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New aromatic monoesters of α-aminoaralkylphosphonic acids as inhibitors of aminopeptidase N/CD13

Renata Grzywa [a], Anna M. Sokol [a,b], Marcin Sieńczyk [a], Magdalena Radziszewicz [a], Beata Kościołek [a], Michael P. Carty [b], Józef Oleksyszyn [a,*]

[a] Division of Medicinal Chemistry and Microbiology, Faculty of Chemistry, Wrocław University of Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland
[b] Centre for Chromosome Biology, School of Natural Sciences, National University of Ireland, Galway, University Road, Galway, Ireland

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ABSTRACT

A series of new aromatic monoesters of α-aminoaralkylphosphonic acids were synthesized by selective hydrolysis of corresponding aromatic diesters of α-aminoaralkylphosphonic acids. New potential inhibitors of aminopeptidase N/CD13, an enzyme important in tumour angiogenesis, were developed. Some derivatives of the homophenylalanine and norleucine related monoaryl phosphonates displayed higher inhibition potency than corresponding α-aminoaralkylphosphonic acids toward aminopeptidase N/CD13. The effect of one of the new inhibitors on the growth of human PANC-1 and HT-1080 cell lines was examined, either alone or in combination with TNF-α.

1. Introduction

Among several proteolytic enzymes responsible for tumour growth, aminopeptidase N/CD13 is a new emerging target for anticancer therapy. Aminopeptidase N (APN/EC 3.4.11.2) is a membrane-bound zinc-dependent proteolytic enzyme expressed in many cell types including kidney, intestinal epithelium, liver, placenta and lung cells.[1,2] It has an important role in inflammation, immunological responses, signal transduction, antigen processing, cytokine degradation and extracellular matrix degradation. As a possible consequence of these activities it also plays an important role in tumour invasion and angiogenesis.[3,4] Inhibition of APN/CD13 by bestatin, or inhibition of APN/CD13 expression by siRNA significantly decreases growth and migratory potential of ovarian carcinoma cells.[5] Impaired angiogenesis was observed in aminopeptidase N/CD13-null mice.[6] The APN/CD13 activity is not essential for embryonic and fetal development including de novo blood vessel formation and normal adult function; it is critical for pathological development of new blood vessels from existing blood vessels (angiogenesis) that leads to disease development and progression. It was shown that transcription of APN/CD13 is regulated by the proto-oncogene c-Maf via the atypical response element and is critical for tumour angiogenesis.[7] Compounds with well-established anticancer properties such as bestatin (ubenimex) and curcumin are good inhibitors of the aminopeptidase N/CD13.[8]

Aminopeptidase activity is essential for survival of the human malaria parasite *Plasmodium falciparum* and it has been demonstrated that bestatin prevents parasite replication.[9] Thus, there is a growing interest in the development of new aminopeptidase inhibitors as novel chemotherapeutics directed against malaria.

Tumour necrosis factor alpha (TNF-α) is a well known apoptotic agent. Nevertheless, its application is limited due to systemic toxicity. Interestingly, the combination of aminopeptidase N/CD13 inhibitors and TNF-α resulted in a synergistic effect in apoptosis induction in cancer cells.[10]

Several potent inhibitors of aminopeptidases were reported in the literature including some derivatives of α-aminoaralkylphosphonates and corresponding phosphinates.[8,11] These are phosphorus amino acid analogues in which the carboxyl moiety is replaced by

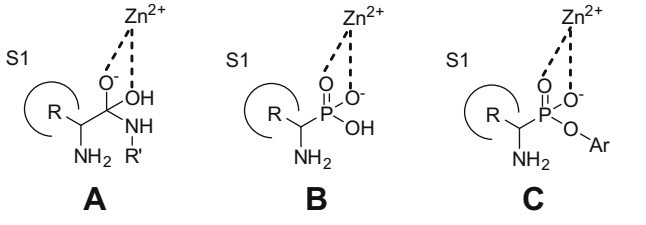

Figure 1. Structural relation between tetrahedral intermediate during peptide bond hydrolysis by aminopeptidase (A), α-aminoaralkylphosphonic acid (B), and aromatic monoester of α-aminoaralkylphosphonic acids (C).

* Corresponding author. Tel.: +48 713204027; fax: +48 713284064.
E-mail address: jozef.oleksyszyn@pwr.wroc.pl (J. Oleksyszyn).

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the phosphonic or phosphinic function. The tetrahedral phosphorus group is a close analog of the gem-diol hydrated carbonyl carbon of the peptide bond, which is the transition state observed during peptide bond hydrolysis catalyzed by, for example, metalloproteinases (Fig. 1). The aminopeptidases are exopeptidases which hydrolyze the *N*-terminal amino acid from longer peptides. A free amino terminal group is essential for proper substrate recognition by this group of proteases.

Phosphonic analogues of amino acid as inhibitors of leucine aminopeptidases were discovered by Giannousis and Bartlett.[12] Phosphonic analogues of L-leucine (L-Leu^P) and L-phenylalanine (L-Phe^P) are good inhibitors of the leucine aminopeptidase (LAP/EC 3.4.11.1) with K_i values of 0.23 μM and 0.42 μM, respectively. However, they are poor inhibitors of aminopeptidase N (EC 3.4.11.2) with K_i = 53 μM for L-Leu^P and K_i = 27.5 μM for L-Phe^P.[11] The aim of our studies was to explore the influence of a simple structural modification within the phosphonic function (incorporation of the additional substituents on one of the acid's oxygens) on the inhibitory properties toward APN. Here, we report the synthesis and biological activity of new aromatic monoesters of α-aminoaralkylphosphonic acids as inhibitors of APN/CD13, and we include some elements of the structure–activity relationship.

2. Materials and methods

2.1. Enzymatic studies

The inhibitory effect of α-aminoaralkylphosphonates and their aromatic monoesters toward aminopeptidase N (from porcine kidney, Sigma–Aldrich) was evaluated using fluorogenic substrate Leu-AMC (Sigma–Aldrich). Sodium phosphate buffer (pH 7.2) was used. The final concentrations were: 0.2 μg/ml APN/CD13 and 12.5 μM Leu-AMC. The inhibitory activity of synthesized compounds was measured during 10 min at 25 °C without pre-incubation. The standard deviation for the presented IC_{50} values is the mean of two independent experiments and is ±20%.

