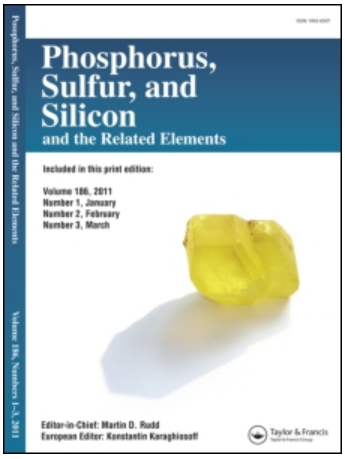


Phosphorus, Sulfur, and Silicon and the Related Elements
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

α-AMINO ACID DERIVED BISPHOSPHONATES. SYNTHESIS AND ANTI-RESORFTIVE ACTIVITY

Dana M. Mizrahi[a]; Trevor Waner[a]; Yoffi Segall[a]
[a] Department of Organic Chemistry, Israel Institute for Biological Research, Ness-Ziona, Israel

To cite this Article Mizrahi, Dana M. , Waner, Trevor and Segall, Yoffi(2001) 'α-AMINO ACID DERIVED BISPHOSPHONATES. SYNTHESIS AND ANTI-RESORFTIVE ACTIVITY', Phosphorus, Sulfur, and Silicon and the Related Elements, 173: 1, 1 — 25
To link to this Article: DOI: 10.1080/10426500108045257
URL: http://dx.doi.org/10.1080/10426500108045257

Phosphorus, Sulfur and Silicon, 2001, Vol. 173, pp. 1-25
Reprints available directly from the publisher
Photocopying permitted by license only

α-AMINO ACID DERIVED BISPHOSPHONATES. SYNTHESIS AND ANTI-RESORPTIVE ACTIVITY

DANA M. MIZRAHI*, TREVOR WANER and YOFFI SEGALL

Department of Organic Chemistry, Israel Institute for Biological Research, P.O. Box 19, Ness-Ziona 74100, Israel

(Received July 11, 2000; In final form November 12, 2000)

Eleven new bisphosphonates were prepared from naturally-occurring l-amino acids. The synthesis required special attention to amino and side-chain protections. The novel compounds were tested in TPTX (thyroparathyroidectomized) rats against arotinoid-induced hypercalcemia and were compared to alendronate. Most of the compounds showed moderate to no anti-resorptive activity. Two compounds were more active than clodronate, but less than alendronate. Limited SAR conclusions were drawn.

INTRODUCTION

Geminal bisphosphonates (BP), characterized by a P-C-P moiety, are pyrophosphate analogs, well-described in the literature. They have been found to inhibit mineralization both in soft-tissue[1,2] and, given in large doses, in bone[3]. Their main therapeutic effect, though, is the inhibition of bone resorption[4–6]. Thus, the extensive research in bisphosphonate field, over the last three decades, has been targeted towards minimizing or partially curing diseases related to bone resorption, such as Paget's disease, tumoral osteolysis and, recently, osteoporosis.

The search for new compounds initiated with simple alkyls, continued with amino alkyls and finally heterocyclic substituted bisphosphonates. First generation bisphosphonates, such as etidronate (hydroxyethylidenebisphosphonate), showed only a small difference between the dose inhibiting bone resorption and the one causing impairment of bone

* Corresponding Author.

mineralization. Second generation bisphosphonates, containing a free amine, such as alendronate (4-amino-1-hydroxybutylidenebisphosphonate) showed a far better therapeutic index.

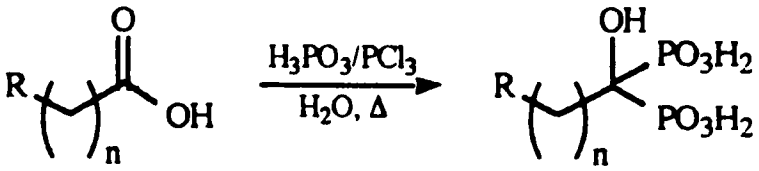
Currently, extensive research is still underway for more efficient bisphosphonates, of which smaller doses can be administered, resulting in diminished side-effects. Among these new compounds are tiludronate (chloro-4-phenylthiomethylenebisphosphonate); risedronate (2-(3-pyridinyl)-1-hydroxyethylydenebisphosphonate) and BM 21.0955 (3-(methylpentylamino)-1-hydroxypropylidenebisphosphonate)[7], which are currently under clinical investigations.

Although bisphosphonates originating from α-amino acids are mentioned briefly in the literature[8], no specific details were given, neither for the synthetic route, nor for their relative activities. In this paper we describe the preparation and activities of several α-amino acid derived 1-hydroxybisphosphonates.

SYNTHESES

Simple alkyl and aminoalkyl substituted 1-hydroxybisphosphonates were prepared by a one-pot reaction, according to numerous publications (Scheme 1). Usually this reaction involved mixing of the amino acid with phosphorus acid at high temperatures, but reactions with PCl_3 (as well as $POCl_3$ and PCl_5) and water were also described[9–16]. The proposed reaction conditions varied largely in reactants ratio, temperature and reaction times[17], due to the fact that no mechanism was determined. Application of this method to α-amino acids was unsuccessful in most cases. The only amino acid that reacted properly with phosphorus acid to give the corresponding BP was l-alanine. All other amino acids did not react and were recovered from the reaction mixture.

A recent publication[18] described bisphosphonate preparation, according to scheme 1, as having "extremely poor physical characteristics". Indeed, in our experience, this reaction which started as a two-phase melt turned into a semisolid whose stirring became difficult and which could not be heated homogeneously. The need for a solvent was obvious, but most of our attempts, using solvents such as chlorobenzene or methanesulfonic acid[18], failed. The authors recommending methanesulfonic acid, indicated that β-substituted amino acids reacted in low yields and more hindered α-amino acids did not react at all.



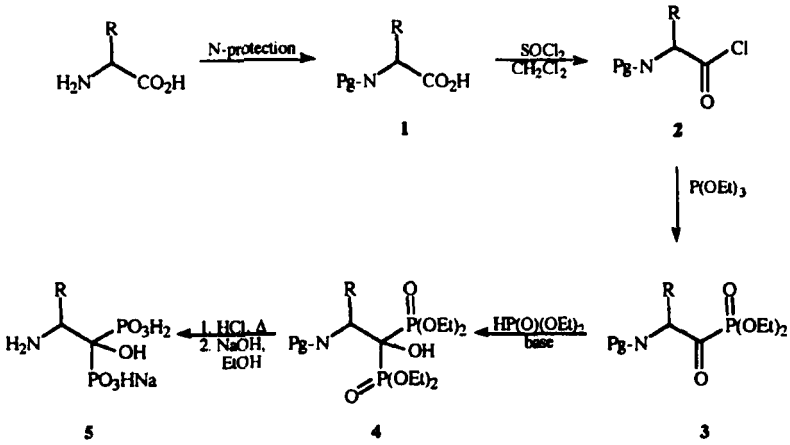
R = alkyl, aminoalkyl

SCHEME 1 Routine Bisphosphonate Synthesis

In our case, l-Proline bisphosphonate was the only compound produced by a reaction carried out in chlorobenzene. It was obtained as a 1:1 mixture with the starting amino acid and was later purified as the mono sodium salt.

In order to carry out bisphosphonate synthesis in organic media, initial protection of the amino acid amine moiety, followed by activation of the acid were required. Insertion of the phosphorus atoms was divided into two steps: initial reaction with a suitable phosphorus reagent to produce the acylphosphonate, and a second phosphorus attack to form the bisphosphonate (scheme 2). Since conversion to acid chloride served as the acid activation process (releasing volatile alkyl chloride upon C-P bond formation), the amine protecting group had to be either Fmoc or phthalimide, both of which are stable in acidic conditions. Various attempts were performed using commercial *N*-Fmoc amino acids. While the acid chlorides **2** and acylphosphonates **3** were obtained, the second C-P bond formation was unsuccessful, resulting in isolation of the starting *N*-Fmoc amino acids.

