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Synthesis and antiproliferative activity of aromatic and aliphatic bis[aminomethylidene(bisphosphonic)] acids

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ABSTRACT

A series of aromatic and aliphatic bis[aminomethylidene(bisphosphonic)] acids was synthesized in the reaction of triethylphosphite with isonitriles followed by hydrolysis or dealkylation. The in vitro anti-proliferative effect of all synthesized tetraphosphonic acids against MCF-7 breast cancer cells, J774E macrophages and HL-60 promyelocytic leukemia cells was determined. Three aromatic derivatives (**5a**, **5f** and **5j**) showed a similar or higher anti-proliferative activity than zoledronic acid.

The commonly used term 'bisphosphonates' is a simplified name for geminal bisphosphonates, that is, compounds containing the P–C–P bonds. The first bisphosphonates were used in the 'non-medical' area,[1–5] however, their most important application is in the treatment of many ailments connected with calcium metabolism disorders such as osteoporosis, tumor-induced hypercalcaemia, Paget's disease or bone metastases of some cancers.[6] Nitrogen-containing bisphosphonates inhibit the mevalonate pathway—a biosynthetic pathway on which depends the prenylation of small GTPases—signaling proteins that regulate a variety of cellular processes.[7] This leads to subsequent inhibition of osteoclast differentiation and promotes apoptosis. Thus, bisphosphonates interrupt bi-directional interactions between tumor cells and osteoclasts.[8] They have become important not only in the treatment of cancer-related bone diseases, but also have been proved to possess anticancer activity, manifested at different levels.[9]

Most of the clinically used bisphosphonates are hydroxybisphosphonates [$RC(OH)(PO_3H_2)_2$], for example the most active third-generation bisphosphonates such as risedronate or zoledronate, and incadronate is the only example of aminomethylidenebisphosphonic acids (Fig. 1).

Despite low or average yield, harsh conditions and problems with separation of pure esters, the reaction of trialkyl orthoformate, dialkylphosphonate and amine is still the most widely used method for the preparation of aminomethylidenebisphosphonic acids.[10] There are several other less useful methods, such as the reaction of dialkylphosphonates with acetals of N-substitute formamides,[11] phosphorylation of the product of the Beckman rearrangement of oxime[12] or reaction of trialkylphosphites with chloroiminium salts.[13,14]

In 2012 we described a very simple method for a synthesis of N-substituted aminomethylidenebisphosphonic acids and their tetraalkyl esters via reaction of the isonitriles and trialkylphosphite in the presence of a stoichiometric amount of hydrogen chloride.[15] This procedure seems to be the most convenient and mild method for the synthesis of N-substituted aminomethylidenebisphosphonic acids, especially their alkyl esters. In continuation of these studies, a reaction of aliphatic and aromatic diisonitriles was performed to extend the applicability of this promising reaction.

The starting diisonitriles were prepared via two step procedure: formylation of the diamine **1** with formic acid in toluene, and next, dehydration of the resulting formamides **2** with phosphorous oxychloride in the presence of triethylamine in dichloromethane as a solvent (Scheme 1).

Diformamides **2** were used in the next step without further purification. The obtained diisonitriles **3** were characterized by

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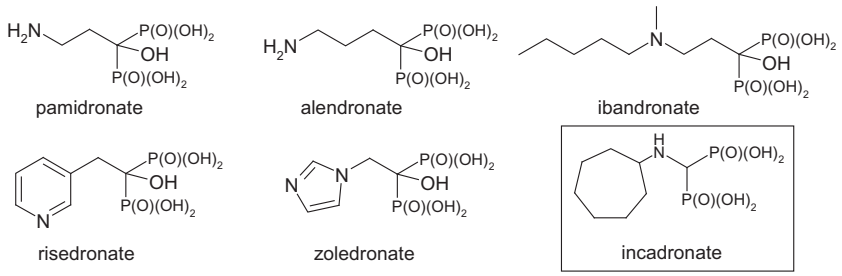
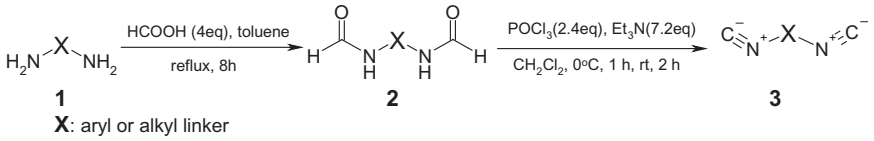


Figure 1. Selected examples of the commercial bisphosphonates.