2.2. Chemistry

General procedure for the synthesis of α-aminoaralkylphosphonic acids and their aryl esters were described previously in literature.[13,14]

2.2.1. General procedure for synthesis of phosphites

0.03 mol of phosphorus trichloride and 0.1 mol of appropriate phenol were dissolved in 40 ml of acetonitrile. The mixture was heated for 4 h at 80 °C, evaporated and crude phosphite was used in the amidoalkylation reaction.

2.2.2. General procedure for synthesis of α-N-(benzyloxycarbonyl)aminoaralkylphosphonate aromatic esters

Crude phosphite (0.03 mol), aldehyde (0.03 mol) and benzyl carbamate (0.03 mol) were dissolved in 15 ml of acetic acid and heated for 2 h at 80 °C, evaporated, re-dissolved in 30 ml of MeOH and crystallized at −20 °C. Resulting product was filtered, washed with MeOH and recrystalized from $CHCl_3$/MeOH system if necessary.

2.2.3. General procedure for synthesis of α-N-(benzyloxycarbonyl)aminoaralkylphosphonate aromatic monoesters[15]

1 mmol of *N*-Cbz-aminoaralkylphosphonate aromatic ester was dissolved in 5 ml of dioxane. 5 ml of 1 M NaOH, 0.35 g of KF and catalytic amount of crown ether (18-crown-6) was added. The mixture was stirred at room temperature for 3 days, then evaporated, re-dissolved in 10 ml of water and crystallized with 2 N HCl. Product was filtered, washed with petroleum ether and dried on air.

2.2.4. General procedure for synthesis of α-aminoaralkylphosphonate aromatic monoesters

0.5 mmol of *N*-Cbz-aminoaralkylphosphonate aromatic monoester was dissolved in 2 ml of 33% HBr in AcOH. After 2 h the mixture was evaporated, dissolved in 1 ml of MeOH and propylene oxide was added to pH 6. The mixture was left for crystallization at 4 °C, the product was filtered and washed with diethyl ether. Some monoesters were obtained as the hydrobromide salts by addition of diethyl ether instead of propylene oxide.

2.3. Cell culture

PANC-1, a human pancreatic carcinoma cell line[16] was obtained from Dr. Alessandro Antoni (National Centre for Biomedical Engineering Science, NUI, Galway), and HT-1080, a human fibrosarcoma cell line[17] was obtained from Dr. Ciaran Morrison (Biochemistry Department, NUI, Galway). PANC-1 cells were grown in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 2 mM L-glutamine, 10% uninactivated FBS and 1% penicillin–streptomycin. HT-1080 cells were grown in DMEM supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS and 1% penicillin–streptomycin. Both cell lines grew as adherent cultures in 75 cm^2 flasks, incubated in an autoflow CO_2 water-jacketed incubator (Nuaire, Plymouth, MN, USA) at 37 °C and 5% CO_2.

2.4. Cell viability assay

Stock solutions of aminopeptidase N inhibitors were prepared in 0.1 M NaOH. On the day of the treatment, solutions of aminopeptidase N inhibitors were prepared in cell culture medium at the indicated doses. Control cell cultures were treated with the equivalent amount of 0.1 M NaOH only. Where indicated, cells were co-treated with tumour necrosis factor α (Sigma–Aldrich).

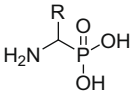
Cell viability was determined using the XTT assay. Cells were treated for 72 h with APN/CD13 inhibitor (0–300 μM), or co-treated with APN/CD13 inhibitor and TNF-α (0–30 μg/ml) diluted with fresh medium. After the treatment, cells were incubated with 150 μl of XTT staining solution (Roche, Mannheim, Germany) for 4 h. Absorbance was measured at 490 nm on a Victor^2 1420 Multilabel Counter (Wallac, MA, USA). Data was normalised to the value for the corresponding untreated cell culture. The average values were plotted as concentration of chemical versus percentage growth relative to control (±one standard deviation, indicated by error bars).

3. Results and discussion

The earlier work of Giannousis and Bartlett[12] suggests that LAP, enzyme related to aminopeptidase N/CD13, prefers leucine and phenylalanine residues in the substrate sequence positioned at the S1 subsite of the enzyme. In a recently reported series of phosphinate dipeptide analogues, the homophenylalanine residue at the S1 subsite of the APN/CD13 was preferred.[18] Our previous studies indicated that norleucine and homophenylalanine side chains are also preferred at the S1 subsite,[19] and these two phosphonic analogues of amino acids were chosen for further modifications. Additionally, derivatives of 1-aminoethylphosphonic acid (Ala^P) and 1-aminopropylphosphonic acid (Abu^P) were included as reference compounds.

Inhibition data for aminopeptidase N/CD13 from porcine kidney (Sigma–Aldrich) by the obtained α-aminoaralkylphosphonic acids is presented in Table 1.

Table 1
Structures and IC_{50} values for inhibition of aminopeptidase N/CD13 by α-aminoaralkylphosphonic acids[a]



Compound	R	IC_{50} (μM)
1a	$-CH_3$ (Ala^P)	42
1b	$-CH_2CH_3$ (Abu^P)[b]	50
1c	$-CH_2CH_2CH_3$ ($nVal^P$)	15
1d	$-CH(CH_3)_2$ (Val^P)	104
1e	$-CH_2CH(CH_3)_2$ (Leu^P)	37
1f	$-CH_2(CH_2)_2CH_3$ (Nle^P)	75
1g	$-CH_2(CH_2)_3CH_3$ ($hNle^P$)	8.3
1h	$-C_6H_5$ (Phg^P)	220
1i	$-CH_2C_6H_5$ (Phe^P)	54
1j	$-CH_2CH_2C_6H_5$ ($hPhe^P$)	46

[a] α-Aminoaralkylphosphonic acids, analogues of natural α-amino acids, are designated by three-letter abbreviation for the amino acid residue and by a superscript P. For example, 1-aminoethanephosphonic acid, which corresponds to alanine is abbreviated as Ala^P.
[b] Abu^P—1-aminopropylphosphonic acid.

