Biochimie 151 (2018) 119–127

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Substituted phosphonic analogues of phenylglycine as inhibitors of phenylalanine ammonia lyase from potatoes

Weronika Wanat [a], Michał Talma [a], Józef Hurek [b], Małgorzata Pawełczak [b], Paweł Kafarski [a, *]

[a] Department of Bioorganic Chemistry, Faculty of Chemistry, Wrocław University of Science and Technology, Wybrzeże Wyspiańskiego 27, 50-370, Wrocław, Poland
[b] Department of Chemistry, University of Opole, ul. Oleska 48, 45-052, Opole, Poland

ARTICLE INFO

Article history:
Received 27 February 2018
Accepted 6 June 2018

Keywords:
PAL inhibitors
Aminophosphonates
Molecular modeling
Structure-activity relationship

ABSTRACT

A series of phosphonic acid analogues of phenylglycine variously substituted in phenyl ring have been synthesized and evaluated for their inhibitory activity towards potato L-phenylalanine ammonia lyase. Most of the compounds appeared to act as moderate (micromolar) inhibitors of the enzyme. Analysis of their binding performed using molecular modeling have shown that they might be bound either in active site of the enzyme or in the non-physiologic site. The latter one is located in adjoining deep site nearby the to the entrance channel for substrate into active site.

1. Introduction

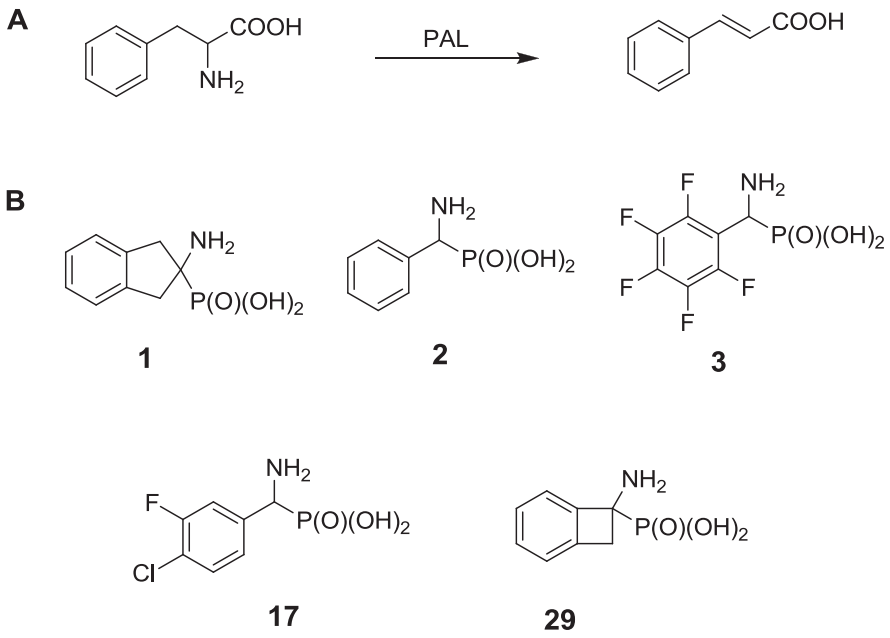
Phenylalanine ammonia lyase (PAL) catalyzes non-oxidative transformation of L-phenylalanine into *trans*-cinnamic acid and ammonia (Scheme 1A). This reaction is a first step for the channeling of carbon from primary metabolism into phenylpropanoid secondary metabolism in plants [1–3]. Since phenylpropanoids play a vital role in development and response to environmental stimuli this enzyme have been intensively studied with respect to: its role in plants, catabolic function in fungi, its mechanism of action, and three-dimensional structure [4–7]. It was also considered as a possible target in the search for new compounds of herbicidal activity, however, phenylopropanoids are so important to plants that blocking the activity of phenylalanine ammonia lyase launches their biosynthesis by alternative pathway [8–10]. Additionally, PAL natural ability to break down L-phenylalanine makes it a reliable treatment for the genetic condition phenylketonuria, an inherited disorder that increases the levels of phenylalanine in the blood [11,12]. Finally, reversed reaction catalyzed by PAL has been successfully utilized for the synthesis of structurally diverse analogues of phenylalanine, useful building blocks in medicinal chemistry [13–15].