Employing the phthalimide protecting group was advantageous. The *N*-phthalimide amino acids were prepared using *N*-(ethoxycarbonyl)phthalimide[19,20], thus avoiding the harsh conditions of reaction with phthalic acid. The condensations of tri- and dialkylphosphites with **2** and **3**, respectively, were initially carried out in dichloromethane. Desired acylphosphonates **3** were usually produced smoothly, but bisphosphonates **4** were obtained as a mixture with the corresponding phosphate-phosphonate rearranged products **6** (scheme 3). This rearrangement has been previously

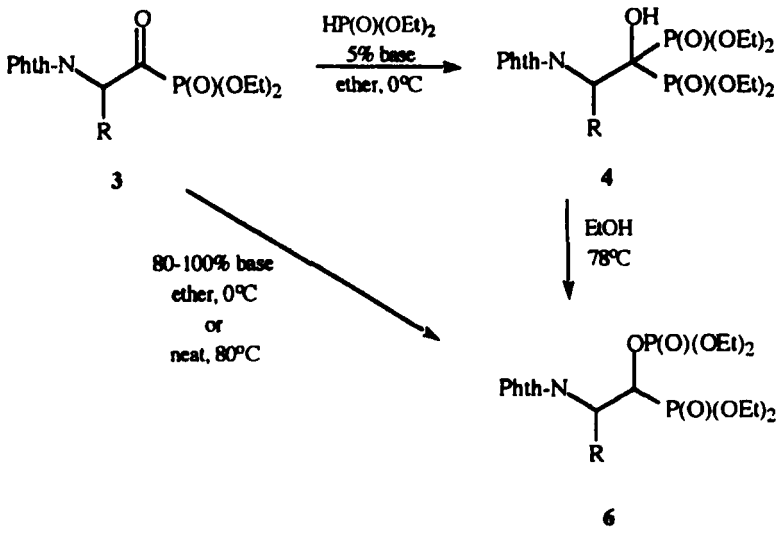


SCHEME 2 Synthetic Route for Amino Acid Derived Bisphosphonates

described in the literature[21] and the products were identified by ^{1}H NMR. In ^{31}P NMR spectrum phosphate-phosphonates **6** appear as a pair of doublets at *ca.* −3 ppm (phosphate) and *ca.* 14 ppm (phosphonate) with a coupling constant of *ca.* 19.5 Hz, whereas the desired bisphosphonate gave peaks at *ca.* 13 ppm. In contrast to the published mechanism (scheme 3), in our case, the rearrangement proceeded smoothly in dichloromethane with no added base, which encouraged us to use a less polar solvent for the condensation reactions.

Performing the reactions in toluene[22] proved beneficial, but the temperature had to be kept close to 0°C. The acylphosphonates **3** were identified by ^{31}P NMR but were not isolated. Addition of diethylphosphite, followed by triethylamine, at the same low temperature, resulted in bisphosphonates **4**. In all cases, the acylphosphonates were obtained in 1 hour, but reaction rate for the second condensation depended greatly on the bulkiness of the amino acid side chain (table I).

In the case of glycine, lacking a β-carbon, the reactions are rapid and the triethylphosphite reacted twice with *N*-phthalimide glycyl chloride to give the desired bisphosphonate, which rearranged immediately to the phosphate-phosphonate **6**. Other amino acids, containing a methylene as the



SCHEME 3 Phosphate-Phosphonate Rearrangement

β-carbon (X=H), showed large quantities of acylphosphonates along with traces of the desired bisphosphonates. These mixtures were converted into almost pure bisphosphonates upon addition of diethylphosphite and base. Isoleucine and valine, comprising a methyl β-substituent (X=Me), were converted into pure acylphosphonates, but the second reaction was very slow. Prolonged reaction times always resulted in partial rearrangement and formation of by-products.

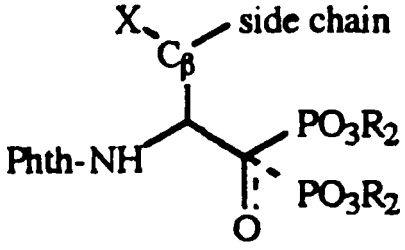


TABLE I β-Substituent Influence on Reaction Profile

Amino Acid	*X*	*1st C-P formation*		*2nd C-P formation*
		Acylphosphonate **3**	*Bisphosphonate* **4**	
Gly	No C_β	–	rearrangement product **6** and BP **4**	rearrangement product **6**
Phe, Leu, Trp...	H	~90%	traces	~90% BP
Ile, Val	Me	pure	–	slow and impure BP

Due to molecular asymmetry, the two phosphorus atoms were not equivalent in ^{31}P NMR. They appeared either as two separate doublets, or, more frequently, as an AB quartet, due to their close chemical shifts. In some cases (e.g. l-alanine, figure 1), the phosphorus atoms were virtually chemically different, so that they gave diverse coupling constants with the adjacent β-CH in H-coupled ^{31}P NMR (W-effect).

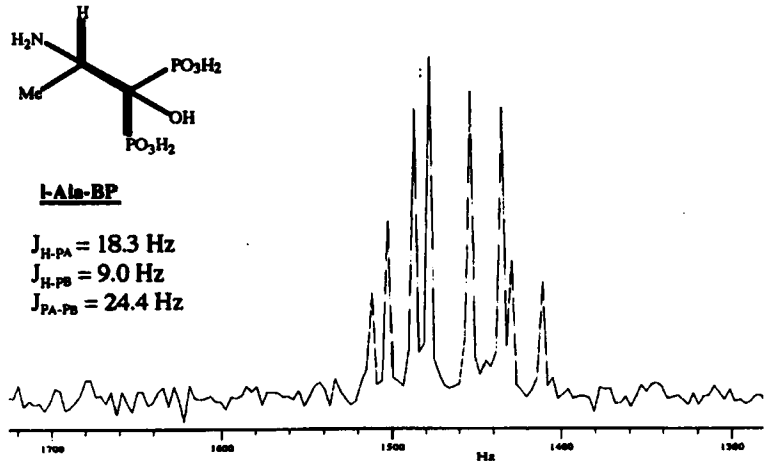


FIGURE 1 ^{31}P NMR spectrum of l-alanine derived bisphosphonate

Eleven tetraethyl 2-*N*-phthalimide-1-hydroxy-1,1-bisphosphonates were prepared, originating from *l*-amino acids (table II). For tryptophane (**f**),

additional protection of the indole amine as the formamide derivative was required. In the case of aspartic and glutamic acids (**i** and **j**), mono β- and γ- esters were used as the starting materials. Two phthalimide groups were introduced to protect both amine moieties of lysine and ornithine (**k** and **l**). All bisphosphonates **4** were purified by column chromatography (2–5% MeOH in $CHCl_3$), which resulted in relatively low yields. Compounds **4** were further hydrolyzed to remove the ester and phthalimide groups. Pure bisphosphonates were isolated as the mono sodium salts **5**, suitable for biological testing. Valine-derived bisphosphonate **5g** was tested as part of a mixture that could not be purified. Methionine-derived bisphosphonate **5h** was obtained as a 1:1 mixture of the desired product and the sulfoxide bisphosphonate. The oxidation of the sulfide occurred during hydrolysis, and the products were inseparable.

BIOLOGICAL TESTS

The bisphosphonate mono sodium salts were tested in Thyroparathyroidectomized (TPTX) rats for bone resorption inhibitory activity, according to the literature[7]. In this model, test compounds treat chemically-induced hypercalcemia (induced by administration of a vitamin A derivative). Removal of the thyroid and parathyroid glands serves to avoid PTH interference.

All bisphosphonates were tested at a dose of 0.1 mg P per kg body weight and compared to the action of alendronate sodium salt at the same concentration. Active bisphosphonates were also tested at a double dose, to establish dose response. The results are described in table III. Graph 1 shows the results in comparison to the activities of known bisphosphonates alendronate, pamidronate and clodronate[7].

Leucine and phenylalanine bisphosphonates (**5d** and **5c**) showed modest antiresorbing activity, as compared to alendronate. These activities were confirmed by testing with a 2-fold concentration. Glutamic acid derived bisphosphonate **5j** showed high activity during the first test, which decreased when tested at double concentration. Repeating the test at both concentrations gave no activity altogether. We suspected that the compound decomposed, but ^{31}P NMR showed no changes in the molecule. Thus we concluded that, probably, the first test result was false. All other compounds showed no activity at all.