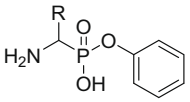
In general α-aminoaralkylphosphonic acids are moderate inhibitors of APN/CD13 with IC_{50} values ranging from 220 μM for Phg^P, **1h** to 8.3 μM for $hNle^P$, **1g**. Although the literature data has reported *slow binding* inhibition kinetics for some α-aminoaralkylphosphonic acids,[11,12] we have not noticed such behaviour in the case of new monoaryl esters shown in Tables 2 and 3. Therefore, the IC_{50} values for α-aminoaralkylphosphonic acids **1a**–**1j** in Table 1 are inhibition values measured directly after mixing the substrate, inhibitor and enzyme, and they are sometimes different than those measured after incubation of the inhibitors with the enzyme.[11,12] For example Val^P (**1d**), Leu^P (**1e**) and Phe^P (**1i**) are slow binding inhibitors of leucine aminopeptidase. However, for APN (described under its old name as microsomal aminopeptidase M) among the 26 α-aminoaralkylphosphonic acids tested, including Val^P (**1d**), Leu^P (**1e**) and Phe^P (**1i**), only five derivatives having a second amino function in the β-position displayed slow binding behaviour. Therefore, under the conditions of our experiments, it was not unexpected that slow binding inhibition kinetics were not observed for the compounds in Tables 1–3.

The synthesis of the phosphonic monoester type of inhibitors starts with the α-amidoalkylation of triaryl phosphite with benzyl carbamate and aldehyde (Scheme 1).[13] Obtained diaryl α-*N*-(benzyloxycarbonyl)aminoaralkylphosphonates **I** were then the subject of selective basic hydrolysis to the corresponding monoesters of α-*N*-(benzyloxycarbonyl)aminoaralkylphosphonates, **II**. Finally, the *N*-benzyloxycarbonyl protective group was removed by application of hydrobromic acid solution in AcOH and the monoesters were isolated as hydrobromide salts or as free monoesters **III**.

Inhibition data for aminopeptidase N/CD13 by unsubstituted phenyl monoesters of α-aminoaralkylphosphonic acids are shown in Table 2. Clearly, the presence of a simple phenyl ester ring in the inhibitor molecules resulted in an extended interaction with the active site of APN/CD13 to the S1′ subsite with significant improvement of the inhibitory potency.

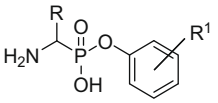
For the most potent α-aminoalkylphosphonic acid, $hNle^P$ (**1g**, IC_{50} = 8.3 μM) only approximately twofold improvement of the inhibitory activity was noticed for its monophenyl ester (**2e**, IC_{50} = 4.8 μM). However, the monophenyl ester of the weakest α-aminoaralkylphosphonic acid Phg^P (**1h**, IC_{50} = 220 μM) shows a 10-fold increase of action (**2f**, IC_{50} = 21 μM). Phenyl monoesters of Nle^P, **2d**, and $hPhe^P$, **2h**,[20] are 14 and 7 times more potent inhibitors than parent phosphonic acids Nle^P (**1f**) and $hPhe^P$ (**1j**), respectively. We have concluded that introduction of additional substituents on phenyl monoester ring **2d** and **2h** could be worth examining.

Table 2
Structures and IC_{50} values for inhibition of aminopeptidase N/CD13 by phenyl monoesters of α-aminoaralkylphosphonic acids

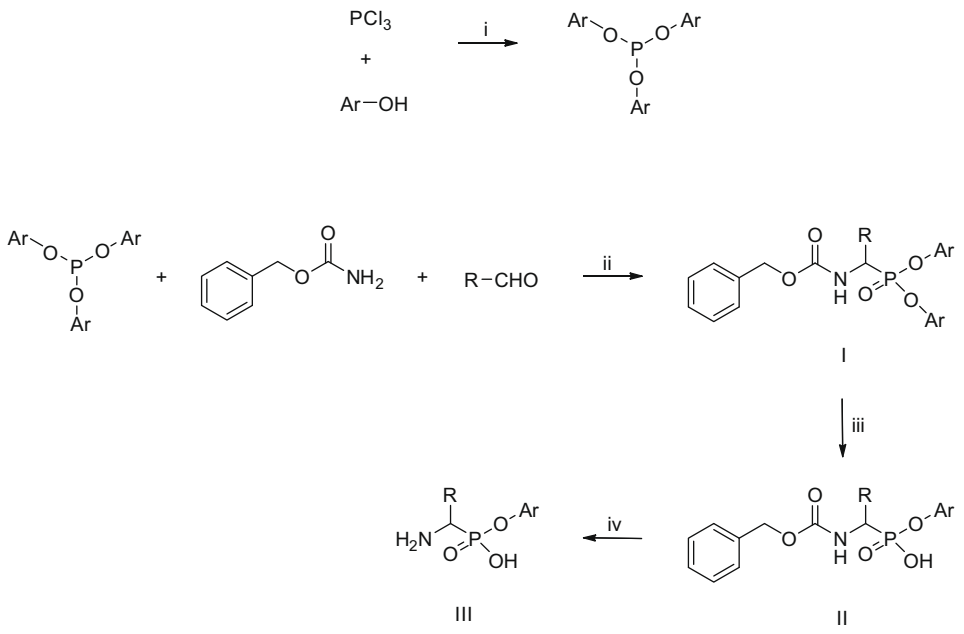


Compound	R	IC_{50} (μM)
2a	$-CH(CH_3)_2$ (Val^P)	14
2b	$-CH(CH_2CH_3)_2$	>100
2c	$-CH_2CH(CH_3)_2$ (Leu^P)	9.3
2d	$-CH_2(CH_2)_2CH_3$ (Nle^P)	5.6
2e	$-CH_2(CH_2)_3CH_3$($hNle^P$)	4.8
2f	$-C_6H_5$ (Phg^P)	21
2g	$-CH_2C_6H_5$ (Phe^P)	8.2
2h	$-CH_2CH_2C_6H_5$ ($hPhe^P$)	6.2

