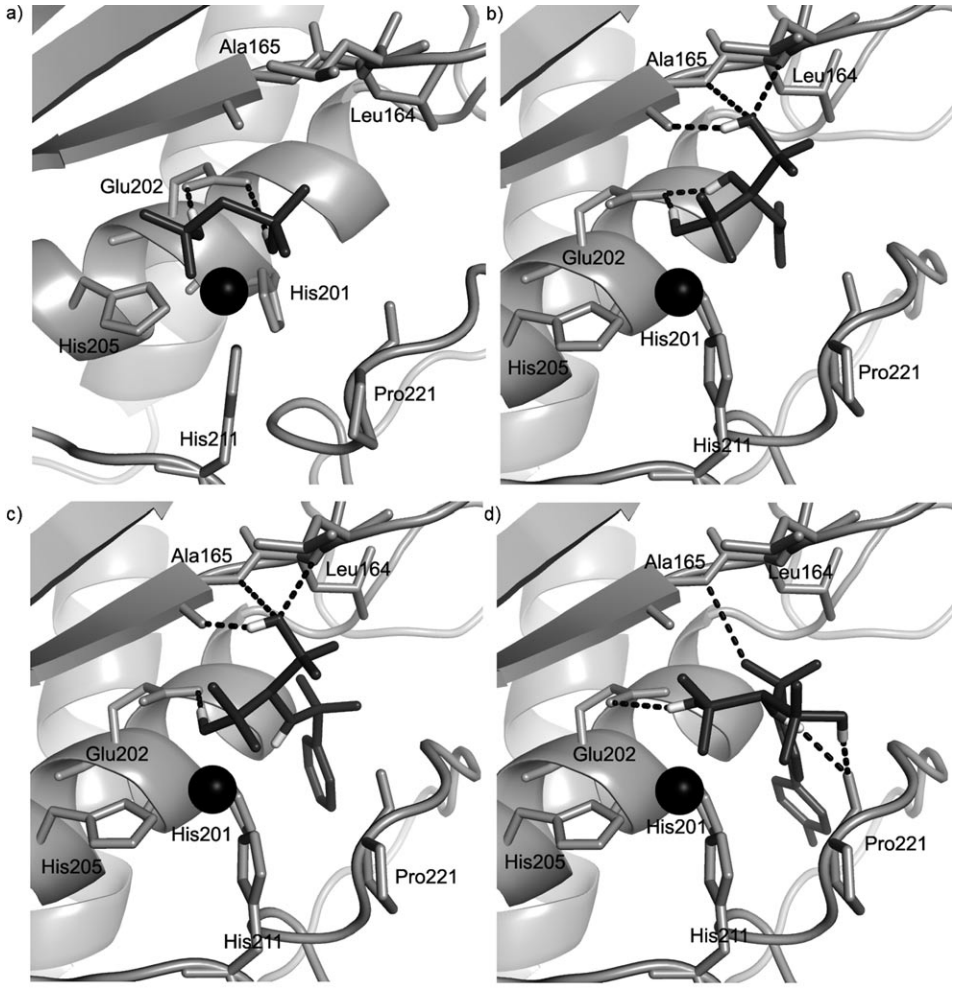


Figure 2. Best docked pose of a) MBP, b) zolendronate, c) **2**, and d) **15** into the active site of MMP-2. In ligands, polar hydrogen atoms are shown as white sticks, while nonpolar hydrogen atoms are not shown for sake of clarity; all other atoms are represented as dark grey sticks. The zinc ion is represented as a sphere, and most relevant residues of MMP-2 are displayed as light grey sticks. Hydrogen bonds are depicted as dashed lines.

The hydroxy group at the α position is hydrogen bonded to the carboxylate of Glu202. In this way, the substituent on the methylene carbon can move towards the entrance of the S_1' subsite (Figure 2b). A more effective interaction at this site could account for the higher activity observed for zolendronate as compared with those of the amino-alkyl derivatives. Interactions provided by alendronate and pamidronate may not be sufficient to counterbalance the conformational energy spent by the flexible alkyl side chain for binding (data not shown). The higher activity of these two compounds against MMP-14 can be ascribed to the relatively different active site arrangement of MMP-14 with respect to the other MMPs (the ω-loop is closer to the upper rim and the catalytic glutamate is moved away from the zinc ion), which allows the terminal amino group of alendronate and pamidronate to form a hydrogen bond with the carboxylate of Glu202.

Data reported in Table 1 show that currently available BPs seem to be slightly selective inhibitors of MMPs and are characterized by an activity far below the most potent hydroxamic or monophosphonic inhibitors.[6,20,23]

Given the low potency of the commercial BPs against MMPs (Table 1), we selected tiludronate as a lead compound and investigated the effects resulting from the replacement of the sulfur atom with sulfone, carboxyamide, or sulfonamide moieties in an attempt to identify more promising inhibitors (Table 2). Sulfone and sulfonamide groups have often been incorporated in MMPIs to improve enzyme–inhibitor binding, not only by forming favorable hydrogen bonds at the S_1' pocket entrance of the MMP catalytic center, where leucine and alanine residues are located, but also by properly directing the hydrophobic substituent to the S_1' pocket and enabling it to plunge in deeply.[40] Moreover, the biphenylsulfonamide moiety has been recently used in a series of non-bisphosphonic inhibitors of bone resorption.[27]

A set of new aryl sulfonamide bisphosphonic derivatives (**1**–**15**), with different P_1' substituents, were developed and evaluated following a simple synthetic approach (Table 2). The replacement of the sulfur atom of tiludronate (Table 1) with the sulfonamide moiety (**1**, Table 2) improved the activity against MMP-8, -9, and -14. The displacement of the chlorine atom from the aromatic ring of this derivative resulted in a less active compound **2**, whereas its substitution with bromine (**3**) improved the activity against MMP-2 and MMP-14. The replacement of the sulfonamide group in **3** with carboxyamide (**4**) caused a decreased inhibition for all tested MMPs. Its substitution with sulfonylmethyl (**5**) had only a slight effect with regard to MMP inhibitory activities, while the removal of the methylene spacer (**6**) caused a decrease in activity, especially against MMP-8.

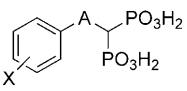
Next, we investigated whether the substitution pattern of the aromatic ring was important for the inhibitory activity of these compounds. The presence of substituents with pronounced electronic effects on the aromatic ring affects the electronic properties of both the nitrogen and sulfur of the sulfonamide group[40] and therefore they could, in fact, improve the interaction of MMPIs with the enzyme. 4-Methyl (**7**), 4-methoxy (**8**), and 3-nitro (**9**) groups had very little effects on the potency, while the introduction of the nitro group in the *para* position (**10**) increased the potency against MMP-2 about threefold as compared to that of unsubstituted analogue **2**.

Docking studies, performed as reported above, show that derivatives **1**–**10** bind MMPs with the bisphosphonate portion as previously described for the commercial BPs: one phosphonic group binds to the zinc ion in a monodentate fashion and forms a hydrogen bond with the catalytic Glu202, while the second one interacts with the backbone NH of Leu164 and backbone NH and CO of Ala165 (Figure 2c).

The sulfone or sulfonamide groups are not involved in hydrogen-bond formation with NH of Ala165 and Leu164; instead, these residues interact with the second phosphonic group of the ligand. In some cases, the sulfonamide NH can form a hydrogen bond with the CO of Pro221. The phenyl ring of these inhibitors interacts with His201 in the S_1' site through π–π stacking.

With the aim of further increasing the inhibitory activity against MMPs, we prepared the biaromatic analogues **11**–**15**, in which the sulfonamide group was maintained, to evaluate the effects of the additional aromatic ring on van der Waals

Table 2. Inhibition data for bisphosphonates **1**–**15** against MMPs.