Scheme 1. Synthesis of isonitriles **3**.

^{1}H, ^{13}C NMR, IR and, in the case of new compounds (**3i** and **3l**), by HRMS spectroscopy. All isonitriles showed a characteristic strong sharp band on the FTIR spectra (in the 2125–2135 cm^{-1} region for aromatic and 2140–2153 cm^{-1} for aliphatic), due to the vibration of the isonitrile group triple bond. On the ^{13}C NMR spectra the characteristic signal was present at about 155–170 ppm from the isonitrile carbon. It was a sharp 1:1:1 triplet in the case of the aliphatic isonitrile and a very broad unresolved triplet in the case of the aromatic isonitrile. The yields of the resultant isonitriles are depicted in Supplementary data (Table 1 at page 3).

Having the diisonitriles **3** in hand, a series of aromatic and aliphatic octaethyl bis[aminomethylidene(bisphosphonates)] **4** were synthesized by the reaction of isonitrile **3** with four equivalents of triethylphosphite in the presence of six equivalents of dry hydrogen chloride in 1,4-dioxane (Scheme 2). The aliphatic octaethyl tetraphosphonates **4k–n** were purified by washing the crude reaction mixture with an aq. solution of $NaHCO_3$ and the products were obtained as a thick oil. Aromatic tetraphosphonates **4a–j** were separated as solids by crystallization from an appropriate solvent (see Table 1 in Supplementary data). All octaethyl tetraphosphonates **4** were characterized by ^{31}P, ^{1}H, ^{13}C NMR, FTIR and by HRMS spectroscopy. In most cases, the reaction was almost quantitative, that is, the yields of tetraphosphonates **4** assayed by ^{31}P NMR were >90% and the only by-products were traces of the diethyl phosphonate resulting from dealkylation of the triethylphosphite and the volatile ethyl chloride (which was easily removed by evaporation).

Next, the octaethyl bis[aminomethylidene(bisphosphonates)] **4** were transformed into free acids **5** by the acid hydrolysis (in the case of the aliphatic tetraphosphonates **4k–n**) with refluxing 6 M hydrochloric acid or by dealkylation (in the case of the aromatic tetraphosphonates **4a–j**) with bromotrimethylsilane (Scheme 2).

A mild dealkylation method was chosen instead of hydrolysis because a cleavage of the Aryl-N bond and formation of aminomethylidenebisphosphonic acid ($H_2NCH(PO_3H_2)_2$) was observed in many cases of aromatic aminomethylidenebisphosphonates. For example, after a refluxing of the octaethyl naphthyl-1,5-diaminobis[aminomethylidene(bisphosphonate)] (**4j**) with 6 M hydrochloric acid, aminomethylidenebisphosphonic acid was obtained with a 96% yield. All bis[aminomethylidene(bisphosphonic)] **5** acids were obtained as crystalline, stable solids with high yields (see Table 1 in Supplementary data).