Table 3
Structures and IC_{50} values for inhibition of aminopeptidase N/CD13 by substituted phenyl monoesters of α-aminoaralkylphosphonic acids



Compound	R	R^1	IC_{50} (μM)
3a	$-CH_3$ (Ala^P)	4-Ethyl	8.3
3b	$-CH_3$ (Ala^P)	4-*t*-Butyl	8.2
3c	$-CH_3$ (Ala^P)	2,3,5-Trimethyl	14
4a	$-CH_2CH_3$ (Abu^P)	4-Methyl	7.0
4b	$-CH_2CH_3$ (Abu^P)	3,4-Dimethyl	3.0
4c	$-CH_2CH_3$ (Abu^P)	2,5-Dimethyl	120
4d	**$-CH_2CH_3$ (Abu^P)**	**4-*t*-Butyl**	**1.1**
4e	$-CH_2CH_3$ (Abu^P)	4-Carboxy	200
5a	**$-CH_2(CH_2)_2CH_3$ (Nle^P)**	**4-Methyl**	**1.0**
5b	$-CH_2(CH_2)_2CH_3$ (Nle^P)	3,4-Dimethyl	1.4
5c	$-CH_2(CH_2)_2CH_3$ (Nle^P)	2,5-Dimethyl	>200
5d	$-CH_2(CH_2)_2CH_3$ (Nle^P)	4-Methoxy	210
5e	$-CH_2(CH_2)_2CH_3$ (Nle^P)	4-Carboxy	55
6a	$-CH_2CH_2C_6H_5$ ($hPhe^P$)	2,5-Dimethyl	15
6b	$-CH_2CH_2C_6H_5$ ($hPhe^P$)	3,4-Dimethyl	2.2
6c	$-CH_2CH_2C_6H_5$ ($hPhe^P$)	2,3,5-Trimethyl	5.0
6d	$-CH_2CH_2C_6H_5$ ($hPhe^P$)	4-Ethyl	13
6e	**$-CH_2CH_2C_6H_5$ ($hPhe^P$)**	**4-Isopropyl**	**0.5**
6f	$-CH_2CH_2C_6H_5$ ($hPhe^P$)	4-*t*-Butyl	1.9
6g	$-CH_2CH_2C_6H_5$ ($hPhe^P$)	4-Methoxy	2.9
6h	$-CH_2CH_2C_6H_5$ ($hPhe^P$)	4-Carboxy	22
6i	$-CH_2CH_2C_6H_5$ ($hPhe^P$)	4-(1,1,3,3-Tetramethyl)butyl	6.3



Scheme 1. Synthesis of aryl monoesters of α-aminoaralkylphosphonic acid III (**2a**–**2h**, **3a**–**3c**, **4a**–**4e**, **5a**–**5e**, **6a**–**6i**). Reagents and conditions: (i) acetonitrile, reflux; (ii) AcOH, 80 °C; (iii) NaOHaq/dioxane, KF, crown ether, rt; (iv) (a) HBr/AcOH; (b) propylene oxide.

The 4-*t*-butyl substituent at the phenyl ring of Abu^P monoester (**4d**, IC_{50} = 1.1 μM) shows the greatest improvement when compared to the parent 1-aminopropylphosphonic acid (**1b**, Abu^P, IC_{50} = 50 μM) at almost 40-fold. In case of Ala^P (**1a**, IC_{50} = 42 μM), its 4-*t*-butyl phenyl monoester derivative **3a** has an IC_{50} = 8.2 μM, which is five times lower.

For the series of substituted phenyl monoesters of Nle^P, **5a**–**5e**, compound **5a** is the most potent inhibitor of APN with IC_{50} = 1 μM. It is a 75-fold improvement if compared to the parent phosphonic amino acid **1f**, and it is five times better than the unsubstituted phenyl monoester **2d**.

It seems that within these series the methyl group in position 4 of the phenyl ring is preferred and APN/CD13 does not tolerate hydrophilic methoxy or free carboxyl functionality. The additional methyl group at the 3 position, such as 3,4-dimethyl in compound **5b** is a comparably good inhibitor. However, the 2,5-dimethyl isomer **5c** has an IC_{50} >200 μM, suggesting that there is no space within the S1′ subsite for binding an additional methyl at the *ortho* position.

Among the monoesters of hPhe^P, **6a**–**6i**, the best inhibitor of APN/CD13 in these studies was found to be the 4-isopropyl substituted monophenyl ester of hPhe^P, **6e**. It showed 90-fold improvement compared to the phosphonic amino acid **1j**, and 12-fold compared to the unsubstituted phenyl monoester **2h**. Compound **6f** with a 4-*t*-butyl substituent shows almost four times less inhibition which suggests a specific interaction of the isopropyl group at this region of APN/CD13. Aromatic monoesters of the α-aminoaralkylphosphonic acids are quite stable. In the enzyme assay buffer (phosphate buffer, pH = 7.2, 20% DMSO-d_6) we have not noticed any change by the ^{31}P NMR after 4 days of incubation. Bestatin, an inhibitor of APN/CD13 with well-established anti-tumour activity, under the conditions of our assay has an IC_{50} = 2.1 μM. Compound **6e**, even as a racemic mixture, is at least four times more potent than bestatin.

In preliminary studies, compound **4d** was found to have the greatest effect on cell growth, among 17 compounds screened. The growth inhibitory effects of compound **4d** and bestatin on the cancer cell lines PANC-1 and HT-1080 were compared, in the absence or presence of TNF-α (Fig. 2).

Growth inhibition for both cell cultures was less than 10% when cells were treated only with bestatin (Fig. 2). Monoester treatment showed a greater dose-dependent effect. Following exposure of cells to 300 μM of compound **4d**, cell growth was inhibited (when compared to untreated cells) by 26% in PANC-1 cells, and by 36% in HT-1080 cells. Combined treatment with bestatin and TNF-α decreased cell growth in the case of both cell lines. However, the highest inhibition of cell growth with this treatment was 31% (Fig. 2A). Compound **4d** clearly shows an additive effect when used with TNF-α, especially in the case of the HT-1080 cell line, where cell growth was inhibited by over 60%.