Compd	X	A	IC_{50} [μM]			
			MMP-2	MMP-8	MMP-9	MMP-14
1	4-Cl	SO_2NH	9.5 ± 0.7	12 ± 4	76 ± 11	25 ± 7
2	H	SO_2NH	15.8 ± 2.4	23 ± 5	> 100	46 ± 4
3	4-Br	SO_2NH	4.8 ± 0.6	13 ± 3	> 100	9.4 ± 1.1
4	4-Br	CONH	30 ± 1.4	19 ± 4	> 100	41 ± 8
5	4-Br	SO_2CH_2	14.8 ± 0.4	5.0 ± 0.9	> 100	6.5 ± 0.5
6	4-Br	SO_2	52 ± 4	> 100	> 100	20.0 ± 1.4
7	4-CH_3	SO_2NH	18 ± 6	50 ± 4	> 100	14.1 ± 0.1
8	4-CH_3O	SO_2NH	19 ± 2.8	15 ± 2	> 100	27.6 ± 2.6
9	3-NO_2	SO_2NH	16 ± 2.8	15.9 ± 2.3	> 100	11.6 ± 2.1
10	4-NO_2	SO_2NH	4.9 ± 1.3	15.4 ± 1.2	> 100	26 ± 5
11	4-C_6H_5	SO_2NH	0.14 ± 0.04	0.40 ± 0.03	> 100	4.6 ± 1.2
12	4-(2-thienyl)	SO_2NH	0.23 ± 0.06	> 1[a]	> 1[a]	> 1[a]
13	4-(4-CH_3O-C_6H_4)	SO_2NH	0.11 ± 0.03	0.49 ± 0.03	> 1[a]	> 1[a]
14	4-(4-NO_2-C_6H_4)	SO_2NH	0.192 ± 0.002	0.36 ± 0.06	> 10	1.3 ± 0.1
15	4-(4-Cl-C_6H_4)	SO_2NH	0.037 ± 0.008	0.32 ± 0.05	> 1[a]	> 1[a]

[a] This compound showed fluorescence above 1 μM.

interactions within the S_1' pocket. The biphenyl analogue **11** was about 110- and 60-fold more active against MMP-2 and -8, respectively, as compared to the phenyl analogue **2**, showing also a very good selectivity against MMP-9. The substitution of the distal phenyl moiety with a thiophene ring (**12**) and the introduction of a methoxy (**13**) or a nitro group (**14**) in the *para* position had a very little effect, while the introduction of a chlorine atom at the same position (**15**) led to a further increase in inhibitory activity against MMP-2 and a very interesting selectivity over MMP-9 and -14.

Docking calculations carried out for compounds **11**–**15** show that the presence of the biaromatic system induces a slightly different binding mode of the bisphosphonate moiety to MMPs: although the first phosphonate group still coordinates the zinc ion, the second one is now directed towards the ω-loop and forms a hydrogen bond with the CO of Pro221. In this configuration, the sulfone group can move closer to Ala165 and Leu164 NH and form a hydrogen bond with these residues of the enzyme. The biphenyl group is inserted into the S_1' site as expected (Figure 2d).

The more extended hydrophobic interactions account for the higher activity observed for these compounds against MMPs with a deep S_1' site (e.g., MMP-2 and MMP-8), while the binding against MMPs with a shorter S_1' site (e.g., MMP-9 and MMP-14) is unfavored; however, it is worth noting that **14** presents a hydrogen bond between the 4-NO_2 group and the NH_2 of Gln262 of MMP-14, yielding the most active ligand towards this enzyme. The nanomolar activity of compound **15** against MMP-2 can be attributed to the already discussed hydrophobic interactions in the S_1' site (the preference for the 4-Cl substituent on the distal aromatic ring has already been observed for this enzyme);[20] moreover, **15** is the only compound among the sulfonamide derivatives examined that forms an additional hydrogen bond between the sulfonamide NH and the CO of Pro221. This interaction has not been observed for other investigated MMPs.

Due to the well-known activity of BPs on osteoclasts, we decided to also evaluate the activity of our new BPs on osteoclast-mediated bone resorption. Since osteoclast inhibition assays are time-consuming and unsuitable for large screening, we conducted an initial screening of our BPs in cultures of the mouse macrophage cell line J774 (Figure 3). This cell line has been already used as a model system to screen the inhibitory effects of BPs on osteoclast activity.[27,28] J774 cells were chosen because the structure–activity relationships of BPs for reducing J774 cell viability closely match the structure–activity relationships for inhibiting bone resorption in vivo.[28]

The inhibitory effects of all bisphosphonates investigated in the present study on osteoclast activity were assessed on J774 cells by using the MTT assay (Table 3). The IC_{50} value obtained for etidronate confirmed its previously reported low antiresorptive potency,[41] while the introduction of nitrogen-containing substituents on the side chain led to higher inhibition of J774 cell viability (pamidronate, alendronate, and zolendronate). Tiludronate showed a fivefold decrease in potency against J774 as compared with zolendronate.

Table 3. IC_{50} values of bisphosphonate derivatives as assessed by J774 and HepG2 survival assay.

Compd	IC_{50} [μM]		Compd	IC_{50} [μM]	
	J774	HepG2		J774	HepG2
Etidronate	>100	n.d.[b]	**6**	75±6	n.d.
Pamidronate[a]	13±1	n.d.	**7**	95±25	n.d.
Alendronate[a]	30±6	n.d.	**8**	67±22	n.d.
Zolendronate	7.8±1.6	8±3	**9**	>100	n.d.
Tiludronate	37±8	>100	**10**	>100	>100
1	70±10	n.d.	**11**	4.9±1.1	>100
2	80±5	n.d.	**12**	15±3	>100
3	>100	n.d.	**13**	12±1	>100
4	>100	n.d.	**14**	32.3±1.1	>100
5	>100	n.d.	**15**	1.7±0.3	>100

[a] sodium salts. [b] n.d., not determined.

The inhibitory effects of these commercial BPs on osteoclast activity appear to reflect quite well the rank order of their kinetic binding affinity to HAP (etidronate < alendronate < pamidronate < zolendronate) and their antiresorptive potency observed in animals.[41] Some of our sulfonamide bisphosphonate derivatives showed an inhibitory activity against the J774 cell line that was higher (**11** and **15**) or comparable (**10**, **12**, and **13**) to that of zolendronate, one of the most potent BPs. We also conducted the MTT assay with the hepatocyte tumor cell line HepG2 for all compounds; the observed lack of activity (Table 3) suggested that the effects of these BPs were selective on J774 and not due to indiscriminate cytotoxicity.

The most promising agents **11** and **15** were then selected for further investigation. The effects of **11** and **15** on the survival of murine osteoclasts (OCs) were studied and compared with those of zolendronate and compound **10** as representatives of less active compounds. Osteoclasts were generated from bone marrow macrophages plated on plastic (Figure 3a) or bone (Figure 3b and c) in the presence of RANKL (100 ng mL^{-1}) and M-CSF. Each of the four compounds was added for 48 h, and osteoclast number and cell morphology were evaluated. Osteoclasts were efficiently killed by treatment with zolendronate, **11**, and **15** (Figure 3a and d); the same compounds also abolished the formation of actin rings (Figure 3b and e), the functional structure typical of resorbing osteoclasts and inhibited bone resorption (Figure 3c and f). Compound **10** reduced the total number of OCs (Figure 3a and d) and the number of OCs with regular actin rings by about 25% (Figure 3b and e); furthermore, this compound reduced bone resorption by about 90% (Figure 3c and f). Our data therefore confirm the good correlation between J774 inhibition and OC viability and functionality.