Enzyme inhibitors are used as tools for studying mechanisms of enzymatic catalysis and as compounds for treating certain physiologic disorders. Phosphonic analogues of amino acids are well-recognized class of inhibitors of enzymes of variable activities and thus are believed to be valuable potential dugs and pesticides [16–18]. Such analogues of aromatic amino acids also rank amongst the most interesting inhibitors of phenylalanine ammonia lyase [19–23], with a strained analogue of substrate - 2-aminoindane-2-phosphonic acid (compound **1** in Scheme 1B) being the most potent [24–26] and with analogues of phenylglycine (compound **2**, Scheme 1B) being the simplest ones with micromolar inhibitory constants (value of which depends on enzyme source) [27]. Therefore, we have synthesized a series of analogues of phenylglycine bearing fluorine atoms in phenyl ring. For comparison, a series of variously substituted phenylglycine analogues was also obtained. All the compounds (see Table 1) were evaluated for their potency to inhibit activity of phenylalanine ammonia lyase isolated from potatoes.

Fluorination has become recently an increasingly popular strategy in medicinal chemistry and protein biochemistry. Replacement of phenylalanine, tyrosine or tryptophan by their fluorinated analogues has been recently described as a mean to study amino acid interactions within the protein fold [28,40], whereas the judicious introduction of fluorine into an inhibitor molecule can productively influence conformation, pK_a, intrinsic

* Corresponding author.
E-mail address: pawel.kafarski@pwr.edu.pl (P. Kafarski).

https://doi.org/10.1016/j.biochi.2018.06.005
0300-9084/



Scheme 1. Reaction catalyzed by L-phenylalanine ammonia lyase (A) and structures of the chosen inhibitors of the enzyme (B).

potency, membrane permeability, metabolic pathways, and pharmacokinetic properties of new drug candidate [29–33]. Although a C-F bond (1.34 Å for an sp^3 carbon) is about 20% longer than a C-H bond (1.09 Å), fluorination has been shown as being well tolerated by a variety of proteins without introducing much steric perturbation to the parent structure.

2. Results and discussion

2.1. Synthesis

Synthesis of the series of racemic ring-substituted 1-aminobenzylphosphonates, analogues of phenylglycine, was performed by applying three-component amidoalkylation reaction designed by Oleksyszyn and Soroka [34–36]. This procedure is simple and straightforward and provided the desired compounds in satisfactory yields.

2.2. Inhibitory activity

Inhibitory potency of the obtained compounds was evaluated using phenylalanine ammonia lyase isolated from potatoes (*Solanum tuberosum* L.) and compared with literature data for buckwheat (*Fagopyrum esculentum*) enzyme [27]. Results presented in Table 1 indicate that, with exception of compounds **3**, **15**, **25** and **26**, which are inactive, all analogues of phenylglycine appeared to be weak or moderate inhibitors of PAL. Generally, analogues of phenylglycine exhibit inhibitory activity towards buckwheat enzyme higher than towards potato PAL. They are also equipotent with or even more active than aminobenzylphosphonic acid **2**, a formal analogue of phenylglycine, against parsley (*Petroselinum crispum*) enzyme [21,23]. Additionally, the most active compounds: **5**, **6**, **17**, **18** and **20** were significantly less active than cyclic derivative **29** (Scheme 1B) towards buckwheat enzyme [22].

It is worth to note that total replacement of hydrogen atoms in phenylglycine analogue **2** leading to perfluorinated compound **3** (Scheme 1B) resulted in total abolishment of inhibitory activity. When analyzing single substitution of phenyl ring it is seen that substitution of parent compound **2** in *para*-position if of choice with bromine, chlorine and methyl group being the most suitable (compounds **5**, **6** and **20**). This suggests that steric effects are playing the most important role here. Analogues substituted with nitrile moiety appeared to be completely inactive showing that there is a certain limit of *para*-substitution.

Moreover, the introduction of fluorine as an additional substituent in position 3 usually resulted in enhanced inhibitory activity (see compounds **16** and **17**), an effect, which is in opposition to introduction of this atom in position 2 of aromatic ring (compounds **14** and **21**). Summing up, introduction of one fluorine atom into phenyl ring of phosphonic analogues did not affect significantly activity of these compounds against PAL.