Thus, we showed that phenyl monoesters of α-aminoaralkyl phosphonates are better APN/CD13 inhibitors than the corresponding acids. Furthermore, simple modifications of the ester aromatic group can increase the inhibitory potency of more than 10-fold. The stability, structural simplicity and in some cases higher activity towards aminopeptidase N/CD13 in comparison to bestatin make them good candidates for future studies of the role of APN/CD13 in tumour development, especially after resolution of the inhibitor racemic mixture into single enantiomers. This was confirmed by our preliminary in vitro study.

Compound **2a**, 1-amino-2-methylpropylphosphonic acid monophenyl ester: White solid; mp 221 °C; HRMS *m*/*z* 229.0868/230.0929 $(M+1)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 23.90 (s); ^{1}H NMR (300 MHz, D_2O, ppm): δ 0.94 (dd, 6H, *J* = 6.90, 9.00 Hz, 2 × C*H*$_3$), 2.03–2.07 (m, 1H, C*H*(CH$_3$)$_2$), 2.76 (dd, 1H, *J* = 4.11, 13.51 Hz, C*H*P), 7.08–7.33 (m, 5H, Ar–*H*).

Compound **2b**, 1-amino-2-ethylbutylphosphonic acid monophenyl ester: White solid; mp 222 °C; HRMS *m*/*z* 257.1181/280.1062 $(M+Na)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 24.35 (s); ^{1}H NMR (300 MHz, D_2O, ppm): δ 0.85–0.91 (m, 6H, 2 × C*H*$_3$), 1.25–1.29 (m, 4H, 2 × C*H*$_2$), 1.38–1.43 (m, 4H, 2 × C*H*$_2$), 1.58–1.63 (m, 4H, 2 × C*H*$_2$), 1.68–1.74 (m, 4H, 2 × C*H*$_2$), 1.75–1.79 (m, 1H, C*H*(CH$_2$)$_2$), 3.52 (d, 1H, *J* = 15.62 Hz, C*H*P), 7.12–7.33 (m, 5H, Ar–*H*).

Compound **2c**, 1-amino-2-methylbutylphosphonic acid monophenyl ester: White solid; mp 214–216 °C; HRMS *m*/*z* 243.1024/266.0923 $(M+Na)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 25.07 (s); ^{1}H NMR (300 MHz, D_2O, ppm): δ 0.82 (dd, 6H, *J* = 6.60, 22.51 Hz, 2 × C*H*$_3$), 1.40–1.48 (m, 2H, C*H*$_2$), 1.70–1.77

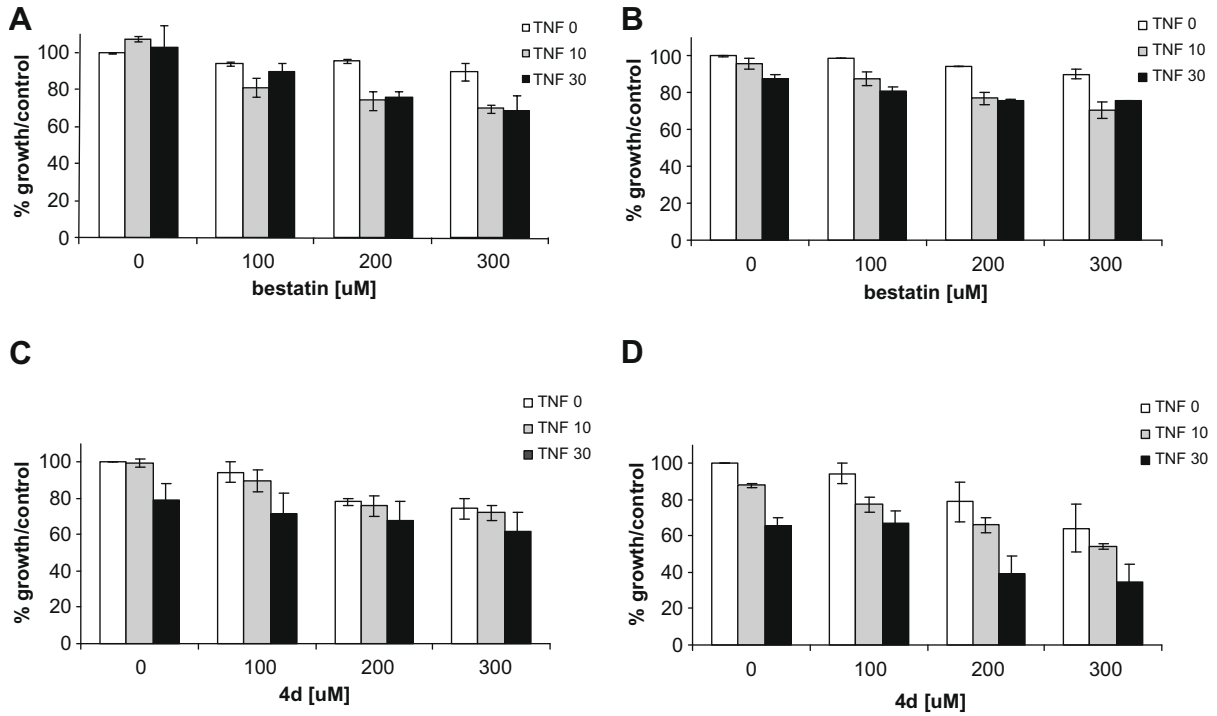


Figure 2. Cell growth inhibition by combined treatment with either TNF-α and bestatin (A, B) or TNF-α and compound **4d** (C, D). A, C—PANC-1 cell line; B, D—HT-1080 cell line. TNF 0, 10, 30: cells were either mock-treated or treated with 10 μg/ml or 30 μg/ml TNF-α.

(m, 1H, C*H*(CH_3)$_2$), 2.90–2.97 (m, 1H, C*H*P), 7.08–7.33 (m, 5H, Ar–*H*).