Among all bis[aminomethylidene(bisphosphonic)] acids **5** synthesized and described in this paper, only benzene-1,4-bis[aminomethylidene(bisphosphonic)] acid (**5a**) is known in the literature.[16] Xie et al. described in a Chinese patent its synthesis in the reaction of triethyl orthoformate, *p*-phenylenediamine and dimethyl phosphonate.[17] It is worth mentioning that this is the only example of the adoption of the diamine in the most widely used 'orthoformate method'. Surprisingly, we found only a few examples of other tetraphosphonic acids (with general formula $(H_2O_3P)_2CHNH\text{-}X\text{-}NHCH(PO_3H_2)_2$) in the literature. From aliphatic tetraphosphonic acids, only a derivative of ethylenediamine ($(H_2O_3P)_2CHNHCH_2CH_2NHCH(PO_3H_2)_2$) had been described as an environmentally friendly bleach fixing stabilizer chelating agent.[18] From aromatic derivatives, Lecercle et al. published a synthesis of four aromatic tetraphosphonates via double N–H insertion of diamines into tetraethyl diphosphonodiazomethane in the presence of $Rh_2(NHCOCF_3)_4$. The resultant octaethyl esters were dealkylated to free acids which showed interesting uranyl-binding properties.[19] Petrov et al. described a preparation of the benzene-1,3-bis[aminomethylidene(bisphosphonic)] acid in the reaction of m-phenylenebisformamide with a mixture of PCl_3/H_3PO_3 as phosphorylation agent and used it as monomer for synthesis of ion exchange resins.[20]

All bis[aminomethylidene(bisphosphonic)] acids **5a–n** were evaluated for their antiproliferative activity against MCF-7 human breast adenocarcinoma cells, HL-60 human promyelocytic leukemia cells and J774E mouse macrophages.[21] Since bisphosphonates accumulate in bones, a strong antiproliferative effect on bone metastatic and hematopoietic tumors can be expected, suggesting their possible clinical application as anticancer drugs. The MCF-7 cell line is a well-established model of breast cancer, which preferentially metastasizes to bone forming predominantly osteolytic lesions.[22] HL-60 cells are derived from hematopoietic lineage and, moreover, under specific culture conditions can differentiate into cells of osteoclast phenotype. Because of a difficulty in isolating and culturing large numbers of osteoclasts, many studies characterizing the pharmacologic properties of bisphosphonates in vitro are performed in osteoclast surrogates, in particular macrophages. J774E macrophages and osteoclasts are derived from the same hematopoietic lineage as well as are highly endocytic and capable of demineralizing bone particles.[23] J774E macrophages are also a model in studies on the influence of bisphosphonates

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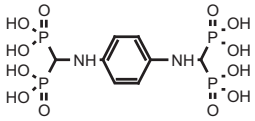
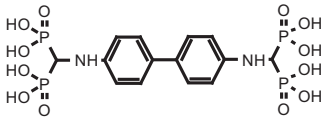
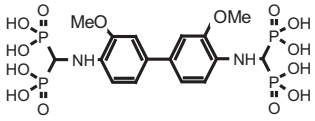
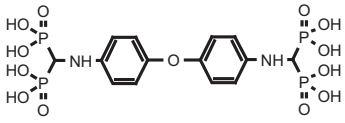
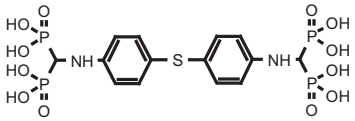
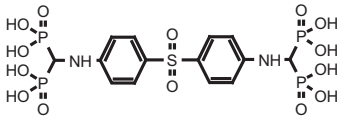
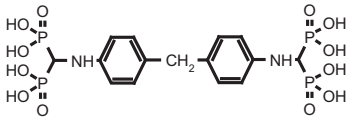
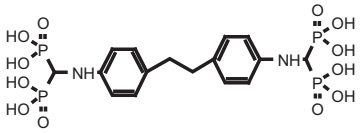
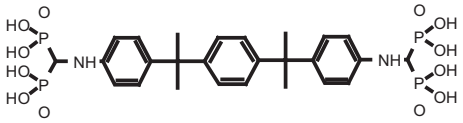
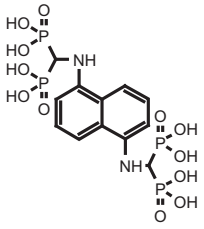
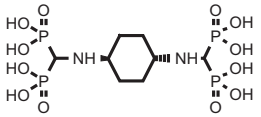
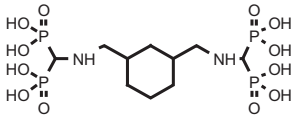
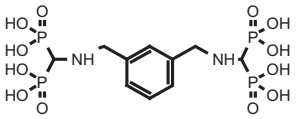
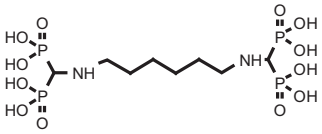
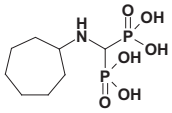
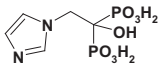
Table 1
In vitro anti-proliferative activity of bis[aminomethylidenebis(phosphonic)] acids **5** against MCF-7 human breast cancer cells, J774E mouse macrophages and HL-60 human promyelocytic leukemia cells