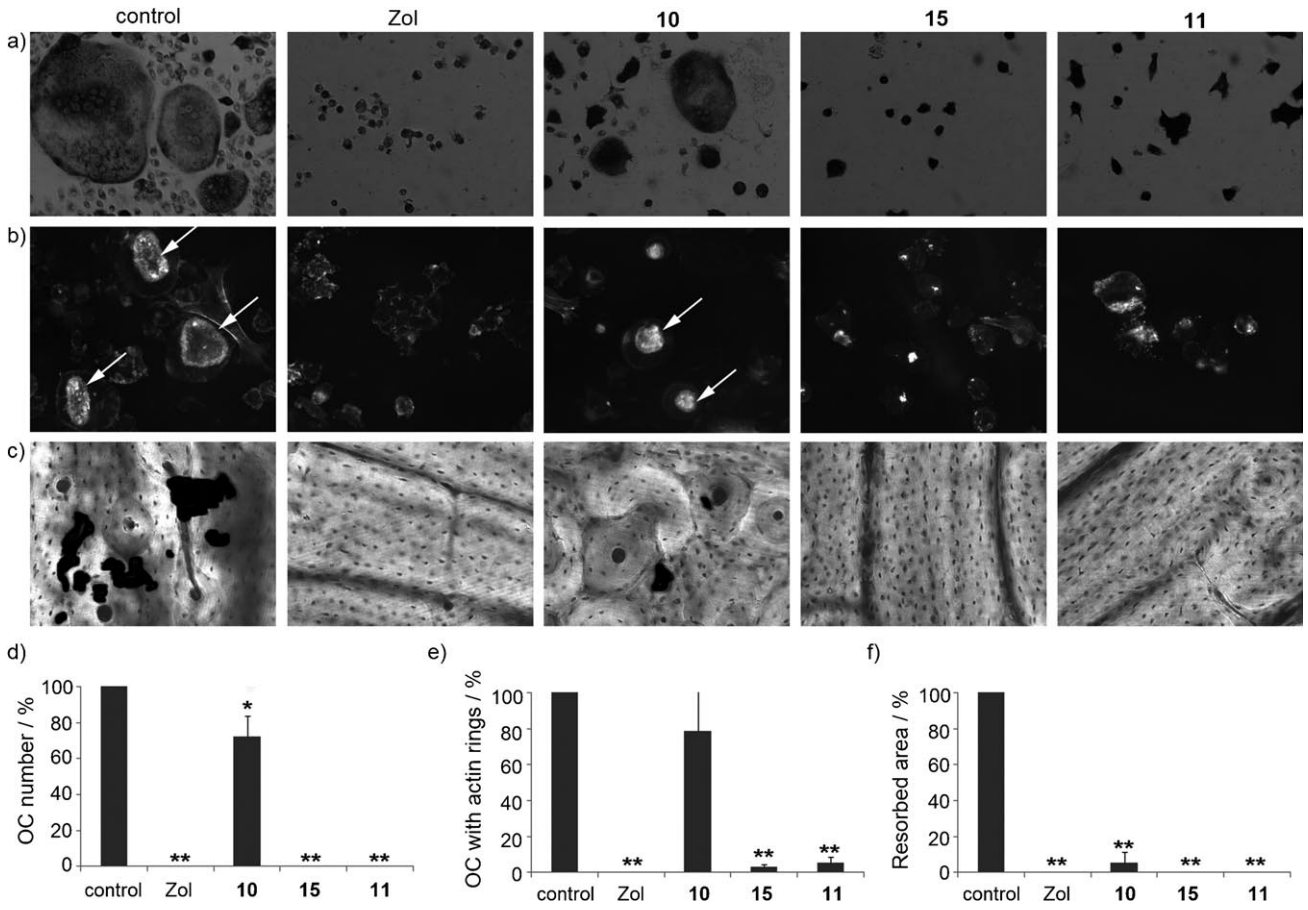
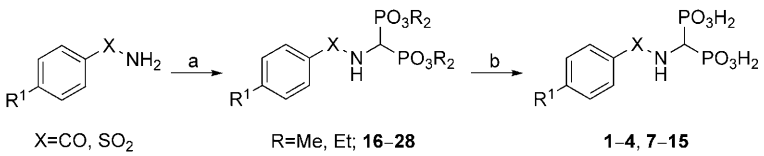


Figure 3. a) Bone marrow macrophages were differentiated into osteoclasts for five days in the presence of 10 ng mL^{-1} M-CSF and 100 ng mL^{-1} RANKL. Drugs at a concentration of 25 μM (or DMSO as a control) were added to mature osteoclasts for two days. Cells were then fixed and TRAP-stained to determine osteoclast viability. b) Pre-osteoclasts were lifted and plated on top of bone slices for two days in the presence of the drugs (or DMSO as a control). Cells were then fixed and stained with FITC-phalloidin to detect actin rings. c) Cells as shown in panel b were removed and resorption pits visualized by staining with peroxidase-conjugated anti-wheat germ agglutinin antibody. Pits were pseudocolored in black. d) Quantification of osteoclast number from data as shown in panel a. e) Quantification of osteoclasts with actin rings from data as shown in panel b. f) Quantification of bone resorption area per visual field, as shown in panel c, was determined using Image J software.

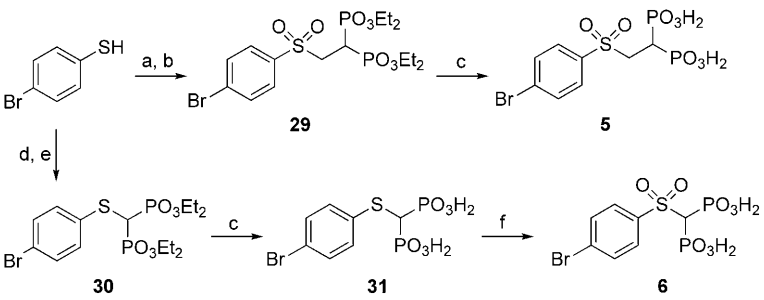
Chemistry

Carboxyamide and sulfonamide bisphosphonates **1**–**4** and **7**–**15** were prepared by treating the appropriate amides with trialkyl orthoformate and dialkyl phosphite to give the tetraalkyl bisphosphonates **16**–**28**.[42] Subsequent deprotection of the bisphosphonate esters under acidic conditions or with BBr_3 afforded the desired bisphosphonic acids (Scheme 1).

Diethylesters of BPs **5** and **6** were prepared by alkylation of 4-bromothiophenol or its corresponding disulfide with tetraethyl ethenylidenebisphosphonate[43] and commercially available tetraethyl methylenebisphosphonate, respectively (Scheme 2). The sulfone bisphosphonate **5** was obtained by oxidation with *m*-chloroperbenzoic acid (*m*-CPBA) of the intermediate sulfide, followed by acid hydrolysis of the resulting tetraethylester **29**. Acid hydrolysis of the intermediate ester **30**, followed by oxidation with dioxirane, gave the sulfone bisphosphonate **6**.



Scheme 1. a) $HC(OR)_3$, $HP(OR)_2$, 150 °C; b) BBr_3, toluene, CH_3OH or dioxane/6 N HCl (2:1), reflux.



Scheme 2. a) Tetraethyl ethenylidenebisphosphonate, $CHCl_3$, 40 °C; b) *m*-CPBA, CH_2Cl_2, 0 °C; c) 4 N HCl, reflux; d) I_2, EtOH, reflux; e) Tetraethyl methylenebisphosphonate, NaH 95%, DMF, 0 °C; f) dimethyldioxirane, acetone.

Conclusions

In conclusion, we have developed a novel class of compounds some of which were able to attain considerable antiresorptive effects and possessed potent MMP-2/MMP-8 inhibitory activity. The most promising compound (**15**) showed nanomolar activity against MMP-2 and good selectivity over MMP-8, −9, and −14; furthermore, it showed a very good antiresorptive activity comparable with that of zolendronic acid. Compound **15** can therefore be considered as a lead compound for the development of new therapeutic agents for the treatment of tumor-induced bone disease characterized by increased osteoclast activity.