Compound **2d**, 1-aminopentylphosphonic acid monophenyl ester: White solid; mp 241 °C; HRMS *m*/*z* 243.1024/266.0872 $(M+Na)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 24.72 (s); ^1H NMR (300 MHz, D_2O, ppm): δ 0.81 (t, 3H, *J* = 6.90 Hz, CH_3), 1.20–1.32 (m, 6H, 3 × CH_2), 1.40–1.47 (m, 6H, 3 × CH_2), 1.71–1.74 (m, 6H, 3 × CH_2), 2.80–2.88 (m, 1H, C*H*P), 7.08–7.33 (m, 5H, Ar–*H*).

Compound **2e**, 1-aminohexylphosphonic acid monophenyl ester: White solid; mp 244 °C; HRMS *m*/*z* 257.1181/280.1056 $(M+Na)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 24.11 (s); ^1H NMR (300 MHz, D_2O, ppm): δ 0.80 (t, 3H, *J* = 7.21 Hz, CH_3), 1.20–1.33 (m, 8H, 4 × CH_2), 1.41–1.52 (m, 8H, 4 × CH_2), 1.70–1.76 (m, 8H, 4 × CH_2), 2.42–2.87 (m, 1H, C*H*P), 7.10–7.33 (m, 5H, Ar–*H*).

Compound **2f**, 1-aminobenzylphosphonic acid monophenyl ester: Obtained as a hydrobromide salt; white solid; mp 169 °C; HRMS *m*/*z* 263.0711/264.0776 $(M+1)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 20.92 (s); ^1H NMR (300 MHz, D_2O, ppm): δ 4.06 (d, 1H, *J* = 18.61 Hz, C*H*P), 6.92–7.31 (m, 10H, Ar–*H*).

Compound **2g**, 1-amino-2-phenylethylphosphonic acid monophenyl ester: Obtained as a hydrobromide salt; white solid; mp 181 °C; HRMS *m*/*z* 277.0868/300.0751 $(M+Na)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 23.98 (s); ^1H NMR (300 MHz, D_2O, ppm): δ 2.37–2.43 (m, 2H, CH_2Ph), 2.53–2.64 (m, 2H, CH_2Ph), 3.53 (d, 1H, *J* = 1.50 Hz, C*H*P), 7.01–7.31 (m, 10H, Ar–*H*).

Compound **2h**, 1-amino-3-phenylpropylphosphonic acid monophenyl ester: White solid; mp >250 °C; HRMS *m*/*z* 291.1024/314.0910 $(M+Na)^+$; ^{31}P NMR (300 MHz, DMSO, ppm): δ 14.45 (s); ^1H NMR (300 MHz, DMSO, ppm): δ 1.85–2.07 (m, 2H, CH_2CH_2Ph), 2.65–2.76 (m, 2H, CH_2CH_2Ph), 3.24–3.31 (m, 1H, C*H*P), 7.03–7.28 (m, 10H, Ar–*H*).

Compound **3a**, 1-aminoethylphosphonic acid mono-(4-ethylphenyl) ester: White solid; mp >250 °C; HRMS *m*/*z* 229.0868/230.0946 $(M+1)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 25.05; ^1H NMR (300 MHz, D_2O, ppm): δ 0.82–0.89 (m, 3H, Ar–CH_3), 1.00 (dd, *J* = 7.22, 16.82 Hz, 3H, CH_3), 2.29–2.36 (m, 2H, Ar–CH_2), 2.69–2.80 (m, 1H, C*H*P), 6.82 (d, *J* = 7.40 Hz, 2H, Ar–*H*), 6.97 (d, *J* = 8.45 Hz, 2H, Ar–*H*).

Compound **3b**, 1-aminoethylphosphonic acid mono-(4-*t*-butylphenyl) ester: White solid; mp >250 °C; HRMS *m*/*z* 257.1181/258.1259 $(M+1)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 25.02; ^1H NMR (300 MHz, D_2O, ppm): δ 0.97–0.88 (m, 3H, CH_3), 1.00–1.10 (m, 9H, *t*-Bu), 2.77–2.86 (m, 1H, C*H*P), 6.89 (d, *J* = 7.72 Hz, 2H, Ar–*H*), 7.23 (d, *J* = 8.82 Hz, 2H, Ar–*H*).

Compound **3c**, 1-aminoethylphosphonic acid mono-(2,3,5-trimethylphenyl) ester: Obtained as a hydrobromide salt; white solid; mp 205–210 °C; HRMS *m*/*z* 243.1024/244.1103 $(M+1)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 24.81; ^1H NMR (300 MHz, D_2O, ppm): δ 1.05–1.23 (dd, *J* = 7.07, 16.89 Hz, 3H, CH_3), 1.92 (s, 6H, 2 × Ar–CH_3), 2.02 (s, 3H, Ar–CH_3), 2.83–2.86 (m, 1H, C*H*P), 6.66 (d, *J* = 13.07 Hz, 2H, Ar–*H*).

Compound **4a**, 1-aminopropylphosphonic acid mono-(4-methylphenyl) ester: White solid; mp 226–227 °C; HRMS 229.0868/230.0946 $(M+1)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 24.52 (s); ^1H NMR (300 MHz, D_2O, ppm): δ 0.85 (t, *J* = 14.71 Hz, 3H, CH_3), 1.31–1.42 (m, 1H, CH_2CH_3), 1.63–1.73 (m, 1H, CH_2CH_3), 2.12 (s, 3H, CH_3), 2.61–2.69 (m, 1H, C*H*P), 6.86–7.04 (m, 4H, Ar–*H*).

Compound **4b**, 1-aminopropylphosphonic acid mono-(3,4-dimethylphenyl) ester: White solid; mp 237–238 °C; HRMS *m*/*z* 243.1024/244.1088 $(M+1)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 24.41 (s); ^1H NMR (300 MHz, D_2O, ppm): δ 0.87 (t, *J* = 14.7 Hz, 3H, CH_3), 1.36–1.39 (m, 1H, CH_2CH_3), 1.67–1.75 (m, 1H, CH_2CH_3), 2.07 (d, *J* = 6.90 Hz, 6H, 2 × CH_3), 2.66–2.68 (m, 1H, C*H*P), 6.77–7.01 (m, 3H, Ar–*H*).