Thus, despite of descriptive analysis, there is no possibility to build-up the simple structure-activity relationship for phosphonic analogues of phenylalanine also because modeling studies (see next paragraph) indicate that they might be bound in two neighboring binding sites of the enzyme – either in the active site (subunit A) or in site, which has no physiological meaning located nearby to the entrance channel of substrate phenylalanine (subunit B).

2.3. Docking of most potent inhibitors to Petroselinum crispum PAL

Docking studies might contribute to understanding of mode of binding of phenylglycine analogues by the enzyme and to identification of amino acids vital for this process. Quite surprisingly, docking studies on parsley enzyme indicated that analogues of phenylalanine could be bound in two subunits, namely in the active site (subunit A marked as green in Fig. 1) and, unexpectedly, in adjoining deep site located in subunit B (marked in red in Fig. 1). The second site is located nearby the to the channel, which forms an entrance for substrate into active site in unit A. Modeling predicts binding of compounds: **5, 6** and **10** (only *S* isomers), and both isomers of compounds: **7**, **11**, **20** and **22** in this site, whereas remaining ones are bound, as expected, in subunit A. It is worth to mention, that in the case of aminophosphonic acids *R*-isomers are mimicking *S*-amino acids. From modeling it is clearly seen that Arg354 plays a major role for binding in site A forming strong electrostatic interactions with phosphonic moiety, while in site B similar role play Lys 336 and Lys345.

Table 1
Structures and *in vitro* inhibitory potency of phosphonic analogues of phenylglycine towards potato PAL.

Compound	Structure	IC_{50} [μM] [*S*-Phe] = 1 mM	K_i [μM]
2	all-H		6.5 [21] 41.5 [23]
3	perfluoro	na[a]	
4	4-F	57.47 ± 2.31	19.33 ± 1.02 1.8 [27] [c]
5	4-Cl	11.86 ± 0.86	1.85 ± 0,23 0.21 [27]
6	4-Br	10.25 ± 1.24	4.65 ± 0,48 0.29 [27]
7	4-I	106.87 ± 6.13	10.08 ± 3.43
8	2-F	628.76 ± 58.24	nd[b] na [27]
9	2-Cl	115.60 ± 6.14	nd na [27]
10	2-Br	135.09 ± 5.96	nd 1.0 [27]
11	3-F	81.86 ± 5.82	5.78 ± 2.34
12	3-Cl	16.86 ± 1.31	1.75 ± 0.40 1.0 [27]
13	2-F,4-F	98,68 ± 6,10	34.21 ± 1.70
14	2-F,4-Cl	76.48 ± 6.61	10.91 ± 1.62
15	2-Cl,4-Cl	na	
16	3-F,4-F	46.51 ± 5.15	4.72 ± 1.05
17	3-F,4-Cl	10.94 ± 0.96	3.14 ± 0.76
18	3-Cl,4-F	8.22 ± 0.59	1.65 ± 0.48
19	2,4,6-F,F,F	476.47 ± 16.35	nd
20	4-CH_3	11,46 ± 1,58	6,00 ± 1,35 0.25 [27]
21	2-F,4-CH_3	101.78 ± 3.55	6.53 ± 1.89
22	4-CF_3	130.11 ± 6,65	nd 8.3 [27]
23	2-F,4-$OCHF_2$	118.02 ± 3.43	nd
24	3-F,5-CF_3	181.79 ± 7.91	nd
25	4-CN	na	
26	2-F,4-CN	na	
27	4-OCH_3	1038.60 ± 41.28	nd
28	4-NH_2	348.83 ± 11.95	nd
29	cyclobutyl derivative		0.047 [22]

[a] na = not active at concentration of 1 mM.
[b] nd = not determined.
[c] Data in references 18 and 20 consider parsley enzyme, whereas data in references 19 and 24 buckwheat enzyme.

Quite interestingly modeling predicts quite effective binding of all the studied compounds with preferable binding of two isomers of compound **1**. However, as seen from Table 1 this compound is inactive. We speculate that the lack of activity might result from restricted ability of this molecule to reach any of the possible binding sites or may reflect the differences between parsley and potato enzymes. The process of movement of substrates and inhibitors along entrance channel has not been, however, analyzed in he literature yet. This speculation is to some extend supported by recent finding that some inhibitors of phenylalanine ammonia lyase act as slow-binding ones [23].