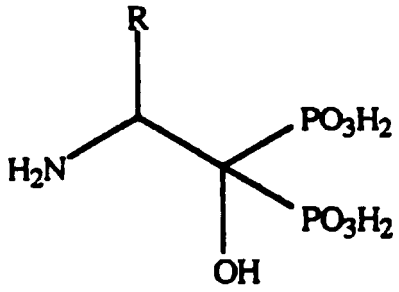


TABLE II Novel Bisphosphonate salts **5**

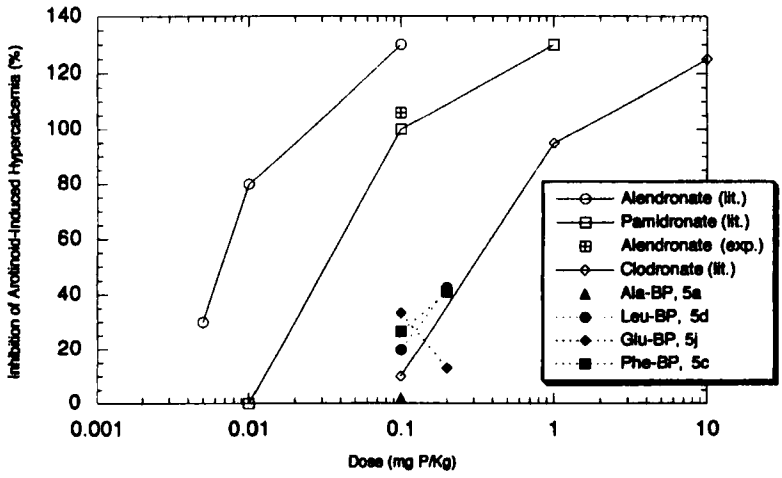
No.	*Amino Acid*	*R*	*Bisphosphonate 5 (%)*[a]	*^{31}P NMR chemical shift of ABq (ppm)*	*P-P Coupling Constant (Hz)*
a	Ala	CH_2Me	20	12.0	24.4
b	Pro	Cyclic	40	11.3	23.0
c	Phe	CH_2Ph	12.5	11.7	19.5
d	Leu	CH_2(i-Pr)	45	12.1	16.7
e	Ile	$CH(Me)CH_2Me$	Impure		
f	Trp	CH_2(indole)	31	11.9	singlet
g	Val	$CH(Me)_2$	mixture		
h	Met	$(CH_2)_2SMe$	42, mixture	14.2, 14.8	16.7, 18.5
i	Asp[b]	CH_2CO_2H	7	11.1	18.2
j	Glu[c]	$(CH_2)_2CO_2H$	24	11.8	24.0
k	Lys	$(CH_2)_4NH_2$	15	12.4	22.2
l	Orn	$(CH_2)_3NH_2$	14	12.2	24.0
m	GABA[d]		43	14.8	12.0

a. Overall yield from **2**, after column chromatography and hydrolysis
b. l-Aspartic acid β-methyl ester
c. l-Glutamic acid γ-ethyl ester
d. leading to Alendronate

TABLE III Inhibition of Induced Hypercalcemia by Novel Bisphosphonates

No.	*Original Amino Acid*	*Inhibition at 0.1 mg P/Kg (%)*	*Repeat*	*Inhibition at 0.2 mg P/Kg (%)*	*Repeat*
5a	Ala	1.7±21.3			
5i	Asp	0			
5j	Glu	33.3±18.2	0[a]	13.0+6	0
5d	Leu	19.7±23		42.5±7.8	
5k	Lys	0			
5h	Met	0			
5l	Orn	0			
5c	Phe	26.5±8.2		41.0±9.2	
5b	Pro	0			
5f	Trp	0			
5g	Val	0			

a. Test was repeated at the same concentration, but no inhibition was detected.



GRAPH 1 Anti-Resorptive Activity of Novel Bisphosphonates 5, compared to known compounds

DISCUSSION

Bisphosphonates are pyrophosphate analogs in which the P-O-P has been replaced by a virtually non-cleavable P-C-P moiety. The P-C-P "hook" of the molecule affords strong affinity to bone and the chain is responsible for inhibition of bone resorption, as well as toxicity.[23] Bone affinity increases if a hydroxy group is also part of the "hook", and small changes in the chain result in largely different effects.

As a result, we concentrated on amino acids as a family of compounds leading to 1-hydroxy-1,1-bisphosphonates which differ by the side chain. Some groups of amino acids show rather small changes in the side-chain (e.g. Ala-Val-Ile-Leu or Phe-Trp), which could afford some SAR. Moreover, most of the bisphosphonates described in the literature feature an amine moiety that is distant from the P-C-P moiety. We were interested in investigating the influence of an α-amine on the activity.

A clear SAR could not be elucidated from our results but some general observations were made. As in less hindered bisphosphonates, the more lipophilic the side chain, the better resorption inhibition was displayed. The most active compounds prepared contained benzyl (Phe) and isobutyl (Leu) side chains. Likewise, we expected valine and isoleucine bisphosphonates to show modest activities, but due to synthetic difficulties they could not be purified and tested. Bisphosphonates displaying more polar side chain functionalities, such as carboxylic acid, amine or indole, showed no activity at all.

Bisphosphonates originating from lysine and ornithine (**5l** and **5m**) were surprisingly inactive. Ornithine-bisphosphonate is, in fact, one carbon longer than alendronate and contains an additional α-amine (figure 2). It seemed that the additional amine increased the total polarity of the molecule, so that antiresorptive activity was diminished.

In general, we postulated that placing the amine at the α-position increased the polarity of the molecule "hook", thus creating a very polar head and non-polar chain, which resulted in increased dipole of the whole molecule. Most reported active bisphosphonates featured a polar head, but the presence of the amine at β- and γ – positions distributed the polarity more evenly.

An additional explanation for the inactivating influence of the α-amine may be attributed to the larger steric hindrance of the "bone hook", which might affect the attachment to hydroxyapatite. Similar low potencies were described for cyclic bisphosphonates, designed to decrease mineralization defects by decreasing hydroxyapatite affinity[23].

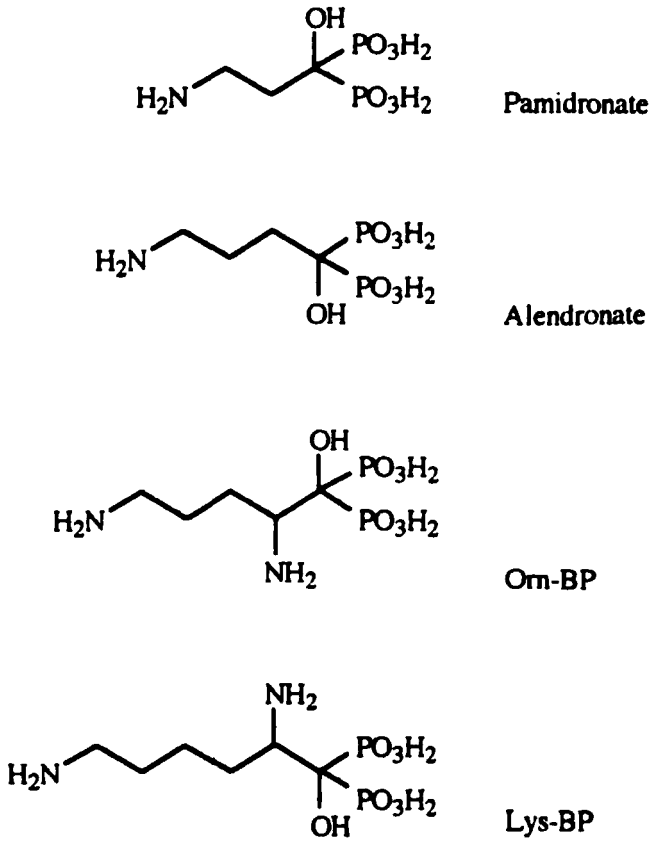


FIGURE 2 Analogous Structures of Known and Novel Bisphosphonates

EXPERIMENTAL SECTION

Chemistry

NMR spectra were obtained on GE GN 300 WB at 300.1 MHz for ^{1}H and 121.65 MHz for ^{31}P. Deuterated solvents were used as standards for ^{1}H NMR. Trimethylphosphate was used as an external standard for ^{31}P NMR. Mass spectra were obtained on VG 70 VSEQ spectrometer, using a Cs gun for FAB spectra. Flash chromatography was carried out on silica gel

(Merck. Art. 9385). Commercially available chemicals were purchased from Aldrich Co., Sigma Co. and Fluka Co. and were used without further purification.