Compound **4c**, 1-aminopropylphosphonic acid mono-(2,5-dimethylphenyl) ester: White solid; mp 221 °C; HRMS *m*/*z* 243.1024/244.1080 $(M+1)^+$; ^{31}P NMR (300 MHz, D_2O, ppm): δ 24.33 (s); ^1H NMR (300 MHz, D_2O, ppm): δ 0.89 (t, *J* = 14.41 Hz,

3H, CH_3), 1.37–1.44 (m, 1H, CH_2CH$_3$), 1.72–1.76 (m, 1H, CH_2CH$_3$), 2.09 (d, J = 18.61 Hz, 6H, 2 × CH_3), 2.71–2.76 (m, 1H, CHP), 6.75–7.01 (m, 3H, Ar–H).

Compound **4d**, 1-aminopropylphosphonic acid mono-(4-*t*-butylphenyl) ester: White solid; mp 236–237 °C; HRMS *m/z* 271.1337/272.1409 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 24.49 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 0.88 (t, J = 15.01 Hz, 3H, CH_3), 1.44 (s, 9H, 3 × CH_3), 1.35–1.40 (m, 1H, CH_2CH$_3$), 1.67–1.73 (m, 1H, CH_2CH$_3$), 2.64–2.72 (m, 1H, CHP), 6.96–7.32 (m, 4H, Ar–H).

Compound **4e**, 1-aminopropylphosphonic acid mono-(4-carboxyphenyl) ester: White solid; mp 180–184 °C; HRMS *m/z* 259.0610/260.0740 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 24.59 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 0.83–0.88 (m, 3H, CH_3), 1.31–1.47 (m, 1H, CH_2CH$_3$), 1.68–1.77 (m, 1H, CH_2CH$_3$), 2.68–2.72 (m, 1H, CHP), 6.40–6.44 (m, 2H, Ar–H), 7.51–7.54 (m, 2H, Ar–H).

Compound **5a**, 1-aminopentylphosphonic acid mono-(4-methylphenyl) ester: White solid; mp 235 °C; HRMS *m/z* 257.1181/258.1259 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 24.76 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 0.68 (t, J = 13.21 Hz, 3H, CH_3(CH$_2$)$_3$CHP), 1.02–1.34 (m, 5H, CH$_3$(CH_2)$_3$CHP), 1.51–1.69 (m, 1H, CH$_3$(CH$_2$)$_2$CH_2CHP), 1.97–2.10 (m, 3H, ArCH_3), 2.67–2.75 (m, 1H, PCHCH$_2$), 6.34–7.02 (m, 4H, Ar–H).

Compound **5b**, 1-aminopentylphosphonic acid mono-(3,4-dimethylphenyl) ester: Obtained as a hydrobromide salt; white solid; mp 224 °C; HRMS *m/z* 271.1337/272.1400 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 24.65 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 0.72 (t, J = 12.31 Hz, 3H, CH_3(CH$_2$)$_3$CHP), 1.12–1.45 (m, 5H, CH$_3$(CH_2)$_3$CHP), 1.57–1.69 (m, 1H, CH$_3$(CH$_2$)$_2$CH_2CHP), 2.05 (d, J = 7.20 Hz, 6H, 2 × ArCH_3), 2.69–2.73 (m, 1H, PCHCH$_2$), 6.72–6.99 (m, 3H, Ar–H).

Compound **5c**, 1-aminopentylphosphonic acid mono-(2,5-dimethylphenyl) ester: White solid; mp >250 °C; HRMS *m/z* 271.1337/272.1416 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 24.59 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 0.63–0.69 (m, 3H, CH_3(CH$_2$)$_3$CHP), 1.07–1.13 (m, 5H, CH$_3$(CH_2)$_3$CHP), 1.22–1.38 (m, 1H, CH$_3$(CH$_2$)$_2$CH_2CHP), 1.96–1.99 (m, 3H, ArCH_3), 2.03–2.07 (m, 3H, ArCH_3), 2.67–2.76 (m, 1H, PCHCH$_2$), 6.70–7.02 (m, 3H, Ar–H).

Compound **5d**, 1-aminopentylphosphonic acid mono-(4-methoxyphenyl) ester: White solid; mp >250 °C; HRMS *m/z* 273.1130/274.1208 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 22.71 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 0.62–0.75 (m, 3H, CH_3(CH$_2$)$_3$CHP), 0.94–1.27 (m, 5H, CH$_3$(CH_2)$_3$CHP), 1.40–1.67 (m, 1H, CH$_3$(CH$_2$)$_2$CH_2CHP), 2.14–2.33 (m, 1H, PCHCH$_2$), 3.49–3.51 (m, 3H, ArOCH_3), 6.31–6.91 (m, 4H, Ar–H).

Compound **5e**, 1-aminopentylphosphonic acid mono-(4-carboxyphenyl) ester: White solid; mp 143–185 °C; HRMS *m/z* 287.0923/288.1502 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 22.63 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 0.68 (t, J = 14.40 Hz, 3H, CH_3(CH$_2$)$_3$CHP), 1.03–1.32 (m, 5H, CH$_3$(CH_2)$_3$CHP), 1.45–1.59 (m, 1H, CH$_3$(CH$_2$)$_2$CH_2CHP), 2.26–2.33 (m, 1H, PCHCH$_2$), 6.36–7.49 (m, 4H, Ar–H).

Compound **6a**, 1-amino-3-phenylpropylphosphonic acid mono-(2,5-dimethylphenyl) ester: White solid; mp 235 °C; HRMS *m/z* 319.1337/320.1400 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 23.85 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 1.58–1.73 (m, 1H, PhCH$_2$CH_2), 1.84–2.06 (m, 1H, PhCH$_2$CH_2), 1.84–2.06 (m, 6H, 2 × ArCH_3), 2.50–2.60 (m, 1H, PhCH_2CH$_2$), 2.71–2.80 (m, 1H, PhCH_2CH$_2$), 2.71–2.80 (m, 1H, PCHCH$_2$), 6.15–7.18 (m, 7H, Ar–H).