For comparison, we have constructed models of pharmacophores for these two binding sites (Fig. 2). These models are quite similar to each other and thus, only small differences decide, which site (A or B) is occupied by inhibitor.

As seen from Fig. 3 both enantiomers of compound **17** are effectively bound to the site A of the enzyme, although in visibly different manner. This is, more or less, typical situation for analogues of phenylglycine studied in this work. It is also worth to mention that *R*-phenylalanine acts as moderate inhibitor of the enzyme [37], while *S*-isomer is its substrate.

Identified interactions of *R*-3-fluoro-4-chlorobenzylphosphonic acid with the active site of the enzyme, shown in Fig. 4, are relatively simple. The Arg 354 electrostatically stabilizes the two oxide atoms of phosphonic acid group when its third oxygen atom interacts with Phe 400 (blue arrow) and Tyr351. Phe116 interacts with phenyl ring of the inhibitor **17** (yellow arrow) by π−π interaction. This interaction, however, is not of vital for inhibitory action since any visible dependence of the inhibitory potential of the full set of inhibitors bound in site A on electron density in their phenyl ring is seen (data not shown).

2.4. Conclusions

As seen, from this work and literature data, phosphonic acid analogues of phenylglycine appear to be, in most cases, moderate inhibitors of phenylalanine ammonia lyase, with inhibitory potential being strongly dependent on the source of the enzyme. Molecular modeling done for parsley enzyme suggest that these inhibitors can be bound in two pockets being in vicinity to each other and interconnected by a channel directing substrate into active site cavity. Therefore, we also modeled binding of the most effective inhibitor of buckwheat enzyme - 1-aminobenzocyclobutene-1-phosphonic acid (**29**) [22]. Modeling predicts that this compound might be effectively bound in four different sites of the enzyme (Fig. 5). This indicates that the determination of binding of phenylglycine analogues require crystallographic studies to be done in order to better understand their action on PAL and to design more effective inhibitors.

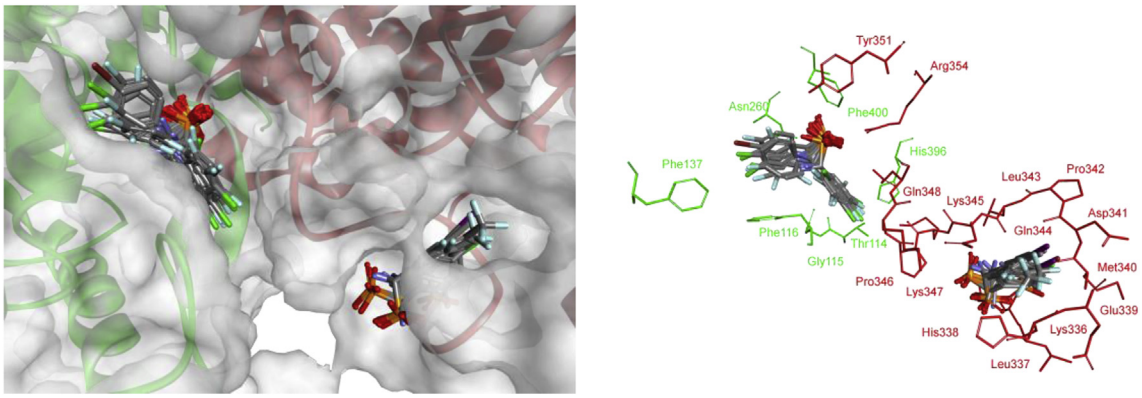


Fig. 1. Binding of the studied analogues by two subunits of parsley PAL. Active site is located in subunit A marked by green tertiary structure, whereas non-physiologic site B is located in subunit B marked in red Mode of binding in these two sites is also shown.