2-Amino-1-hydroxypropylidene-1,1-bisphosphonic acid (5a acid)

A mixture of *l*-alanine (4.45 g, 0.05 mol) and H_3PO_3(8.2 g, 0.1 mol) was heated to 90°C under N_2, until a homogeneous mixture was achieved. PCl_3 (6.55 mL, 10.3 g, 0.075 mol) was added dropwise and the gradually yellowing mixture was stirred at ~100°C for 1.5 h. The resulting strong yellow gummy mixture was cooled to room temperature and further by an ice bath. Dist. H_2O (~30 mL) was added and the resulting slurry was refluxed for 4.5 h. The yellow mixture was cooled and filtered to give a faint yellow filtrate and a strongly yellow cake. The filtrate was concentrated rotarily, MeOH and acetone were added and the mixture was stirred at rt overnight. Heavy precipitation occurred and the white powder was collected by filtration. Yield: ~20%. ^1H NMR: D_2O δ 1.48 (d, J = 6.7 Hz, 3H, Me), 3.75 (m, 1H, CH). ^{31}P NMR {^1H}: D_2O δ 12.0 (ABq, J = 24.4 Hz); MS: Derivatized with diazomethane, to give the ester 274 (51), 259 (5), 243 (8), 165 (24), 109 (100); FAB+: 236 (100); FAB: 234 (100); desired 235 (M^+).

1-Hydroxy-1-(2-pyrrolidine)-1,1-bisphosphonic acid (5b acid)

A mixture of *l*-proline (5.75 g, 0.05 mol), H_3PO_3 (6.15 g, 0.075 mol) and chlorobenzene (25 mL) was heated to 100°C under N_2, until a homogeneous mixture was achieved. PCl_3 (6.55 mL, 10.3 g, 0.075 mol) was added dropwise and stirring was maintained at ~100°C for 2 h. The mixture solidified and heating was stopped. Dist. H_2O (25 mL) was added and the resulting homogeneous mixture was refluxed for 2 h. The mixture was filtered, the phases separated and the aqueous phase was concentrated. Treatment with EtOH precipitated a 1:1 mixture of *l*-proline and desired bisphosphonate.

1-Hydroxy-1-(2-pyrrolidine)-1,1-bisphosphonate mono sodium salt (5b)

The above mixture was suspended in dist. H_2O and brought to pH=4.4 with 5N NaOH. The suspension became clear and was added dropwise to a large amount of EtOH (~ 100 mL). The resulting suspension was aged overnight and filtered quickly. The desired bisphosphonate salt was

obtained as a very hygroscopic solid. Yield: ~40%. ^{1}H NMR: D_2O δ 1.99 and 2.09 (two m, 2H, CH_2CH), 2.22 (m, 2H, CH_2CH_2N), 3.27 (m, 2H, CH_2N), 4.00 (m, 1H, CH). ^{31}P NMR {^{1}H}: D_2O δ 11.3 (ABq, J_{AB} = 23 Hz). FAB+: 284 (32), 185 (30), 171 (46), 149 (100); desired 283 (M^+).

N-phthalimide amino acids[19]

These compounds were prepared according to the literature, except for diamino acids, for which two equivalents of both *N*-(ethoxycarbonyl)phthalimide and $NaHCO_3$ were used.

N-Phthalimide glycine

White crystals; yield: 63%. ^{1}H NMR ($CDCl_3$ and CD_3OD): δ 4.37 (s, 2H, CH_2), 7.70 (m, 2H, phth), 7.82 (m, 2H, phth).

N-Phthalimide l-phenylalanine (1c)

White powder; yield: 88%. ^{1}H NMR (acetone-d_6): δ 3.58 (ABq, J_{AB} = 15.9 Hz, 1H, CH_2), 3.60 (s, 1H, CH_2), 5.22 (dd, J = 9.5, 7.4 Hz, 1H, CH), 7.18 (m, 5H, phenyl), 7.65 (m, 2H, phth), 7.82 (m, 2H, phth).

N-Phthalimide l-leucine (1d)

White crystals; yield: 81%. ^{1}H NMR ($CDCl_3$): δ 0.93 (t, J = 6.8 Hz, 6H, *Me*$_2$CH), 1.45 (m, 1H, Me$_2$C*H*), 1.95 (ddd, J = 14.4, 10.3, 4.4 Hz, 1H, CH_2), 2.36 (ddd, J = 14.4, 11.7, 4.15 Hz, 1H, CH_2), 4.97 (dd, J= 11.7, 4.4 Hz, 1H, CHN), 7.72 (m, 2H, phth), 7.85 (m, 2H, phth).

N-Phthalimide l-isoleucine (1e)

White crystals; yield: 61%. ^{1}H NMR ($CDCl_3$): δ 0.84 (t, J = 7.3 Hz, 3H, *Me*CH_2), 1.10 (d, J = 6.8 Hz, 3H, *Me*CH), 1.05 (m, 2H, CH_2), 2.50 (m, 1H, C*H*Me), 4.65 (d, J = 8.6 Hz, 1H, CHN), 7.72 (m, 2H, phth), 7.85 (m, 2H, phth).

N-Phthalimide l-tryptophane (1f)

Bright yellow powder; yield: 95%. ^{1}H NMR ($CDCl_3$): δ 3.76 (ABq of d, J_{AX} = 5.2, 0.7 (long range) Hz, J_{BX} = 11.0 Hz, J_{AB} = 15.0 Hz, 2H, CH_2), 5.33 (dd, J = 11.0, 5.3 Hz, 1H, C*H*CH_2), 7.00 (d, J = 2.2 Hz, 1H, C*H*NH), 7.06 (td, J_t = 7.4 Hz, J_d = 1.2 Hz, 1H, indole), 7.13 (td, J_t = 7.5 Hz, J_d = 1.5 Hz, 1H, indole), 7.27 (d, J = 8.1 Hz, 1H, indole), 7.60 (d, J = 8.1 Hz, 1H, indole), 7.65 (m, 2H, phth), 7.75 (m, 2H, phth), 7.95 (Brs, 1H, NH).

N-Phthalimide l-methionine (1h)

White crystals; yield: 92%. ^{1}H NMR ($CDCl_3$): δ 2.06 (s, 3H, SMe), 2.52 (m, 4H, two CH_2), 5.12 (m, 1H, CH), 7.75 (m, 2H, phth), 7.85 (m, 2H, phth).

N-Phthalimide l-aspartic acid β-methyl ester (1i),

White crystals; yield: 55%. ^{1}H NMR ($CDCl_3$): δ 3.05 (dd, J_{AB} = 16.5 Hz, J_d = 8.9 Hz, 1H, CH_2CO_2), 3.27 (dd, J_{AB} = 16.5 Hz, J_d = 5.8 Hz, 1H, CH_2CO_2), 3.62 (s, 3H, OMe), 5.29 (dd, J = 8.9, 5.8 Hz, 1H, CH), 7.70 (m, 2H, phth), 7.82 (m, 2H, phth).

N-Phthalimide l-glutamic acid γ-ethyl ester (1j)

Thick, colorless oil; yield: 83%. ^{1}H NMR ($CDCl_3$): δ 1.20 (t, J = 7.2 Hz, 3H, Me), 2.39 (d, J = 7.5 Hz, 2H, CH_2CO_2), 2.55 (two m, 2H, $CHCH_2$), 4.06 (q, J = 7.2 Hz, 2H, OCH_2), 4.97 (dd, J = 10.1, 5.3 Hz, 1H, CH), 7.73 (m, 2H, phth), 7.86 (m, 2H, phth).

4-N-Phthalimide butyric acid (1m)

White crystals; yield: 62%. ^{1}H NMR ($CDCl_3$): δ 2.00 (quintet, J = 7.2 Hz, 2H, $CH_2CH_2CO_2$), 2.40 (t, J = 7.4 Hz, 2H, CH_2CO_2), 3.75 (t, J = 6.8 Hz, 2H, CH_2N), 7.70 (m, 2H, phth), 7.83 (m, 2H, phth).