Experimental Section

Biological methods

MMP inhibition assays: Recombinant human progelatinase A (pro-MMP-2) and enzymes consisting of the catalytic domain of MMP-9 and MMP-14 were purchased from Calbiochem, while catalytic domain of MMP-8 was purchased from Biomol. Pro-MMP-2 was activated immediately prior to use with *p*-aminophenylmercuric acetate (APMA, 2 mM) for 1 h at 37 °C. The assays were performed in triplicate in a total volume of 100 μL per well in 96-well microtitre plates (Corning, white, NBS). For assay measurements, inhibitor stock solutions (DMSO, 10 mM) were diluted to six different concentrations (0.1 nM–100 μM) in fluorometric assay buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.05% NaN_3, 0.05% Brij-35, 9% CH_3CN, and 1% DMSO). Activated enzyme and inhibitor solutions were incubated in the assay buffer for 30 min at 25 °C before the addition of the fluorogenic substrate solution (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, Calbiochem, 2.5 μM final concentration). After further incubation for 2–4 h at 37 °C, the hydrolysis was stopped by the addition of a 3% acetic acid solution, and the fluorescence was measured (λ_{ex} = 340 nm, λ_{em} = 405 nm) using a PERKIN–ELMER Victor V^3 plate reader. Control wells lacked inhibitor. The MMP inhibition activity was expressed in relative fluorescent units (RFU). Percent of inhibition was calculated from control reactions without the inhibitor, and IC_{50} values were determined using GraphPad PRISM version 5.0 software.[44]

MTT assay for cell viability:[28,45] The murine macrophage-like J774 A.1 and human liver carcinoma HepG2 cell lines were obtained from the ITCC (Genova, Italy). Cells were grown in DMEM (J774 A.1) or MEM (HepG2) medium supplemented with 10% fetal bovine serum, 10 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, and 2 mM L-glutamine in a 5% CO_2 atmosphere at 37 °C.

Cells were seeded at a density of 1×10^4 cells/well into 96-well flat bottom culture plates containing 50 μL of the test compound, previously half-log serially diluted (from 0.316 mM to 100 nM), in a final volume of 100 μL. The bisphosphonates were dissolved in DMSO (1% final concentration; DMSO carrier had no effect on cell proliferation). Control wells lacked inhibitor. After 48 h of incubation at 37 °C in a 5% CO_2 atmosphere, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg mL^{-1} stock solution) was added to a final concentration of 0.5 mg mL^{-1}. To control for background absorbance, six wells of cells were lysed by adding Triton X-100 (0.1% *v/v* final concentration) immediately prior to the addition of MTT reagent. After incubation under the same conditions for further 3–4 h, the culture medium was removed, the insoluble product dissolved by the addition of 100 μL of solvent (50% DMSO, 50% EtOH *v/v*), and the absorbance of the well was measured at 570 nm using a PERKIN–ELMER Victor V^3 plate reader. Cell growth inhibition was then calculated using Equation (1), where *I* is the percentage of cell growth inhibition, *A* is the absorbance of treated cultures, *Ab* is the absorbance of background control, and *Ac* is the absorbance of control cultures. IC_{50} values were determined from dose-response curves using GraphPad PRISM version 5.0.

$$I = \left(1 - \frac{A - Ab}{Ac - Ab}\right) x100 \quad (1)$$

Osteoclast assay: OCs were generated in vitro on plastic dishes or bone slices by plating murine bone marrow macrophages in the presence of M-CSF (10 ng mL^{-1}) and RANKL (100 ng mL^{-1}). After 5 days, compounds or DMSO as control were added to mature OCs at a final concentration of 25 μM. Two days later, cells were fixed and stained with TRAP to count the OC number or with FITC-Phalloidin to detect the presence of actin rings. In some circumstances, cells were removed from bone slices by gentle scraping, and bone resorptive areas were identified by using anti-germ agglutinin Ab. Quantifications of OC number and of the percentage of cells with actin rings and resorbed areas were performed by a blinded operator in triplicate.

Computational methods

All calculations were performed on a Fujitsu Siemens Celsius R550 workstation, equipped with two Intel Quad-Core Xeon E5410 2.33 GHz processors. All compounds in Table 1 and Table 2 were manually built in Maestro version 9.0,[46] exploiting the Built facility. The protonation state of all the studied BPs was predicted using the Major Macrospecies Calculator plugin available in Marvin version 5.2,[47] that predicted the di-deprotonated state (one deprotonated OH on each phosphonic group, total formal charge −2) as the most stable state at pH 7.4. Ligand structures were minimized to a derivative convergence of 0.001 kJ Å^{-1} mol^{-1}, using the Truncated Newton Conjugate Gradient (TNCG) minimization algorithm, the OPLS2005 force field, and the GB/SA water solvation model implemented in MacroModel version 9.7.[48]

Conformational searches, applying the Mixed torsional/Low-mode sampling and the automatic set-up protocol, were carried out on all minimized ligand structures in order to obtain the global minimum geometry of each molecule, which was then used as the starting conformation for docking calculations with Glide version 5.5.[38,49]

Three-dimensional coordinates of MMP-2, MMP-8, MMP-9, and MMP-14 were taken from the Brookhaven Protein Data Bank[50] (PDB codes: 1QIB,[51] 1ZVX,[25] 1GKC,[52] and 1BQQ,[53] respectively). Structures of all MMPs were superimposed in order to align the binding sites with the same frame of reference (1QIB was selected as a reference structure) exploiting the superimposition tool of Maestro. All structures were submitted to the Protein Preparation routine in Maestro that allows fixing of receptor structures, eliminating water molecules and possible ligands, fixing bond orders, adding hydrogen atoms, and ionizing lysine, arginine, glutamate, and aspartate residues. To optimize the hydrogen bond network, histidine tautomers and ionization states were predicted, 180° rotations of the terminal χ angle of Asn, Gln, and His residues were assigned, and hydroxyl and thiol hydrogen atoms were sampled. For each structure, a brief relaxation was performed using an all-atom constrained minimization carried out with the Impact Refinement

module version 5.5[54] and the OPLS-2005 force field to reduce steric clashes that may exist in the original PDB structures. The minimization was terminated when the energy converged or the root mean square deviation (RMSD) reached a maximum cut-off of 0.30 Å.

Glide energy grids were generated for each of the prepared proteins. The receptor grid generation was performed for a rectangular box with a center in the centroid of the 1ZVX crystallographic ligand. The size of the box was determined automatically. Binding to the catalytic zinc ion was favored imposing an energy constraint.

The global minimum geometry of ligands and MMP-2/MMP-8/MMP-9/MMP-14 grids were used for subsequent docking studies. The same procedure was applied for all ligands in each protein. The van der Waals radii for non-polar ligand atoms were scaled by a factor of 0.8, thereby decreasing penalties for close contacts. Receptor atoms were not scaled. A first docking run was carried out applying the Standard Precision settings of Glide. Ten poses were saved and re-submitted to docking with the Extra Precision (XP) settings;[39] one pose was saved in this second run. The XP scoring function contains a number of additional terms beyond those present in GlideScore, including terms for hydrophobic enclosure and large desolvation penalties that result in a better prediction of the docking poses and a closer correlation with the experimental results. The best ranking pose for each ligand in each protein was considered for the following analysis.

Chemical methods

Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus and are uncorrected. Mass spectra were recorded on a HP MS 6890–5973 MSD spectrometer, electron impact 70 eV, equipped with a HP ChemStation or with an Agilent LC-MS 1100 Series LC-MSD Trap System VL spectrometer, electrospray ionization (ESI). ^{1}H and ^{31}P NMR spectra were recorded using the suitable deuterated solvent on a Varian Mercury 300 NMR Spectrometer. Chemical shifts (δ) are expressed as parts per million (ppm) and the coupling constants (*J*) in Hertz (Hz). Microanalyses of solid compounds were carried out with an Eurovector Euro EA 3000 model analyzer. Flash column chromatography was performed using Geduran silica gel 60 Å (45–63 μm). Chemicals were purchased from Aldrich Chemicals (Milan, Italy) or Lancaster Synthesis (Ward Hill, USA), and were used without any further purification.

General procedure for the preparation of tetraalkyl(aryl-amido)-methyl-1,1-bisphosphonates 16–28:[42] A mixture of the suitable carboxyamide or sulfonamide (3.17 mmol) with the appropriate trialkyl orthoformate (3.78 mmol) and dialkyl phosphite (10.54 mmol) was stirred at 150 °C for 2–4 h with continuous removal of the solvent formed. After cooling, the volatiles were removed under reduced pressure. The residue was chromatographed on silica gel using EtOAc and EtOAc/*i*PrOH 9:1 as eluent, affording the desired esters (15–92% yields).