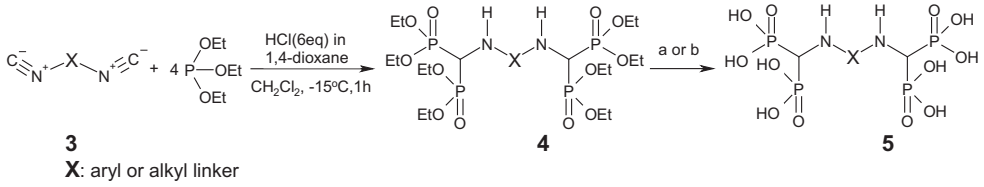
Compound no.	Compound structure	IC_{50}[a] [μM]		
		MCF-7	J774E	HL-60
5a		96.5 ± 24.8	90.1 ± 15.1	91.7 ± 21.5
5b		UN[b]	1242.8 ± 105.4	UN[b]
5c		UN[b]	541.9 ± 104.6	UN[b]
5d		1056.2 ± 436.5	667.3 ± 1.1	UN[b]
5e		625.5 ± 82.1	489.7 ± 49.5	1160.5 ± 13.5
5f		560.2 ± 0.8	97.7 ± 3.3	600.5 ± 1.7
5g		764.1 ± 216.7	654.2 ± 182.6	850.4 ± 30.8
5h		—[c]	—[c]	—[c]
5i		—[c]	—[c]	—[c]
5j		309.9 ± 25.7	5.8 ± 1.6	—[d]
5k		529.2 ± 38.3	749.6 ± 4.5	914.7 ± 38.3
5l		640.4 ± 15.9	604.7 ± 5.5	608.8 ± 11.2

(*continued on next page*)

Table 1 (*continued*)

Compound no.	Compound structure	IC_{50}[a] [μM]		
		MCF-7	J774E	HL-60
5m		1069.8 ± 350.6	632.0 ± 113.6	639.0 ± 28.7
5n		526.7 ± 48.3	786.0 ± 22.4	680.2 ± 254.3
Incadronic acid		233.8 ± 220.9	628.6 ± 126.8	105.9 ± 38.3
Zoledronic acid		23.0 ± 8.4	92.4 ± 54.5	595.8 ± 262.9

[a] IC_{50} values were determined at concentrations in the range 1–1000 μg/ml. Values are mean ± standard deviation.
[b] Compound was inactive in the concentration range tested.
[c] Compounds **5h** and **5i** were insoluble in the test conditions.
[d] It was impossible to estimate the activity of colored **5j** against HL-60 cells because of the limitations associated with the test technique.



Scheme 2. Synthesis of the bis[aminomethylidene(bisphosphonic)] acids **5** and their octaethyl esters **4**. Reagents and conditions: (a) TMSBr (10 equiv), CH_2Cl_2, 0 °C, 1 h, rt, overnight, MeOH (used for **4a**–**j**); (b) $6MHCl_{aq}$, reflux 8 h (used for **4k**–**n**).

on the proliferation and activity of tumor-associated macrophages (TAMs).[24]

The results of substance screening are summarized in Table 1.