Di-N-phthalimide l-lysine (1k)

Thick, colorless oil; yield: 80%. ^{1}H NMR ($CDCl_3$): δ 1.37 (m, 2H, CH_2CH_2CH), 1.72 (m, 2H, CH_2CH_2N), 2.30 (m, 2H, $CHCH_2$), 3.63 (t, J = 7.2 Hz, 2H, CHIN), 4.86 (dd, J = 10.0, 6.0 Hz, 1H, CH), 7.68 (m, 2H, phth), 7.73 (m, 2H, phth), 7.78 (m, 2H, phth), 7.83 (m, 2H, phth).

Di-N-phthalimide l-ornithine (1l)

White powder; yield: 26%. ^{1}H NMR ($CDCl_3$): δ 1.69 (quintet, J = 7.5 Hz, 2H, CH_2CH_2CH), 2.27 (m, 2H, $CHCH_2$), 3.70 (d quintet, J_q = 7.1 Hz, J_d = 3.7 Hz, 2H, CH_2N), 4.96 (dd, J = 9.8, 5.8 Hz, 1H, CH), 7.68 (m, 2H, phth), 7.72 (m, 2H, phth), 7.80 (m, 2H, phth), 7.84 (m, 2H, phth).

Alternative method for preparing N-phthalimide amino acids

In some cases, the phthalimide protecting group could not be attached by the common procedure. Thus, an alternative method was used, employing an organic solvent[20].

N-Phthalimide l-valine (1g)

White crystals; yield: 81%. ^{1}H NMR ($CDCl_3$): δ 0.91 (d, J = 6.8 Hz, 3H, Me_2CH), 1.16 (d, J = 6.6 Hz, 3H, Me_2CH), 2.76 (d quintet, J_d = 8.4, J_q = 6.8, 1H, CH_β), 4.61 (d, J = 8.4 Hz, 1H, CHN), 7.75 (m, 2H, phth), 7.85 (m, 2H, phth).

N-Phthalimide-N(in)-formyl l-tryptophane

HCl(g) was bubbled into a mixture of *N*-phthalimide l-tryptophane (5 g, 15 mmol) in formic acid (60 mL). The mixture turned dark red and was stirred at rt for 5 h. Formic acid was evaporated under reduced pressure and the resulting red solid was dissolved in EtOAc and recrystallized with ether. The desired product was obtained by filtration as yellow crystals. Yield: 90%. ^{1}H NMR ($CDCl_3$): δ 3.70 (m, 2H, CH_2), 5.33 (m, 1H, $CHCH_2$), 7.27 (Brs, 1H, indole), 7.30 (m, 2H, indole), 7.65 (m, 2H, indole), 7.65 (m, 2H, phth), 7.85 (m, 2H, phth), 8.29 (Brd, J = 4.0 Hz, 1H, CHO).

General procedure for the preparation of N-phthalimide amino acid chlorides

A mixture of an *N*-phthalimide amino acid (20 mmol) and $SOCl_2$ (12 ml, 164 mmol) in dry CH_2Cl_2 (40 mL) was refluxed for 5 h. Excess $SOCl_2$ and solvent were evaporated under reduced pressure and the residue was washed twice with hexane and reevaporated. The product was used without further purification.

N-Phthalimide glycinyl chloride

Off-white powder; yield: 85%. ^{1}H NMR (DMSO-d_6): δ 4.30 (s, 2H, CH2), 7.90 (m, 4H, phth). MS (CI *i*-Bu): 224 (MH^+, 100)

N-Phthalimide l-phenylalaninyl chloride (2c)

Yellow powder; yield: 88%. ^{1}H NMR ($CDCl_3$): δ 3.60 (ABq of d, J_{AB} = 14.2 Hz, J_{AX} = 10.8 Hz, J_{BX} = 5.4 Hz, 1H, CH_2), 5.32 (dd, J = 10.8, 5.3 Hz, 1H, CH), 7.00–8.00 (several m, 9H, phenyl and phth).

***N*-Phthalimide l-leucinyl chloride (2d)**

Oil; yield: 90%. ^{1}H NMR ($CDCl_3$): δ 0.94 (t, J = 6.6 Hz, 3H, Me_2CH), 0.96 (t, J = 6.6 Hz, 3H, Me_2CH), 1.51 (m, 1H, Me_2CH), 2.04 (ddd, J = 14.4, 10.0, 4.4 Hz, 1H, CH_2), 2.37 (ddd, J = 14.4, 11.2, 4.15 Hz, 1H, CH_2), 5.12 (dd, J = 11.2, 4.4 Hz, 1H, CHN), 7.79 (m, 2H, phth), 7.91 (m, 2H, phth).

***N*-Phthalimide l-isoleucinyl chloride (2e)**

Oil; yield: 88%. ^{1}H NMR ($CDCl_3$): δ 0.87 (t, J = 7.3 Hz, 3H, $MeCH_2$), 1.07 (m, 1H, CH_2), 1.13 (d, J = 6.6 Hz, 3H, $MeCH$), 1.50 (m, 1H, CH_2), 2.54 (m, 1H, $CHMe$), 4.81 (d, J = 8.8 Hz, 1H, CHN), 7.80 (m, 2H, phth), 7.93 (m, 2H, phth).

***N*-Phthalimide-*N*(in)-formyl l-tryptophanyl chloride (2f)**

Light brown powder; yield: 93%. ^{1}H NMR ($CDCl_3$): δ 3.72 (ABq of d, J_{AX} = 6.0 Hz, J_{BX} = 10.0 Hz, J_{AB} = 15.0 Hz, 2H, CH_2), 5.45 (Brt, 1H, $CHCH_2$), 7.20–7.65 (several m, 5H, indole), 7.75 (m, 2H, phth), 7.85 (m, 2H, phth), 8.32 (Brd, 1H, CHO).

***N*-Phthalimide l-methioninyl chloride (2h)**

Yellow oil; yield: 85%. ^{1}H NMR ($CDCl_3$): δ 2.08 (s, 3H, SMe), 2.48 (m, 1H, CH_2S), 2.58 (m, 1H, CH_2S), 2.59 (m, 2H, CH_2CH), 5.34 (m, 1H, CH), 7.80 (m, 2H, phth), 7.93 (m, 2H, phth).

***N*-Phthalimide l-aspartyl chloride β-methyl ester (2i)**

Orange oil; yield: 88%. ^{1}H NMR ($CDCl_3$): δ 3.13 (dd, J_{AB} = 17.0 Hz, J_d = 8.1 Hz, 1H, CH_2CO_2), 3.43 (dd, J_{AB} = 17.0 Hz, J_d = 6.2 Hz, 1H, CH_2CO_2), 3.70 (s, 3H, OMe), 5.57 (dd, J = 8.0, 6.1 Hz, 1H, CH), 7.80 (m, 2H, phth), 7.93 (m, 2H, phth).

***N*-Phthalimide l-glutamyl chloride γ-ethyl ester (2j)**

Brown oil; yield: 89%. ^{1}H NMR ($CDCl_3$): δ 1.20 (t, J = 7.2 Hz, 3H, Me), 2.38 (m, 2H, CH_2CO_2), 2.50 and 2.65 (two m, 2H, $CHCH_2$), 4.07 (dq, J_q = 7.2 Hz, J_d = 1.1 Hz, 2H, OCH_2), 5.16 (dd, J = 10.0, 4.8 Hz, 1H, CH), 7.80 (m, 2H, phth), 7.92 (m, 2H, phth).

4-N-Phthalimide butyryl chloride (2m)

White crystals; yield: 92%. ^{1}H NMR ($CDCl_3$): δ 2.08 (quintet, J = 7.0 Hz, 2H, $CH_2CH_2CO_2$), 2.99 (t, J = 7.2 Hz, 2H, CH_2CO_2), 3.77 (t, J = 6.6 Hz, 2H, CH_2N), 7.74 (m, 2H, phth), 7.86 (m, 2H, phth).

Di-N-phthalimide l-lysinyl chloride (2k)

Brown oil; yield: 80%. ^{1}H NMR ($CDCl_3$): δ 1.41 (m, 2H, CH_2CH_2CH), 1.75 (m, 2H, CH_2CH_2N), 2.38 (m, 2H, $CHCH_2$), 3.68 (t, J = 7.2 Hz, 2H, CH_2N), 5.04 (dd, J = 8.3, 6.8 Hz, 1H, CH), 7.73 (m, 2H, phth), 7.82 (m, 4H, phth), 7.93 (m, 2H, phth).