Tetraethyl (4-chloro-phenylsulfonylamino)methyl-1,1-bisphosphonate (16): White solid, 77% yield; mp: 129–131 °C; ^{1}H NMR ($CDCl_3$): $\delta = 1.24$–1.31 (m, 12H, CH_2CH_3), 4.01–4.27 (m, 8H, CH_2CH_3 and 1H, PCHP), 5.32–5.35 (m, 1H, NH), 7.46–7.49, 7.81–7.85 ppm (m, 2H, 2H, aromatics); MS (ESI): *m/z*: 500 $[M+Na]^+$; MS^2: *m/z* (%): 450 (100).

Tetraethyl (benzensulfonylamino)methyl-1,1-bisphosphonate (17): Yellow oil, 42% yield; ^{1}H NMR ($CDCl_3$): $\delta = 1.21$–1.29 (m, 12H, CH_2CH_3), 3.97–4.06 (m, 1H, PCHP), 4.07–4.26 (m, 8H, CH_2CH_3), 5.43 (br, 1H, NH), 7.46–7.60, 7.87–7.90 ppm (m, 3H, 2H, aromatics); MS (ESI): *m/z*: 466 $[M+Na]^+$; MS^2: *m/z* (%): 416 (100).

Tetraethyl (4-bromo-phenylsulfonylamino)methyl-1,1-bisphosphonate (18): White solid, 71% yield; mp: 133–136 °C; ^{1}H NMR ($CDCl_3$): $\delta = 1.23$–1.38 (m, 12H, CH_2CH_3), 4.00–4.24 (m, 8H, CH_2CH_3 and 1H, PCHP), 5.58–5.61 (m, 1H, NH), 7.62–7.65, 7.74–7.78 ppm (m, 2H, 2H, aromatics); MS (ESI): *m/z*: 546 $[M+2+Na]^+$, 544 $[M+Na]^+$; MS^2: *m/z* (%): 408 (98), 406 (100).

Tetramethyl [(4-bromo-phenylcarboxyamido)-methyl]-1,1-bisphosphonate (19): Yellowish solid, 15% yield; mp: 158–161 °C; ^{1}H NMR ($CDCl_3$): $\delta = 3.81$–3.90 (m, 12H, CH_3), 5.31 (td, $J_{HH} = 9.9$, $J_{HP} = 21.7$, 1H, PCHP), 6.91 (d, $J_{HH} = 9.3$, 1H, NH), 7.59–7.0, 7.71–7.73 ppm (m, 2H, 2H, aromatics); MS (ESI): *m/z*: 454 $[M+2+Na]^+$, 452 $[M+Na]^+$; MS^2: *m/z* (%): 422 (83), 420 $[C_{11}H_{15}BrNO_6P_2+Na]^+$ (100).

Tetramethyl (4-methyl-phenylsulfonylamino)methyl-1,1-bisphosphonate (20): Yellowish oil, 29% yield; ^{1}H NMR ($CDCl_3$): $\delta = 2.42$ (s, 3H, CH_3), 3.66–3.77 (m, 12H, OCH_3), 4.24 (td, $J_{HH} = 9.6$, $J_{HP} = 22.2$, 1H, PCHP), 5.33–5.44 (m, 1H, NH), 7.29–7.31, 7.74–7.77 ppm (m, 2H, 2H, aromatics); MS (ESI): *m/z*: 424 $[M+Na]^+$; MS^2: *m/z* (%): 314 (100).

Tetramethyl (4-methoxy-phenylsulfonylamino)methyl-1,1-bisphosphonate (21): Yellowish solid, 40% yield; mp: 122–125 °C; ^{1}H NMR ($CDCl_3$): $\delta = 3.70$–3.78 (m, 12H, CH_3), 3.86 (s, 3H, OCH_3), 4.23 (td, $J_{HH} = 9.6$, $J_{HP} = 22.1$, 1H, PCHP), 5.25–5.31 (m, 1H, NH), 6.96–6.99, 7.79–7.82 ppm (m, 2H, 2H, aromatics); MS (ESI): *m/z*: 440 $[M+Na]^+$; MS^2: *m/z* (%): 330 (100).

Tetraethyl (3-nitro-phenylsulfonylamino)methyl-1,1-bisphosphonate (22): Yellow oil, 92% yield; ^{1}H NMR ($CDCl_3$): $\delta = 1.22$–1.29 (m, 12H, CH_2CH_3), 4.03–4.25 (m, 8H, CH_2CH_3 and 1H, PCHP), 7.70–7.75, 8.23–8.27, 8.40–8.44, 8.73–8.75 ppm (m, 4H, aromatics); MS (ESI): *m/z*: 511 $[M+Na]^+$; MS^2: *m/z* (%): 373 (100).

Tetraethyl (4-nitro-phenylsulfonylamino)methyl-1,1-bisphosphonate (23): Yellowish solid, 47% yield; mp: 122–125 °C; ^{1}H NMR ($CDCl_3$): $\delta = 1.23$–1.31 (m, 12H, CH_2CH_3), 4.11–4.21 (m, 8H, CH_2CH_3) , 4.25 (td, $J_{HH} = 9.6$, $J_{HP} = 22.1$, 1H, PCHP), 6.06–6.11 (m, 1H, NH), 8.08–8.12, 8.32–8.35 ppm (m, 2H, 2H, aromatics); MS (ESI): *m/z*: 511 $[M+Na]^+$; MS^2: *m/z* (%): 373 (100).

Tetraethyl (biphenyl-4-sulfonylamino)methyl-1,1-bisphosphonate (24): Pale yellow oil, 68% yield; ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.11$–1.16 (m, 12H, CH_2CH_3), 3.83–4.06 (m, 8H, CH_2CH_3), 4.07–4.25 (td, $J_{HH} = 9.6$, $J_{HP} = 23.1$, 1H, PCHP), 7.37–7.52, 7.69–7.79, 7.81–7.90 (m, 3H, 2H, 4H, aromatics), 8.78 ppm (d, $J_{HH} = 9.6$, 1H, NH); MS (ESI): *m/z*: 542 $[M+Na]^+$; MS^2: *m/z* (%): 404 $[C_{17}H_{20}NO_5PS+Na]^+$ (100).

Tetraethyl [4-(2-thienyl)phenylsulfonylamino]methyl-1,1-bisphosphonate (25): Yellow solid, 15% yield; mp: 101–103 °C; ^{1}H NMR ($CDCl_3$): $\delta = 1.23$–1.31 (m, 12H, CH_2CH_3), 4.09–4.19 (m, 8H, CH_2CH_3), 4.21 (td, $J_{HH} = 9.6$, $J_{HP} = 22.1$, 1H, PCHP), 5.30 (br, 1H, NH), 7.11–7.14, 7.38–7.42, 7.69–7.73, 7.86–7.89 ppm (m, 1H, 2H, 2H, 2H, aromatics); MS (ESI): *m/z*: 548 $[M+Na]^+$; MS^2: *m/z* (%): 410 $[C_{15}H_{18}NO_5PS_2+Na]^+$ (100).

Tetramethyl [(4′-methoxy-biphenyl-4-sulfonylamino)methyl)]-1,1-bisphosphonate (26): Yellowish solid, 21% yield; mp: 180–183 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta = 3.53$–3.58 (m, 12H, CH_3), 3.79 (s, 3H, OCH_3), 4.27 (td, $J_{HH} = 9.9$, $J_{HP} = 23.1$, 1H, PCHP), 7.02–7.05, 7.65–7.70, 7.74–7.85 ppm (m, 2H, 2H, 4H, aromatics); MS (ESI): *m/z*: 516 $[M+Na]^+$; MS^2: *m/z* (%): 406 (100).