Compound **6b**, 1-amino-3-phenylpropylphosphonic acid mono-(3,4-dimethylphenyl) ester: White solid; mp 223 °C; HRMS *m/z* 319.1337/320.1367 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 23.85 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 1.58–1.67 (m, 1H, PhCH$_2$CH_2), 1.84–1.95 (m, 1H, PhCH$_2$CH_2), 2.02 (s, 6H, 2 × ArCH_3), 2.47–2.70 (m, 1H, PhCH_2CH$_2$), 2.64–2.77 (m, 1H, PhCH_2CH$_2$), 2.64–2.77 (m, 1H, PCHCH$_2$), 6.59–7.20 (m, 7H, Ar–H).

Compound **6c**, 1-amino-3-phenylpropylphosphonic acid mono-(2,3,5-trimethylphenyl) ester: Obtained as a hydrobromide salt; white solid; mp 201 °C; HRMS *m/z* 333.1494/356.1361 (M+Na)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 23.70 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 1.55–1.71 (m, 1H, PhCH$_2$CH_2), 1.89–2.02 (m, 1H, PhCH$_2$CH_2), 1.89–2.02 (m, 9H, 3 × ArCH_3), 2.47–2.57 (m, 1H, PhCH_2CH$_2$), 2.69–2.78 (m, 1H, PhCH_2CH$_2$), 2.69–2.78 (m, 1H, PCHCH$_2$), 6.57–7.17 (m, 6H, Ar–H).

Compound **6d**, 1-amino-3-phenylpropylphosphonic acid mono-(4-ethylphenyl) ester: White solid; mp 213 °C; HRMS *m/z* 319.1337/320.1416 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 21.91 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 0.89–0.94 (m, 3H, ArCH$_2$CH_3), 1.31–1.49 (m, 1H, PhCH$_2$CH_2), 1.77–1.91 (m, 1H, PhCH$_2$CH_2), 2.28–2.53 (m, 1H, PhCH_2CH$_2$), 2.28–2.53 (m, 2H, ArCH_2CH$_3$), 2.58–2.73 (m, 1H, PhCH_2CH$_2$), 2.58–2.73 (m, 1H, PCHCH$_2$), 6.34–7.19 (m, 9H, Ar–H).

Compound **6e**, 1-amino-3-phenylpropylphosphonic acid mono-(4-isopropylphenyl) ester: Obtained as a hydrobromide salt; white solid; mp 231–233 °C; HRMS *m/z* 333.1494/334.1558 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 23.89 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 1.00 (d, J = 6.90 Hz, 6H, 2 × CHCH_3), 1.54–1.70 (m, 1H, PhCH$_2$CH_2), 1.84–1.98 (m, 1H, PhCH$_2$CH_2), 2.46–2.56 (m, 1H, PhCH_2CH$_2$), 2.63–2.77 (m, 1H, PhCH_2CH$_2$), 2.63–2.77 (m, 1H, PCHCH$_2$), 2.63–2.77 (m, 1H, ArCH), 6.78–7.20 (m, 9H, Ar–H).

Compound **6f**, 1-amino-3-phenylpropylphosphonic acid mono-(4-*t*-butylphenyl) ester: Yellow oil; HRMS *m/z* 347.1650/348.1967 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 23.70 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 1.00 (s, 9H, ArC(CH_3)$_3$), 1.35–1.50 (m, 1H, PhCH$_2$CH_2), 1.75–1.89 (m, 1H, PhCH$_2$CH_2), 2.28–2.50 (m, 1H, PhCH_2CH$_2$), 2.62–2.76 (m, 1H, PhCH_2CH$_2$), 2.28–2.50 (m, 1H, PCHCH$_2$), 6.34–7.17 (m, 9H, Ar–H).

Compound **6g**, 1-amino-3-phenylpropylphosphonic acid mono-(4-methoxyphenyl) ester: Obtained as a hydrobromide salt; white solid; mp 236 °C; HRMS *m/z* 321.1130/322.1170 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 24.14 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 1.56–1.71 (m, 1H, PhCH$_2$CH_2), 1.86–2.00 (m, 1H, PhCH$_2$CH_2), 2.42–2.58 (m, 1H, PhCH_2CH$_2$), 2.68–2.79 (m, 1H, PhCH_2CH$_2$), 2.68–2.79 (m, 1H, PCHCH$_2$), 3.59 (d, J = 18.9 Hz, 3H, ArOCH_3), 6.31–7.21 (m, 9H, Ar–H).

Compound **6h**, 1-amino-3-phenylpropylphosphonic acid mono-(4-carboxyphenyl) ester: White solid; mp 164–166 °C; HRMS *m/z* 335.0923/336.1001 (M+1)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 21.86 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 1.40–1.53 (m, 1H, PhCH$_2$CH_2), 1.83–1.96 (m, 1H, PhCH$_2$CH_2), 2.35–2.53 (m, 1H, PhCH_2CH$_2$), 2.35–2.53 (m, 1H, PCHCH$_2$), 2.69–2.79 (m, 1H, PhCH_2CH$_2$), 6.40–7.52 (m, 9H, Ar–H).

Compound **6i**, 1-amino-3-phenylpropylphosphonic acid mono-(4-(1,1,3,3-tetramethyl)butylphenyl) ester: Yellow solid; mp 120–122 °C; HRMS *m/z* 403.2276/426.2174 (M+Na)$^+$; ^{31}P NMR (300 MHz, D$_2$O, ppm): δ 21.85 (s); ^{1}H NMR (300 MHz, D$_2$O, ppm): δ 0.57 (s, 9H, C(CH_3)$_3$), 1.13 (s, 6H, ArC(CH_3)$_2$CH$_2$), 1.39–1.51 (m, 2H, ArC(CH$_3$)$_2$CH_2), 1.39–1.51 (m, 1H, PhCH$_2$CH_2), 1.84–1.97 (m, 1H, PhCH$_2$CH_2), 2.36–2.54 (m, 1H, PhCH_2CH$_2$), 2.36–2.54 (m, 1H, PCHCH$_2$), 2.64–2.79 (m, 1H, PhCH_2CH$_2$), 6.38–7.20 (m, 9H, Ar–H).

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