Di-N-phthalimide l-ornithinyl chloride (2l)

Off white powder; yield: 92%, ^{1}H NMR ($CDCl_3$): δ 1.72 (quintet, J = 7.0 Hz, 2H, $CHCH_2$), 2.33 (m, 2H, CH_2CH_2CH), 3.72 (d quintet, J_q = 7.0 Hz, J_d = 4.3 Hz, 2H, CH_2N), 5.16 (dd, J = 8.2, 7.1 Hz, 1H, CH), 7.71 (m, 2H, phth), 7.79 (m, 2H, phth), 7.82 (m, 2H, phth), 7.91 (m, 2H, phth).

N-Phthalimide l-valinyl chloride (2g)

Tan powder; yield: 85%. ^{1}H NMR ($CDCl_3$): δ 0.91 (d, J= 6.8 Hz, 3H, Me_2CH), 1.16 (d, J = 6.6 Hz, 3H, Me_2CH), 2.75 (d septet, J_d = 8.2, J_s = 6.8, 1H, CH_β), 4.74 (d, J = 8.4 Hz, 1H, CHN), 7.81 (m, 2H, phth), 7.92 (m, 2H, phth).

General Procedure for the preparation of bisphosphonates via acylphosphonates

Triethylphosphite (5 g, 30 mmol) was added to an ice-cold solution of the acyl chloride (30 mmol) in toluene (18 mL) under N_2. The mixture was stirred at 0°C for 1 h and acylphosphonate was identified by ^{31}P NMR. Diethylphosphite (4.15 g, 30 mmol) was added, followed by dropwise addition of Et_3N (3 g, 30 mmol). The mixture was stirred at 0–5° until all acylphosphonate disappeared (usually 1–3 h for non-hindered acids). Toluene was distilled under reduced pressure at rt. The desired bisphosphonate was purified by column chromatography (2–5% MeOH in $CHCl_3$).

N-Phthalimide glycyl phosphonate diethyl ester

^{31}P NMR {^{1}H} (C_6D_6): δ – 6.10.

2-N-Phthalimide-1-phosphoro-1-phosphonoethylidene tetraethyl ester (6)

(major, rearranged product from *N*-phthalimide glycyl phosphonate) Light yellow oil; yield: 30%. ^{1}H NMR (C_6D_6): δ 0.84 (td, J_t= 7.1 Hz, J_d = 1 Hz, 3H, *Me*CH_2O), 0.98 (td, J_t = 7.1 Hz, J_d = 1 Hz, 3H, *Me*CH_2O), 1.05 (td, J_t = 7.1 Hz, J_d = 1 Hz, 6H, two *Me*CH_2O), 3.80 (m, 2H, CH_2O), 4.05 (m, 7H, three CH_2O and CHN), 4.43 (ddd, J = 14.3, 10.6, 7.1 Hz, 1H, CHN), 5.42 (dddd, J = 14.2, 10.5, 2.9, 0.7 Hz, 1H, CH), 6.90 (m, 2H, phth), 7.50 (m, 2H, phth). ^{31}P NMR {^{1}H} (C_6D_6): δ −3.36 (d, J = 18.5 Hz, phosphate), 13.81 (d, J = 20.3 Hz, phosphonate).

N-Phthalimide phenylalanyl phosphonate diethyl ester (3c)

^{31}P NMR {^{1}H} (C_6D_6): δ −6.66.

Tetraethyl 2-N-phthalimide-3-phenyl-1-hydroxypropylidene-1,1-bisphosphonate (4c)

Oil; yield: 25%. ^{1}H NMR ($CDCl_3$): δ 1.09 (t, J = 7.0 Hz, 3H, *Me*CH_2O), 1.17 (t, J= 7.0 Hz, 3H, *Me*CH_2O), 1.39 (t, J = 7.2 Hz, 6H, two *Me*CH_2O), 3.36 (dd, J = 13.7, 12.6 Hz, 2H, CHC*H*$_2$), 3.92 (m, 2H, OCH_2), 4.09 (m, 2H, OCH_2), 4.33 (quintet, J = 7.3 Hz, 4H, two OCH_2), 5.14 (ddd, J = 12.2, 5.6, 4.0 Hz, 1H, CH), 7.10 (m, 5H, phenyl), 7.57 (m, 2H, phth), 7.78 (m, 2H, phth). ^{31}P NMR {^{1}H} (C_6D_6): δ 14.38 (ABq, J = 12.95 Hz).

N-Phthalimide leucinyl phosphonate diethyl ester (3d)

^{31}P NMR {^{1}H} (C_6D_6): δ −6.26.

Tetraethyl 2-N-phthalimide-3-isopropyl-1-hydroxypropylidene-1,1-bisphosphonate (4d)

Oil; yield: 50%. ^{31}P NMR {^{1}H} (C_6D_6): δ 14.22 (ABq, J = 12.95 Hz). MS (CI-*i*-Bu): 520 (MH^+, 100), 373 (33).

N-Phthalimide N(in)-formyl tryptophanyl phosphonate diethyl ester (3f)

^{31}P NMR {^{1}H} (C_6D_6): δ −6.99.

Tetraethyl 2-N-phthalimide-3-(N-formyl indole)-1-hydroxypropylidene-1,1-bisphosphonate (4f)

Brown solid; yield: 35%. ^{31}P NMR {^{1}H} (C_6D_6): δ 13.84.

N-Phthalimide methioninyl phosphonate diethyl ester (3h)

^{31}P NMR {^{1}H} (C_6D_6): δ –6.54.

Tetraethyl 2-N-phthalimide-4-methylthio-1-hydroxybutylidene-1,1-bisphosphonate (4h)

Yellow oil; yield: 44%. ^{1}H NMR ($CDCl_3$): δ 1.14 (t, J = 7.2 Hz, 6H, two CH_2*Me*), 1.20 (t, J = 7.2 Hz, 6H, two CH_2*Me*), 2.04 (s, 3H, SMe), 2.37 (m, 2H, CH_2S), 2.45 (m, 1H, CHC*H*$_2$), 2.95 (m, 1H, CHC*H*$_2$), 4.30 (m, 8H, four OCH_2), 5.06 (m, 1H, CH), 7.72 (m, 2H, phth), 7.84 (m, 2H, phth). ^{31}P NMR {^{1}H} ($CDCl_3$): δ 13.98 (ABq, J = 12.95 Hz).

N-Phthalimide aspartyl phosphonate β-methyl diethyl ester (3i)

^{31}P NMR {^{1}H} (C_6D_6): δ –6.86.

Tetraethyl 2-N-phthalimide-3-methoxycarbonyl-1-hydroxypropylidene-1,1-bisphosphonate (4i)

Yellow oil; yield: 18%. ^{1}H NMR ($CDCl_3$): δ 1.16 and 1.23 (two t, 12H, four CH_2*Me*), 3.55 (s, 3H, OMe), 3.58 (m, 2H, CH_2CO_2), 4.15 (m, 8H, four OCH_2), 5.40 (m, 1H, CH), 7.71 (m, 2H, phth), 7.85 (m, 2H, phth). ^{31}P NMR {^{1}H} ($CDCl_3$): δ 13.60 (ABq, J = 11.10 Hz).

N-Phthalimide glutamyl phosphonate γ-ethyl diethyl ester (3j)

^{31}P NMR {^{1}H} (C_6D_6): δ –6.61.

Tetraethyl 2-N-phthalimide-4-ethoxycarbonyl-1-hydroxybutylidene-1,1-bisphosphonate (4j)

Yellow oil; yield: 24%. ^{1}H NMR ($CDCl_3$): δ 1.14 (dt, J_t = 7.1 Hz, J_d = 5.3 Hz, 6H, two $POCH_2$*Me*), 1.21 (t, J= 7.1 Hz, 3H, CH_2*Me*), 1.39 (t, J = 7.1 Hz, 6H, two $POCH_2$*Me*), 2.21 (m, 1H, CH_2CO_2), 2.30 (m, 1H, CH_2CO_2), 2.54 (m, 1H, C*H*$_2$CH), 2.93 (m, 1H, C*H*$_2$CH), 3.98 (m, 4H, two POC*H*$_2$Me), 4.16 (m, 2H, OCH_2), 4.32 (m, 4H, two POC*H*$_2$Me), 4.94 (ddd, J = 9.0, 6.2, 3.0 Hz, 1H, CH), 7.73 (m, 2H, phth), 7.84 (m, 2H, phth). ^{31}P NMR {^{1}H} ($CDCl_3$): δ 13.55 (d, J = 14.8 Hz), 14.07 (d, J = 12.95 Hz).