Tetraethyl [(4′-nitro-biphenyl-4-sulfonylamino)methyl)]-1,1-bisphosphonate (27): Yellow solid, 75% yield; mp: 146–147 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.10–1.19 (m, 12H, CH_2CH_3), 3.85–4.06 (m, 8H, CH_2CH_3), 4.07–4.26 (td, J_{HH} = 9.6, J_{HP} = 23.1, 1H, PCHP), 7.9–8.02, 8.30–8.35 (m, 6H, 2H, aromatics), 8.88 ppm (d, J_{HH} = 9.6, 1H, NH); MS (ESI): *m/z*: 587 [*M*+Na]$^+$; MS^2: *m/z* (%): 449 (100).

Tetraethyl [(4′-chloro-biphenyl-4-sulfonylamino)methyl)]-1,1-bisphosphonate (28): White solid, 39% yield; mp: 124–126 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.23–1.29 (m, 12H, CH_2CH_3), 4.01–4.20 (m, 8H, CH_2CH_3), 4.24 (td, J_{HH} = 9.6, J_{HP} = 22.1, 1H, PCHP), 5.21–5.26 (m, 1H, NH), 7.43–7.53, 7.65–7.69, 7.93–7.98 ppm (m, 4H, 2H, 2H aromatics); MS (ESI): *m/z*: 578 [*M*+2+Na]$^+$, 576 [*M*+Na]$^+$; MS^2: *m/z* (%): 410 (100).

Tetraethyl [2-(4-bromo-phenylsulfonyl)ethyl]-1,1-bisphosphonate (29): 4-Bromothiophenol (6.4 mmol) was added to a solution of tetraethyl ethenylidenebisphosphonate[43] (2.5 mmol) in $CHCl_3$ (6 mL). After heating for 40 h at 40 °C, the organic solvent was evaporated and the resulting yellow oil was purified by flash chromatography on silica gel (eluent from $CHCl_3/CH_2Cl_2$ 1:1 to $CHCl_3$) to give the tetraethyl [2-(4-bromo-phenylthiol)ethyl]-1,1-bisphosphonate as a yellow oil (0.67 g, 56%): ^{1}H NMR ($CDCl_3$): δ = 1.32 (td, J_{HH} = 7.1, J_{HP} = 1.9, 12H, CH_2CH_3), 2.59 (tt, J_{HH} = 6.3, J_{HP} = 23.6, 1H, PCHP), 3.42 (td, J_{HH} = 6.3, J_{HP} = 15.7, 2H, CH_2CHP), 4.08–4.23 (m, 8H, CH_2CH_3), 7.24–7.28, 7.38–7.43 ppm (m, 2H, 2H, aromatics); MS (ESI): *m/z*: 513 [*M*+2+Na]$^+$, 511 [*M*+Na]$^+$; MS^2: *m/z* (%): 437 (100).

m-CPBA (70%, 0.53 g, 3.16 mmol) was added to an ice-cooled solution of this sulfide (0.3 g, 0.61 mmol) in CH_2Cl_2 (10 mL). After stirring for 4 h, the organic phase was washed with 0.5 N NaOH and brine, dried over Na_2SO_4, and evaporated to dryness to afford a solid that was purified by silica gel chromatography (EtOAc) to give **29** (0.28 g, 88%): ^{1}H NMR ($CDCl_3$): δ = 1.28–1.38 (m, 12H, CH_2CH_3), 3.03 (tt, J_{HH} = 4.6, J_{HP} = 24.7, 1H, PCHP), 3.64–3.76 (m, 2H, CH_2CHP), 4.08–4.26 (m, 8H, CH_2CH_3), 7.69–7.73, 7.77–7.81 ppm (m, 2H, 2H, aromatics); MS (ESI): *m/z*: 545 [*M*+2+Na]$^+$, 543 [*M*+Na]$^+$; MS^2: *m/z* (%): 323 (100).

Tetraethyl (4-bromo-phenylthio)methyl-1,1-bisphosphonate (30): A solution of iodine (2.09 g, 8.22 mmol) in ethanol (10 mL) was added dropwise to a solution of 4-bromothiophenol (1.34 g, 7.1 mmol) in the same solvent (4.5 mL) at reflux until a persistent brown color was observed. After stirring for 10 min, a saturated solution of $Na_2S_2O_3{\cdot}5H_2O$ (20 mL) was added. The organic solvent was removed under reduced pressure, and the residue was extracted with EtOAc. The organic phase was dried over Na_2SO_4 and evaporated to dryness to obtain 1,1′-(4-bromodiphenyl)disulfide as a yellowish solid in quantitative yield. mp: 88–90 °C; ^{1}H NMR ($CDCl_3$): δ = 7.31–7.35, 7.41–7.45 ppm (m, 4H, 4H, aromatics); GC-MS: *m/z* (%): 378 [*M*+4]$^+$ (40), 376 [*M*+2]$^+$ (74), 374 [*M*]$^+$ (36), 108 (100).

To an ice-cooled suspension of 95% NaH (0.05 g, 2.1 mmol) in anhydrous DMF (3 mL) a solution of tetraethyl methylenebisphosphonate (0.12 mL, 0.48 mmol) in anhydrous DMF (4 mL) was added under N_2 atmosphere. After stirring for 30 min at 0 °C, a solution of the previous disulfide (0.2 g, 0.53 mmol) in anhydrous DMF (5 mL) was added dropwise. The resulting mixture was stirred for 24 h, then the solvent was removed under reduced pressure. The resulting oily residue was purified by flash chromatography on silica gel (eluent: $CHCl_3$/*i*PrOH 98:2) to give the desired compound **30** as a yellowish oil (0.21 g, 89%): ^{1}H NMR ($CDCl_3$): δ = 1.32 (t, J_{HH} = 7.1, 12H, CH_2CH_3), 3.34 (t, J_{HP} = 21.45, 1H, PCHP), 4.16–4.28 (m, 8H, CH_2CH_3), 7.40–7.48 ppm (m, 4H, aromatics); GC-MS: *m/z* (%): 476 [*M*+2]$^+$ (97), 474 [*M*]$^+$ (91), 201 (100).

General procedure for the preparation of 1,1-bisphosphonic acids 1–15. Method A: A solution of the appropriate tetraalkyl bisphosphonate (**29** or **30**) (0.6 mmol) in 4 N HCl (48 mmol) was kept at reflux for 8–10 h. After removal of the aqueous phase under reduced pressure, the crude bisphosphonic acids were triturated with Et_2O and filtered to afford the final compounds as white solids.

(4-Bromophenylthio)methyl-1,1-bisphosphonic acid (31): 98% yield; mp: 207 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ = 3.23–3.36 (m, 1H, PCHP), 5.39 (br, 4H, OH), 7.39–7.5 ppm (m, 4H, aromatics); ^{31}P NMR ([D$_6$]DMSO): δ = 14.9 ppm (d, J_{PH} = 18.3, 2P, PCHP); MS (ESI): *m/z*: 363 [*M*+2−H]$^-$ (100), 361 [*M*−H]$^-$ (85); MS^2: *m/z* (%): 345 (100).

[2-(4-Bromo-phenylsulfonyl)ethyl]-1,1-bisphosphonic acid (5): 79% yield; mp: 200–203 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 2.43 (tt, J_{HH} = 4.6, J_{HP} = 12.4, 1H, PCHP), 3.61 (td, J_{HH} = 4.6, J_{HP} = 6.5, 2H, CH_2CHP), 7.75–7.78, 7.82–7.85 (m, 2H, 2H, aromatics), 8.04 ppm (br, 4H, OH); ^{31}P NMR ([D$_6$]DMSO): δ = 17.8 ppm (dt, J_{PH} = 24.1, J_{PH} = 15.2, 2P, PCH_2CHP); MS (ESI): *m/z*: 409 [*M*+2−H]$^-$, 407 [*M*−H]$^-$; MS^2: *m/z* (%): 391 [*M*+2−H−H_2O]$^-$ (97), 389 [*M*−H−H_2O]$^-$ (100); Anal. calcd for $C_8H_{11}BrO_8P_2S$: C 23.49%, H 2.71%, found: C 23.25%, H 2,63%.