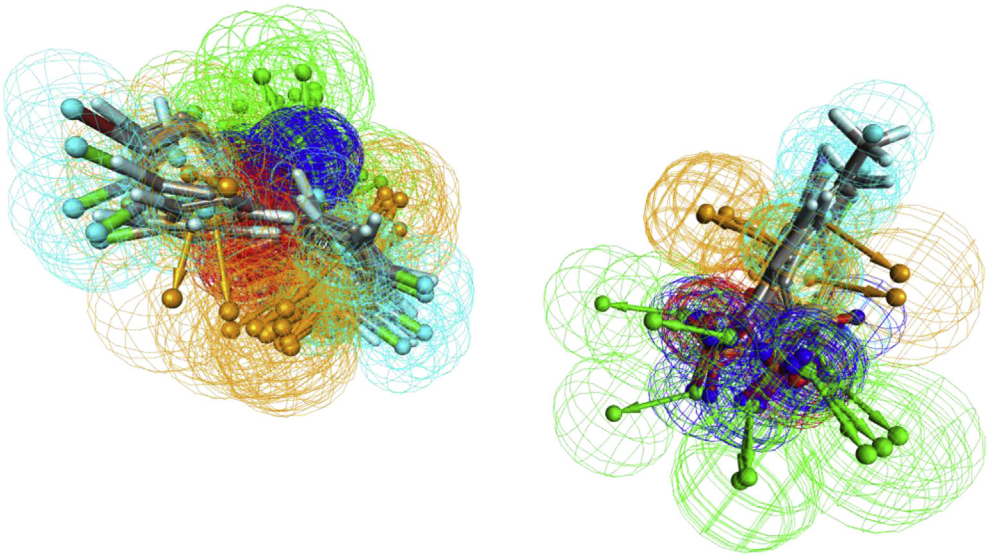


Fig. 2. Pharmacophore models for binding sites A (left panel) and B (right panel). The colors denote regions where: acceptors of hydrogen bonds are present (green), hydrophobic interactions resulting from he presence of aromatic ring are seen (orange), other hydrophobic interactions appear (light blue), and positively (red) and negatively (dark blue) charged fragments of inhibitor appear.

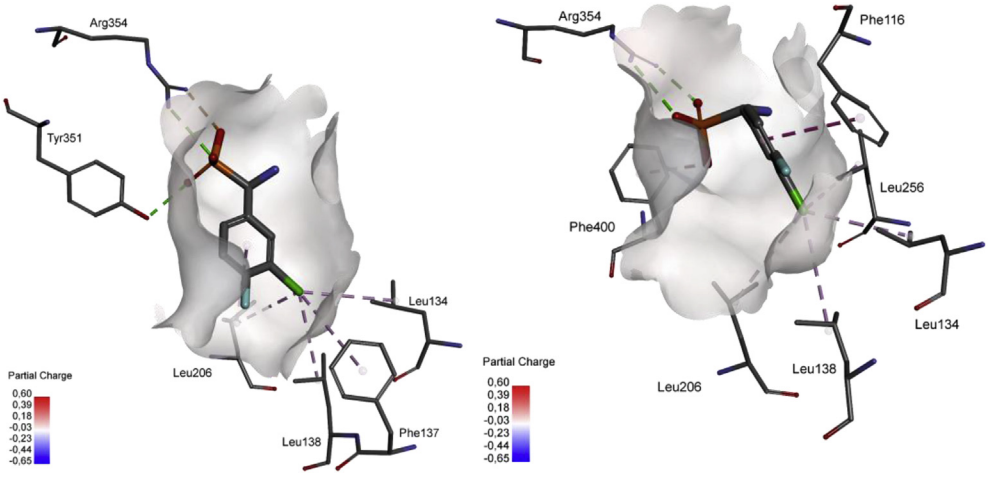


Fig. 3. Modes of binding of *R*- (left panel) and *S*- (right panel) enantiomers of 3-fluoro-4-chlorobenzylphosphonic acid (compound **17**) by parsley PAL.

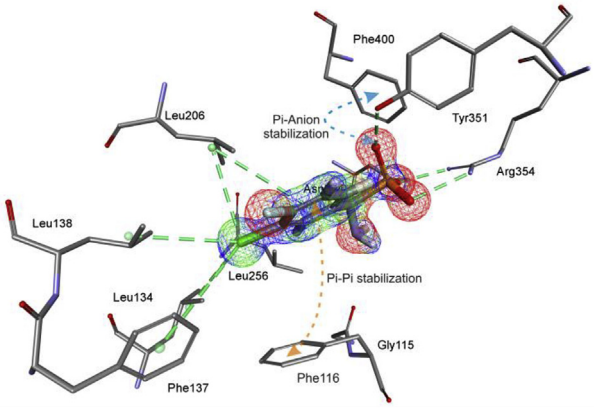


Fig. 4. Interactions of *R*-3-fluoro-4-chlorobenzylphosphonic acid with active site of parsley PAL.