Diethyl 4-N-phthalimidebutyryl phosphonate (3m)

^{31}P NMR {^{1}H} (C_6D_6): δ –5.76.

Tetraethyl 4-N-phthalimide- –1-hydroxybutylidene-1,1-bisphosphonate (Alendronate acid, 4m)

White powder; yield: 48%. ^{1}H NMR ($CDCl_3$): δ 1.32 (dt, 12H, four CH_2*Me*), 2.03 (m, 2H, NCH_2CH_2), 2.51 (m, 1H, CH_2C), 3.18 (t, 1H, OH), 3.70 (m, 2H, CH_2N), 3.80 (m, 1H, CH_2C), 4.20 (m, 8H, four OCH_2), 7.75 (m, 2H, phth), 7.85 (m, 2H, phth). ^{31}P NMR {^{1}H} ($CDCl_3$): δ 17.00.

Di-N-phthalimide lysinyl phosphonate diethyl ester (3k)

^{31}P NMR {^{1}H} (C_6D_6): δ –6.48.

Tetraethyl 2,6-di-N-phthalimide –1-hydroxyhexylidene-1,1-bisphosphonate (4k)

Yellow oil; yield: 18%. ^{1}H NMR ($CDCl_3$): δ 1.15 (t, J = 7.2 Hz, 3H, CH_2*Me*), 1.21 (t, J = 7.2 Hz, 3H, CH_2*Me*), 1.36 (t, J = 7.2 Hz, 6H, two CH_2*Me*), 1.38 (m, 2H, CH_2CH_2CH), 1.77 (m, 2H, CH_2CH_2N), 2.28 (m, 1H, CH_2CH), 2.63 (m, 1H, CH_2CH), 3.58 (t, J = 7.3 Hz, 1H, CH_2N), 4.01 (m, 2H, OCH_2), 4.13 (q, J = 7.2 Hz, 2H, OCH_2), 4.16 (q, J = 7.1 Hz, 2H, OCH_2), 4.31 (m, 2H, OCH_2), 4.90 (ddd, J = 11.3, 6.2, 3.0 Hz, 1H, CH), 7.67 (m, 2H, phth), 7.72 (m, 2H, phth), 7.76 (m, 2H, phth), 7.85 (m, 2H, phth). ^{31}P NMR {^{1}H} ($CDCl_3$): δ 13.86 (d, J = 12.95 Hz), 14.25 (d, J = 12.95 Hz).

Di-N-phthalimide ornithinyl phosphonate diethyl ester (3l)

^{31}P NMR {^{1}H} (C_6D_6): δ –6.68.

Tetraethyl 2,5-di-N-phthalimide –1-hydroxypentylidene-1,1-bisphosphonate (4l)

Yellow oil; yield: 15%. ^{1}H NMR ($CDCl_3$): δ 1.14 (t, J = 7.0 Hz, 3H, CH_2*Me*), 1.20 (t, J = 7.0 Hz, 3H, CH_2*Me*), 1.37 (t, J = 7.2 Hz, 3H, CH_2*Me*), 1.38 (t, J = 7.2 Hz, 3H, CH_2*Me*), 1.47 (m, 1H, CH_2CH_2N), 1.65 (m, 1H, CH_2CH_2N), 2.31 (m, 1H, CH_2CH), 2.59 (m, 1H, CH_2CH), 3.64 (t, J= 6.8 Hz, 1H, CH_2N), 4.00 (m, 2H, OCH_2), 4.14 (m, 2H, OCH_2), 4.30 (m, 4H, two OCH_2), 4.93 (ddd, J= 9.5, 6.2, 3.0 Hz, 1H, CH), 7.66 (m, 2H, phth), 7.70 (m, 2H, phth), 7.75 (m, 2H, phth), 7.82 (m, 2H, phth). ^{31}P NMR {^{1}H} ($CDCl_3$): δ 13.64 (d, J = 14.8 Hz), 14.21 (d, J = 14.8 Hz).

N-Phthalimide valinyl phosphonate diethyl ester (3g)

^{31}P NMR {^{1}H} (C_6D_6): δ –6.40.

Tetraethyl 2-N-phthalimide-3-methyl-1-hydroxybutylidene-1,1-bisphosphonate (4g)

Oil; yield: 18%. ^{31}P NMR {^{1}H} (C_6D_6): δ 13.74 (ABq, J = 14.8 Hz). MS (CI-*i*-Bu): 506 (MH^+, 100), 359 (33).

General procedure for the preparation of mono sodium salts of bisphosphonates

The protected bisphosphonate was suspended in 6N HCl and refluxed overnight. Phthalic acid precipitated and was removed by filtration. The filtrate was evaporated to dryness and dissolved in a small amount of H_2O. The solution was brought to pH=4.3–4.4 using 5N NaOH. The resulting solution was added dropwise to a large volume of EtOH, which resulted in precipitation of the desired salts. Bisphosphonate salts were kept in an anhydrous environment.

2-Amino-3-phenyl-1-hydroxypropylidene-1,1-bisphosphonate sodium salt (5c)

White powder; yield: 50%. ^{31}P NMR {^{1}H} (D_2O): δ 11.72 (ABq, J = 19.5 Hz).

2-Amino-3-isopropyl-1-hydroxypropylidene-1,1-bisphosphonate sodium salt (5d)

Off-white powder; yield: 90%. ^{31}P NMR {^{1}H} (D_2O): δ 12.12 (ABq, J = 16.7 Hz).

2-Amino-3-indole-1-hydroxypropylidene-1,1-bisphosphonate sodium salt (5f)

Yellow powder; yield: 88%. ^{31}P NMR {^{1}H} (D_2O): δ 11.92 FAB+: 360 (12), 185 (33), 171 (27), 149 (46), 115 (95), 93 (100); desired 359 (M^+).

2-Amino-4-methylthio-1-hydroxybutylidene-1,1-bisphosphonate sodium salt (5h)

(a 1:1 mixture with the corresponding diastereomeric sulfoxides). White powder; yield: 95%. ^{31}P NMR {^{1}H} (D_2O): δ 14.19 (ABq, J = 16.7 Hz), 14.21 (ABq, J = 16.7 Hz), 14.77 (ABq, J = 18.5 Hz).

2-Amino-3-carboxylic acid-1-hydroxypropylidene-1,1-bisphosphonate sodium salt (5i)

White powder; yield: 40%. ^{31}P NMR $\{^1H\}$ (D_2O): δ 11.14 (ABq, J = 18.2 Hz).

2-Amino-4-carboxylic acid-1-hydroxybutylidene-1,1-bisphosphonate sodium salt (5j)

White powder; yield: 99%. ^1H NMR (D_2O): 1.97 (m, 2H, CH_2CH), 2.38 (m, 2H, CH_2CO_2), 3.60 (m, 1H, CH). ^{31}P NMR $\{^1H\}$ (D_2O): δ 11.83 (ABq, J = 24 Hz).

4-Amino-1-hydroxybutylidene-1,1-bisphosphonate sodium salt (Alendronate, 5m)

White powder; yield: 90%. ^1H NMR (D_2O): δ 1.98 (two m, 4H, CH_2CH_2C), 3.02 (brt, 2H, CH_2N). ^{31}P NMR $\{^1H\}$ (D_2O): δ 14.87. ^{31}P NMR (D_2O): δ 14.85 (t, J = 12 Hz). FAB+: 272 (25), 185 (65), 171 (32), 149 (47), 115 (99), 93 (100); desired 271 (M^+).