Method B: A solution of the suitable tetraalkyl bisphosphonate (**16–18**, **20**, **21**, **24–26**, **28** and commercially available tetraethyl methylenebisphosphonate) (0.86 mmol) in 6 N HCl/dioxane (1:2, 15 mL) was refluxed for 7–30 h. After removal of the solvents under reduced pressure, the crude bisphosphonic acids were crystallized or triturated with EtOAc or Et_2O and filtered affording the final compounds as white solids in 53–98% yields.

Methylen-1,1-bisphosphonic acid (MBP): 98% yield; mp: 192–195 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 2.13 (t, J_{HP} = 20.6, 2H, PCH_2P), 8.12 ppm (br, 4H, OH); ^{31}P NMR ([D$_6$]DMSO): δ = 17.73 ppm (dt, J_{PH} = 21.3, 2P, PCH_2P); MS (ESI): *m/z*: 175 [*M*−H]$^-$; MS^2: *m/z* (%): 157 [*M*−H−H_2O]$^-$ (100); Anal. calcd for $CH_6O_6P_2$: C 6.82%, H 3.44%, found: C 7.04%, H 3.48%.

(4-Chloro-phenylsulfonylamino)methyl-1,1-bisphosphonic acid (1): 98% yield; mp: 240–243 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 3.79 (td, J_{HH} = 9.6, J_{HP} = 21.7, 1H, PCHP), 6.68 (br, 4H, OH), 7.5–7.55, 7.79–7.83 (m, 2H, 2H, aromatics), 8.06 ppm (d, J_{HH} = 9.6, 1H, NH); ^{31}P NMR ([D$_6$]DMSO): δ = 14.95 ppm (d, J_{PH} = 21.3, 2P, PCHP); MS (ESI): *m/z* (%): 366 [*M*+2−H]$^-$; MS^2: *m/z* (%): 346 [*M*−H−H_2O]$^-$ (100); Anal. calcd for $C_7H_{10}ClNO_8P_2S{\cdot}0.5H_2O$: C 22.44%, H 2.96%, N 3.74%, found: C 22.74%, H 3.30%, N 3.37%.

(Benzensulfonylamino)methyl-1,1-bisphosphonic acid (2): 92% yield; mp: 217 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ = 3.84 (td, J_{HH} = 9.6, J_{HP} = 21.7, 1H, PCHP), 6.68 (br, 4H, OH and 1H, NH), 7.42–7.55, 7.80–7.95 ppm (m, 3H, 2H, aromatics); ^{31}P NMR ([D$_6$]DMSO): δ = 15.15 ppm (d, J_{PH} = 21.3, 2P, PCHP); MS (ESI): *m/z*: 330 [*M*−H]$^-$; MS^2: *m/z* (%): 312 [*M*−H−H_2O]$^-$ (100); Anal. calcd for $C_7H_{11}NO_8P_2S{\cdot}H_2O$: C 24.08%, H 3.75%, N 4.01%, found: C 24.21%, H 4.14%, N 3.68%.

(4-Bromo-phenylsulfonylamino)methyl-1,1-bisphosphonic acid (3): 60% yield; mp: 234–235 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 3.79 (td, J_{HH} = 9.9, J_{HP} = 21.7, 1H, PCHP), 6.83 (br, 4H, OH), 7.64–7.66, 7.71–7.74 (m, 2H, 2H, aromatics), 8.07 ppm (d, J_{HH} = 9.3, 1H, NH); ^{31}P NMR ([D$_6$]DMSO): δ = 14.94 ppm (d, J_{PH} = 24.4, 2P, PCHP); MS (ESI): *m/z*: 410 [*M*+2−H]$^-$, 408 [*M*−H]$^-$; MS^2: *m/z* (%): 392 [*M*+2]$^+$ (100), 390 [*M*] $^+$ (90); Anal. calcd for $C_7H_{10}BrNO_8P_2S$: C 20.50%, H 2.46%, N 3.42%, found: C 20.75%, H 2.56%, N 3.36%.

(4-Methyl-phenylsulfonylamino)methyl-1,1-bisphosphonic acid (7): 90% yield; mp: 218–222 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ = 2.33

(s, 3H, CH_3), 3.82 (td, $J_{HH}=9.6$, $J_{HP}=21.7$, 1H, PCHP), 7.23–7.26, 7.68–7.71 (m, 2H, 2H, aromatics), 6.21 ppm (br, 4H, OH and 1H, NH); ^{31}P NMR ([D$_6$]DMSO): $\delta=15.19$ ppm (d, $J_{PH}=21.3$, 2P, PCHP); MS (ESI): *m/z*: 344 $[M-H]^-$; MS^2: *m/z* (%): 326 (100); Anal. calcd for $C_8H_{13}NO_8P_2S\ H_2O$: C 26.45%, H 4.16%, N 3.86%, found: C 26.23%, H 4.25%, N 3.86%.

(4-Methoxy-phenylsulfonylamino)methyl-1,1-bisphosphonic acid (8): 83% yield; mp: 200–203 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=3.78$ (s, 3H, OCH_3), 3.80 (td, $J_{HH}=9.6$, $J_{HP}=21.7$, 1H, PCHP), 5.47 (br, 4H, OH), 7.50 (d, $J_{HH}=9.3$, 1H, NH), 6.95–6.98, 7.73–7.75 ppm (m, 2H, 2H aromatics); ^{31}P NMR ([D$_6$]DMSO): $\delta=14.22$ ppm (d, $J_{PH}=21.3$, 2P, PCHP); MS (ESI): *m/z*: 360 $[M-H]^-$; MS^2 *m/z* (%): 342 $[M-H-H_2O]^-$ (100); Anal. calcd for $C_8H_{13}NO_9P_2S{\cdot}2H_2O$: C 24.19%; H 4.31%; N 3.53%, found: C 24.59%; H 4.37%; N 3.59%.

(Biphenyl-4-sulfonylamino)methyl-1,1-bisphosphonic acid (11): 93% yield; mp: 236–237 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=3.87$ (td, $J_{HH}=9.6$, $J_{HP}=21.7$, 1H, PCHP), 5.5 (br, 4H, OH and 1H, NH), 7.37–7.51, 7.69–7.77, 7.88–7.91 ppm (m, 3H, 4H, 2H aromatics); ^{31}P NMR ([D$_6$]DMSO): $\delta=15.12$ ppm (d, $J_{PH}=21.3$, 2P, PCHP); MS (ESI): *m/z*: 406 $[M-H]^-$; MS^2: *m/z* (%): 388 (100); Anal. calcd for $C_{13}H_{15}NO_8P_2S{\cdot}3/2H_2O$: C 35.95%, H 4.18%, N 3.23%, found: C 35.81%, H 4.29%, N 3.25%.

[4-(2-Thienyl)phenylsulfonylamino]methyl-1,1-bisphosphonic acid (12): 53% yield; mp: 228 °C (dec); ^{1}H NMR ([D$_6$]DMSO): $\delta=$ 3.82 (td, $J_{HH}=9.6$, $J_{HP}=21.7$, 1H, PCHP), 5.32 (br, 4H, OH and 1H, NH), 7.14–7.17, 7.62–7.67, 7.71–7.74, 7.81–7.84 ppm (m, 1H, 2H, 2H, aromatics); ^{31}P NMR ([D$_6$]DMSO): $\delta=14.97$ ppm (d, $J_{PH}=$ 21.3, 2P, PCHP); MS (ESI): *m/z:* 412 $[M-H]^-$; MS^2: *m/z* (%): 394 (100); Anal. calcd for $C_{11}H_{13}NO_8P_2S_2{\cdot}2H_2O$: C 29.40%, H 3.81%, N 3.12%; found: C 29.07%, H 3.42%, N 3.12%.