The in vitro anti-proliferative activity of the presented compounds against tumor cells is differentiated. All aliphatic bis[aminomethylidene(bisphosphonic)] acids (**5k**–**n**) showed poor anti-proliferative activity towards the investigated cells. The IC_{50} values ranged from 530 μM to over 1 mM. However, in the case of the aromatic bis[aminomethylidene(bisphosphonic)] acids (**5a**–**j**), three of them showed a similar or higher activity than the reference bisphosphonates. Compounds **5a**, **5f** and **5j** proved to be the most active (Table 1, Fig. 2).

Compound **5a** showed a broad anti-proliferative activity towards all cells applied in the study. It inhibited the proliferation of MCF-7, J774E and HL-60 cells with IC_{50} values approx. 90 μM (Table 1). The IC_{50} value of **5a** for MCF-7 was two-fold lower than the corresponding value for incadronate and about four times higher than zoledronic acid. In the case of J774E macrophages compound **5a** showed a comparable anti-proliferative activity to zoledronate and a six times higher activity than incadronate. The derivatives of **4**,**4′**-diaminodiphenyl sulfone (**5f**) and 1,5-diaminonaphthalene (**5j**) exhibited a specificity of action against J77E cells. Compound **5f** showed comparable, while compound **5j** a 16 times stronger anti-proliferative activity in comparison to zoledronic acid.

Taking into account the possible clinical application, compounds **5a** and **5j** are the most interesting of all the compounds tested. Compound **5a** shows a broad nonspecific activity, relatively higher as compared to incadronic acid. Whereas compound **5j** specifically and strongly influence the proliferation of J774E

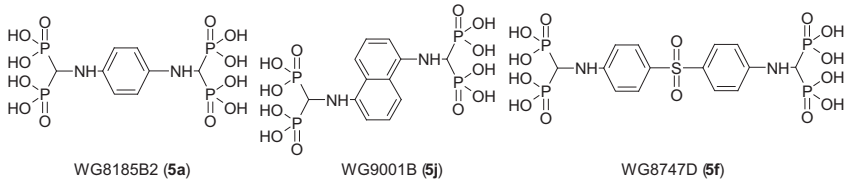


Figure 2. Bis[aminomethylidenebis(phosphonic)] acids **5** showing the highest anti-proliferative activity.

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macrophages. This is especially interesting in the context of anti-osteolytic treatment and blocking of interactions and mutual activation of osteoclasts and tumor metastatic cells in the bone microenvironment.

In summary, it was found that the reaction of triethylphosphite with aliphatic and aromatic diisonitriles in the presence of hydrogen chloride at a low temperature is a very simple and effective method for the preparation of bis[aminomethylidene (bisphosphonic)] acids and their octaalkyl esters. Our protocol is competitive with those described in the literature, mainly the 'orthoformate method' based on reaction of alkyl orthoformates, amines and dialkyl phosphonates. Moreover, in contrast to the esters obtained by literature methods, crude octaethyl esters are pure enough for other modifications, without the necessity for additional costly and time-consuming steps such as chromatographic purification. Using above procedure we obtained fourteen tetraphosphonic acids with diverse activity towards MCF-7, HL-60 and J774E cells. Three of them revealed an exceptionally interesting activity, especially compound **5j** seems to be of particular interest in the context of anti-osteolytic therapy, as it showed a powerful inhibitory activity towards J774E cells, with an IC_{50} value 16 and over a hundred times lower than zoledronate and incadronate, respectively.

Acknowledgments

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Supplementary data

Supplementary data (experimental procedures, spectroscopic data of the synthesized compounds **3i**, **3j**, **4a**–**n** and **5a**–**n** and copies of ^{31}P, ^{1}H, ^{13}C NMR spectra) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.05.071.

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16. In fact, the synthesis of acids and octaethyl esters of the biphenyl derivatives (**4,5 b**–**i**), are the subject of our two Polish patents: Soroka, M.; Goldeman, W. Polish Patent PL212792, 2012; *Chem. Abstr.* **2013**, 158, 158791; (b) Soroka, M.; Goldeman, W. Polish Patent PL212752, 2012; *Chem. Abstr.* **2013**, 158, 158793.
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