2,6-di-Amino-1-hydroxyhexylidene-1,1-bisphosphonate sodium salt (5k)

Off-white powder; yield: 82%. ^1H NMR (D_2O): δ 1.40–2.00 (several m, SH, $CH_2CH_2CH_2N$ and CH_2CH), 2.25 (m, 1H, CH_2CH), 3.08 (t, J = 8.8 Hz, 1H,CH_2N), 3.73 (t, J=7.7 Hz, 1H, CH). ^{31}P NMR $\{^1H\}$ (D_2O): δ 12.38 (d, J = 22.2 Hz). FAB+: 315 (25), 185 (30), 171 (42), 149 (55), 115 (100), 93 (80); desired 314 (M^+).

2,5-di-Amino-1-hydroxypentylidene-1,1-bisphosphonate sodium salt (5l)

Off-white powder; yield: 90%. ^1H NMR (D_2O): δ 1.70–2.06 (m, 3H, CH_2CH_2N and CH_2CH), 2.30 (m, 1H, CH_2CH), 3.12 (t, J = 6.9 Hz, 1H, CH_2N), 3.74 (t, J =6.9 Hz, 1H, CH). ^{31}P NMR $\{^1H\}$ (D_2O): δ 12.16 (d, J = 24.0 Hz). FAB+: 301 (10), 185 (15), 171 (69), 149 (32), 115 (100), 93 (27); desired 300 (M^+).

BIOLOGICAL METHODS

Animals and diets

Male Wistar rats (Crl:(WI)BR), virus antibody free, were purchased from Charles River at about 40 days of age. The rats were held in groups of 5 in

polypropylene cages. The rats were cared for according to the Guidelines for the Care and Use of Laboratory Animals (US Department of Health and Human Services, 1996). The institute's Animal Care and Use Committee approved the study protocol. The animals were held inside a semi-barriered rodent facility. The room target temperature and humidity ranges were set at 21±2°C and 55±15%, respectively. A commercially available rodent laboratory animal diet (Altromin, Lage, Germany) was fed *ad libitum* throughout the study, except before bleeding, when the rats were starved. Water was available *ad libitum*.

Bisphosphonates

All compounds were dissolved in 0.9% NaCl solution and the pH was adjusted to 7.4. Besides the eleven compounds tested, alendronate (4-amino-1-hydroxybutylidene-1,1-bisphosphonate) was prepared and investigated in this work.

Thyroparathyroidectomy (TPTX)

TPTX was performed under halothane anesthesia. After TPTX all rats were supplemented with 2 μg thyroxin injected subcutaneously three times a week. The success of the TPTX surgical procedure was evaluated by determining plasma Ca concentration in a blood sample obtained 5–7 days after the operation. Plasma calcium concentrations were determined using the VetTest VT8008 chemistry analyzer (IDEXX Laboratories, USA). TPTX was considered successful if plasma calcium levels were below 75% of the concentrations found in sham-operated animals performed at the same time.

Blood sampling

Blood was obtained after overnight fast by nicking the rats' tails.

Inhibition of stimulated bone resorption assessed by the arotinoid assay

The TPTX rats were randomly distributed into treatment groups. Bone resorption in TPTX rats was induced as previously described[7,24] with the arotinoid ethyl *p*-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naph-

thyl)phenyl]-benzoate (RO 13–6298, a generous gift from Hoffmann-La Roche AG, Basel, Switzerland). The arotinoid was administered subcutaneously at about 10 am at a dose of 25 μg per rat in 0.1 mL of polyethylene glycol 300 containing 10% ethanol. It was given for three consecutive days (days 1, 2, and 3), five days after TPTX. Bisphosphonates were injected at the same time in a different location. Blood was obtained 24 h before the first and 24 h after the last injection of arotinoid (days 0 and 4).

One group of TPTX rats received only the arotinoid. All other test groups received arotinoid and a test compound. A control group receiving both arotinoid and alendronate was included in each experiment. The inhibitory effect on bone resorption was calculated according to the following equation:

$$\frac{(\Delta_{Ar}(\text{day 4-day 0}) - \Delta_{BP}(\text{day 4-day 0})) \bullet 100}{\Delta_{Ar}(\text{day 4-day 0})}$$

Δ_{BP} = the increment between Ca levels at day 0 (pretreatment) and day 4 (24 h after treatment).

Δ_{Ar} = the increment between Ca levels at day 0 and day 4 for TPTX rats receiving only arotinoid.

Values of 100% inhibition indicated full blocking of the hypercalcemia induced by the arotinoid; 0% inhibition indicated no inhibition at all; values larger than 100% inhibition indicated both full inhibition of arotinoid-induced hypercalcemia and inhibition of endogenous bone resorption.

Data presentation

Mean values ± the standard error of the mean (x ± SEM) are given in the results part of this paper.

Acknowledgements

We thank Dr. Frieder Bauss of Boehringer-Mannheim GmbH. for many fruitful discussions and helpful insights regarding the biological assay. We also thank Dr. Eva-Maria Gutknecht and Mr. Pierre Weber of Hoffmann-La Roche Ltd., for the generous gift of RO-6298.

References

[1] M. D. Francis, R. G. G. Russell and H. Fleisch, *Science* **165**, 1264–1266 (1969).

[2] H. Fleisch, R. G. G. Russell, S. Bisaz, R. C. Mühlbauer and D. A. Williams, *Eur. J. Clin. Invest.* **1**, 12–18 (1970).
[3] R. Schenk, W. A. Merz, R. Mühlbauer, R. G. G. Russell and H. Fleisch, *Calcif. Tissue Res.* 11, 196- 214 (1973).
[4] H. Fleisch, R. G. G. Russell and M. D. Francis, *Science* **165**, 1262–1264 (1969).
[5] R. G. G. Russell, R. Mühlbauer, S. Bisaz, D. A. Williams and H. Fleisch, *Calcif. Tissue Res.* **6**, 183–196 (1970).
[6] O. L. M. Bijvoet, H. A. Fleisch, R. E. Canfield and R. G. G. Russell, Bisphosphonate on Bones (Elsevier, Amsterdam, 1995).
[7] R. C. Mühlbauer, F. Bauss, R. Schenk, M. Janner, E. Bosies, K. Strein and H. Fleisch, *J. Bone Miner. Res.* **6**(9), 1003–1011 (1991).
[8] J. F. Hartmann and D. Farcasiu, PCT Int. Appl. WO 96/40156 (1996).
[9] H. Blum and K. Worms, US Pat. 4, 054,598 (1977).
[10] H. Blum, H. Hempel and K. Worms, US Pat. 4, 267,108 (1981), CAS 91: 20718x.
[11] J. Jary, V. Rihakova and A. Zobacova, US Pat. 4, 304,734 (1981).
[12] H. Blum and K. Worms, US Pat. 4, 327,039 (1982), CAS 95: 150888w.
[13] H. Blum and K. Worms, US Pat. 4, 407,761 (1983), CAS 96: 52503t.
[14] G. Staibano, GB Pat. 2 166 741 A (1986), CAS 105: 191395b.
[15] S. Rosini and G. Staibano, US Pat. 4, 621,077 (1986), CAS 111: 187624g.
[16] E. Bosies and R. Gall, US Pat. 4, 687,767 (1987), CAS 105: 134140r.
[17] G. Guanai-Ricci, S. Rosini and M. Mian, PCT Int. Appl. WO 93,00348, (1993).
[18] G. R. Kieczykowski, R. B. Jobson, D. G. Melillo, D. F. Reinhold, V. J. Grenda, and I. Shinkai, *J. Org. Chem.* **60**, 8310–12 (1995).
[19] G. H. L. Nefkens, G. I. Tesser and R. J. F. Nivard, *Rec. Trav. Chim. Pays-Bas* **79**, 688 (1960).
[20] C. M. McArthur, P. M. Worster and A. U. Okon, *Synth. Comm.* **13**(4), 311–8 (1983).
[21] L. M. Nguyen, E. Niesor and C. L. Bentzen, *J. Med. Chem.* **30**, 1426–33 (1987).
[22] G.R. Kieczykowski, GB Pat. 2 248 061 (1992).
[23] F. H. Ebetino and S. M. Dansereau, Bisphosphonate on Bones (Elsevier, Amsterdam, 1995), Chap. 9, pp. 139–153.
[24] U. Trechsel, A. Stutzer and H. Fleisch, *J. Clin. Invest.*, **80**, 1679–1686 (1987).