(4′-Methoxy-biphenyl-4-sulfonylamino)methyl-1,1-bisphosphonic acid (13): 85% yield; mp: 230 °C (dec); ^{1}H NMR ([D$_6$]DMSO): $\delta=$ 3.71–38.5 (m, 4H, OCH_3 and 1H, PCHP), 6.36 (br, 4H, OH and 1H, NH), 7.01–7.06, 7.64–7.70, 7.80–7.87 ppm (m, 2H, 4H, 2H, aromatics); ^{31}P NMR ([D$_6$]DMSO): $\delta=14.96$ ppm (d, $J_{PH}=21.3$, 2P, PCHP); MS (ESI): *m/z:* 436 $[M-H]^-$; MS^2 *m/z* (%): 418 (100); Anal. calcd for $C_{14}H_{17}NO_9P_2S{\cdot}1.5H_2O$: C 36.21%, H 4.34%, N 3.02%, found: C 36.01%, H 4.06%, N 3.07%.

(4′-Chloro-biphenyl-4-sulfonylamino)methyl-1,1-bisphosphonic acid (15): 90% yield; mp: 252 °C (dec); ^{1}H NMR ([D$_6$]DMSO): $\delta=$ 3.86 (td, $J_{HH}=9.6$, $J_{HP}=21.7$, 1H, PCHP), 7.32 (br, 4H, OH and 1H, NH), 7.52–7.55, 7.72–7.77, 7.88–7.91 ppm (m, 2H, 4H, 2H, aromatics); ^{31}P NMR ([D$_6$]DMSO): 15.04 ppm (d, $J_{PH}=24.4$, 2P, PCHP); MS (ESI): *m/z*: 442 $[M+2-H]^-$, 440 $[M-H]^-$; MS^2 *m/z* (%): 422 (100); Anal. calcd for $C_{13}H_{14}ClNO_8P2S{\cdot}1.5H_2O$: C 33.31%, H 3.66%, N 2.99%, found: C 33.09%, H 3.67%, N 3.16%.

Method C: A solution of BBr_3 (1 M, 1.85 mmol) in CH_2Cl_2 was carefully added dropwise under N_2 atmosphere to a cooled (−30 °C) solution of the appropriate tetraalkyl bisphosphonate (**19**, **22**, **23**, or **27**) (1.02 mmol) in anhydrous toluene (11 mL). After 6 h at 75 °C, the reaction mixture was cooled at room temperature, then CH_3OH (5 mL) was added and stirring was continued for additional 30 min. The organic solvent was removed under reduced pressure, and the crude solids were triturated with Et_2O affording the desired bisphosphonic acids as yellowish solids (50–80% yields).

(4-Bromo-phenylcarboxyamido)methyl-1,1-bisphosphonic acid (4): 63% yield; mp: 240 °C (dec); ^{1}H NMR ([D$_6$]DMSO): $\delta=4.78$ (td, $J_{HH}=9.9$, $J_{HP}=21.4$, 1H, PCHP), 5.42 (br, 4H, OH), 7.66–7.69, 7.79–7.86 (m, 2H, 2H, aromatics), 8.13 ppm (d, $J_{HH}=9.9$, 1H, NH); ^{31}P NMR ([D$_6$]DMSO): $\delta=14.50$ ppm (d, $J_{PH}=21.3$, 2P, PCHP); MS (ESI): *m/z*: 374 $[M+2-H]^-$, 372 $[M-H]^-$; MS^2: *m/z* (%): 356 $[M+2-H-H_2O]^-$ (100), 354 $[M-H-H_2O]^-$ (92); Anal. calcd for $C_8H_{10}BrNO_7P_2{\cdot}3H_2O$: C 22.45%, H 3.77%, N 3.27%, found: C 22.15%, H 3.37%, N 3.03%.

(3-Nitro-phenylsulfonylamino)methyl-1,1-bisphosphonic acid (9): 55% yield; mp: 226–228 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=3.77$ (td, $J_{HH}=$ 9.6, $J_{HP}=21.7$, 1H, PCHP), 7.73–7.78 (m, 1H, aromatic), 8.03 (br, 4H, OH), 8.17–8.20, 8.34–8.39 (m, 1H, 1H, aromatics), 8.48 (d, $J_{HH}=9.6$, 1H, NH), 8.59–8.60 ppm (m, 1H, aromatic); ^{31}P NMR ([D$_6$]DMSO): $\delta=14.62$ ppm (d, $J_{PH}=21.3$, 2P, PCHP); MS (ESI): *m/z*: 375 $[M-H]^-$; MS^2: *m/z* (%): 357 $[M-H-H_2O]^-$ (100); Anal. calcd for $C_7H_{10}N_2O_{10}P_2S{\cdot}H_2O$: C 21.33%, H 3.07%, N 7.11%, found: C 21.15%, H 3.22%, N 6.84%.

(4-Nitro-phenylsulfonylamino)methyl-1,1-bisphosphonic acid (10): 80% yield; mp: 252 °C (dec); ^{1}H NMR ([D$_6$]DMSO): $\delta=3.78$ (td, $J_{HH}=9.6$, $J_{HP}=21.7$, 1H, PCHP), 8.03–8.06, 8.28–8.30 (m, 2H, 2H, aromatics), 8.32–8.53 ppm (br, 4H, OH and 1H, NH); ^{31}P NMR ([D$_6$]DMSO): $\delta=14.59$ ppm (d, $J_{PH}=21.3$, 2P, PCHP); MS (ESI): *m/z*: 375 $[M-H]^-$; MS^2: *m/z* (%): 357 $[M-H-H_2O]^-$ (100); Anal. calcd for $C_7H_{10}N_2O_{10}P_2S{\cdot}H_2O$: C 21.33%, H 3.07%, N 7.11%, found: C 21.05%, H 3.30%, N 6.76%.

(4′-Nitro-biphenyl-4-sulfonylamino)methyl-1,1-bisphosphonic acid (14): 50% yield; mp: 242 °C (dec); ^{1}H NMR ([D$_6$]DMSO): $\delta=$ 3.87 (td, $J_{HH}=9.9$, $J_{HP}=22.0$, 1H, PCHP), 5.79 (br, 4H, OH and 1H, NH,), 7.86–8.01, 8.30–8.34 ppm (m, 6H, 2H, aromatics); ^{31}P NMR ([D$_6$]DMSO): $\delta=14.5$ ppm (d, $J_{PH}=21.3$, 2P, PCHP); MS (ESI): *m/z*: 451 $[M-H]^-$; MS^2: *m/z* (%): 433 $[M-H-H_2O]^-$ (100); Anal. calcd for $C_{13}H_{14}N_2O_{10}P_2S{\cdot}H_2O$: C 33.20%, H 3.43%, N 5.96%, found: C 33.24%, H 3.31%, N 5.59%.

(4-Bromo-phenylsulfonyl)methyl-1,1-bisphosphonic acid (6): An aliquot of a standardized cold solution of dimethyldioxirane (8 mL of 0.09 M) in acetone was rapidly added to a solution of **31** (0.03 g, 0.08 mmol) in 1 mL acetone. After stirring for 4 h, the solvent was evaporated under reduced pressure to give the crude solid that was triturated with Et_2O and filtered to afford the desired bisphosphonic acid (38% yield): mp: 186–189 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=$ 4.32 (t, $J_{HP}=20.6$, 1H, PCHP), 7.73–7.76, 7.86–7.89 ppm (m, 2H, 2H, aromatics); MS (ESI): *m/z*: 395 $[M+2-H]^-$, 393 $[M-H]^-$; MS^2: *m/z* (%): 377 $[M+2-H-H_2O]^-$ (100), 375 $[M-H-H_2O]^-$ (89); Anal. calcd for $C_7H_9BrO_8P_2S$: C 21.28%, H 2.30%, found: C 21.02%, H 2.50%.

Supporting Information

Synthesis and spectroscopic data of etidronate, pamidronate, alendronate, zolendronate, tiludronate, and biphenylsulfonamides are available as Supporting Information on the WWW at http://dx.doi.org/10.1002/cmdc.201000540.

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