3. Experimental section

3.1. Materials and methods

All chemicals were purchased from commercial Polish suppliers (Sigma Aldrich, Trimen Chemicals and POCh), and used without further purification. ^{1}H NMR spectra were recorded on a Bruker Avance II Ultrashield Plus or on a Bruker Avance III 500 MHz (Bruker, Rheinstetten, Germany) spectrometersv c/operating at 600.58 MHz and 400 MHz, ^{19}F NMR at 565.00 MHz and 376 MHz, ^{13}C NMR and ^{31}P NMR{1H} (100.61MHz/151 MHz and 162.01MHz/243 MHz), respectively. Samples of the products were diluted in D_2O (99.8% at % D) with addition of D_2SO_4 or NaOD in cases of poor solubility. Deuterated solvents were supplied by ARMAR AG (Dottingen, Switzerland). Chemical shifts are reported relative to internal TMS (^{1}H NMR), $CFCl_3$ (^{19}F NMR) and 85% H_3PO_4 (^{31}P NMR) standards and are given in parts per million (ppm), while coupling constant are reported in Herz. Multiplicities are shown as the

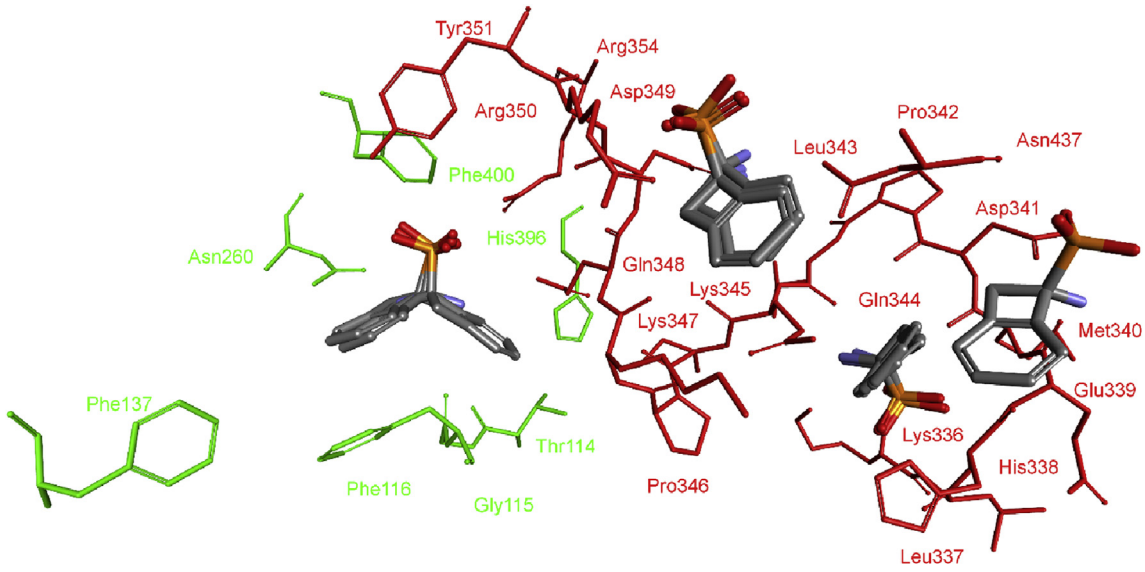


Fig. 5. Multisite binding of two enantiomers of 1-aminobenzocyclobutene-1-phosphonic acid (**29**) by parsley PAL, as predicted by molecular modeling.

abbreviations: s (singlet), bs (broad singlet), d (doublet), t (triplet), m (multiplet). Mass spectra were recorded at the Faculty of Chemistry, Wroclaw University of Science and Technology by using a Waters LCT Premier XE mass spectrometer (electrospray ionization, ESI) (Waters, Milford, MA, USA). Melting points were determined on an SRS Melting Point Apparatus OptiMelt MPA 100 (Stanford Research Systems, Sunnyvale, CA, USA) and reported uncorrected.

3.2. Synthesis of aminobenzylphosphonic acids – general procedure

Synthetic procedure was based on this elaborated earlier by Oleksyszyn and Soroka [34–36]. Thus, acetamide 1.18 g (0.02 mol) was dissolved in glacial acetic acid (4 mL, 4.20 g, 0.07 mol). The solution was cooled in an ice-bath at 0 °C and acetyl chloride (0.71 mL, 0.78 g, 0.01 mol) was added observing the formation of a crystalline by-product. After a few minutes appropriate aldehyde (0.01 mol) was added, and the mixture was kept in ice-bath for 30 min and then left for 1 day at room temperature with constant stirring. Then the mixture was cooled again to 0 °C and phosphorus trichloride was added dropwise (0.87 mL, 1.37 g, 0.01 mol). After stirring for 30 min the mixture was allowed to warm to room temperature, and finally heated for 1–1.5 h at 75–80 °C. Evaporation of the volatile components of the reaction mixture resulted in an oily product, which was refluxed for 8 h in concentrated hydrochloric acid (50 mL). After the removal of volatile components of the reaction mixture oily product was dissolved in ethanol (10 mL) and left for crystallization. Resulting aminobenzylphosphonic acid was then recrystallized from ethanol or ethanol-water.

3.2.1. Amino-[(2,3,4,5,6-pentafluorophenyl)methyl]phosphonic acid (3)

White solid, m p. 247 °C; yield: 40%; **^{1}H NMR (600 MHz, $D_2O + D_2SO_4$)** δ, ppm: 3.94 (d, $J = 18.4$ Hz, 1H, CHP), 5.06 (bs, 1H, NH); **^{13}C NMR [^{19}F] (101 MHz, D_2O + NaOD)** δ, ppm: 145.37 (s, C_{ar}), 144.45 (s, C_{ar}), 139.92 (s, C_{ar}), 137.89 (s, C_{ar}), 136.95 (s, C_{ar}), 115.66 (s, C_{ar}), 48.17 (d, $J = 131.0$ Hz, CHP); **^{19}F NMR (376 MHz, $D_2O + D_2SO_4$)** δ, ppm: −161.90 (qd, $J = 8.3$, 3.6 Hz, 2F), −152.73 (tdd, $J = 21.1$, 4.9, 2.4 Hz, 1F), −141.95 (dd, $J = 12.3$, 8.2, 5.0 Hz, 2F); **^{31}P NMR (162 MHz, $D_2O + D_2SO_4$)** δ, ppm: 8.36–8.23 (m); **HRMS (ESI-MS)** *m/z* $[MH]^+$ calcd. for $C_7H_5F_5NO_3P$: 278.0005, Found: 278.0010.

3.2.2. Amino-[(4-fluorophenyl)methyl]phosphonic acid (4)

White solid, m p. 293 °C (lit [27]. m p. 273–275 °C); yield: 55%; **^{1}H NMR (600 MHz, D_2O)** δ, ppm: 4.27 (d, $J = 15.5$ Hz, 1H, CHP), 4.71 (bs, 1H, NH), 7.11 (t, $J = 8.8$ Hz, 2H, 2xCHar), 7.41 (dd, $J = 7.9$, 4.5 Hz, 2H 2xCHar); **^{13}C NMR (101 MHz, D_2O)** δ, ppm: 163.50 (d, $J = 2.3$ Hz, C_{ar}), 161.88 (d, $J = 2.1$ Hz, C_{ar}), 129.82 (dd, $J = 8.6$, 5.1 Hz, C_{ar}), 128.84–128.71 (m, C_{ar}), 115.85 (d, $J = 22.0$ Hz, 2xC_{ar}), 52.81 (d, $J = 139.5$ Hz, CHP); **^{19}F NMR (565 MHz, D_2O)** δ, ppm: −113.54 (d, $^{6}J = 3.4$ Hz, 1F, FP); **^{31}P NMR (162 MHz, D_2O)** δ ppm: 9.75 (d, $^{6}J = 3.3$ Hz, 1P, PF); **HRMS (ESI-MS)** *m/z* $[MH]^+$ calcd. for $C_7H_9FNO_3P$: 206.0382, found: 206.0361.

3.2.3. Amino-[(4-chlorophenyl)methyl]phosphonic acid (5)

White solid, m p. 270 °C (lit [27]. m p. 265–268 °C); yield: 66%; **^{1}H NMR (600 MHz, D_2O)** δ, ppm: 3.77 (d, $J = 17.0$ Hz, CHP), 5.06 (bs, 1H, NH), 6.54 (dd, $J = 8.6$ Hz, 1.9 Hz, 2H, 2xCHar), 6.59 (d, $J = 8.6$ Hz, 2H, 2xCHar); **^{13}C NMR (151 MHz, D_2O)** δ, ppm: 134.17 (d, $J = 2.8$ Hz, C_{ar}), 131.53 (d, $J = 4.7$ Hz, C_{ar}), 129.24 (d, $J = 5.0$ Hz, 2xC_{ar}), 128.98 (d, $J = 1.7$ Hz, 2xC_{ar}), 52.90 (d, $J = 138.3$ Hz, CHP); **^{31}P NMR (162 MHz, D_2O)** δ, ppm: 11.72 (s, 1P); **HRMS (ESI-MS)** *m/z* $[MH]^-$ calcd. for $C_7H_9ClNO_3P$: 219.9930, found: 219.9919.

3.2.4. Amino-[(4-bromophenyl)methyl]phosphonic acid (6)

White solid, m p. 290 °C (lit [27]. m p. 290–296 °C); yield: 67%; **^{1}H NMR (600 MHz, D_2O)** δ, ppm: 4.28 (d, $J = 16.0$ Hz, 1H, CHP), 4.68 (bs, 1H, NH), 7.23 (dd, $J = 8.5$,1.8 Hz, 2H, CH_{ar}), 7.49 (d, $J = 8.5$ Hz, 2H, CH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 141.10 (s, C_{ar}), 130.84 (d, $J = 2.0$ Hz, 2xC_{ar}), 129.48 (d, $J = 4.9$ Hz, 2xC_{ar}), 119.29 (dd, $J = 3.2$,0.7 Hz, C_{ar}), 55.10 (d, $J = 130.1$ Hz, CHP); **^{31}P NMR (162 MHz, D_2O)** δ, ppm: 10.80 (s, 1P); **HRMS (ESI-MS)** *m/z* $[MH]^-$ calcd. for $C_7H_9BrNO_3P$: 263.9425, found: 263.9417.

3.2.5. Amino-[(4-iodophenyl)methyl]phosphonic acid (7)

White solid, m p. 280 °C, yield: 63%; **^{1}H NMR (600 MHz, D_2O + NaOD)** δ, ppm: 3.61 (d, $J = 15.9$ Hz, 1H, CHP), 4.67 (bs, 1H, NH), 7.02 (dd, $J = 8.3$,1.7 Hz, 2H, 2xCH_{ar}), 7.56 (d, $J = 8.3$ Hz, 2H, 2xCH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 141.91 (d, $J = 2.6$ Hz, C_{ar}), 136.86 (d, $J = 2.1$ Hz, 2xC_{ar}), 129.71 (d, $J = 4.9$ Hz, 2xC_{ar}), 90.65 (d, $J = 3.5$ Hz, C_{ar}), 55.17 (d, $J = 129.9$ Hz, CHP); **^{31}P NMR (162 MHz, D_2O + NaOD)** δ ppm: 17.85 (s, 1P); **HRMS (ESI-MS)** *m/z* $[MH]^+$ calcd. for $C_7H_9INO_3P$: 313.9443, found: 313.9448.

3.2.6. Amino-[(2-fluorophenyl)methyl]phosphonic acid (8)

White solid, m p. 253 °C (lit [27]. m p. 257–260 °C); yield: 95%; **^{1}H NMR (600 MHz, D_2O)** δ, ppm: 4.30 (d, J = 17.5 Hz, 1H, CHP), 4.95 (bs, 1H, NH), 6.61 (t, J = 9.6, 1H, CH_{ar}), 6.68 (t, J = 7.6, 1H, CH_{ar}), 6.87 (dt, J = 13.9,7.5 Hz, 2H, 2xCH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 161.33 (d, J = 5.8 Hz, C_{ar}), 158.91 (d, J = 5.8 Hz, C_{ar}), 129.17–128.79 (m, C_{ar}), 127.89 (dd, J = 8.4,2.4 Hz, C_{ar}), 124.04 (dd, J = 3.2,2.3 Hz, C_{ar}), 115.11 (dd, J = 22.9,1.8 Hz,C_{ar}), 48.38 (dd, J = 132.0,1.6 Hz, CHP); **^{19}F NMR (376 MHz, D_2O)** δ, ppm: −117.14 to −117.26 (m); **^{31}P NMR (162 MHz, D_2O)** δ ppm: 12.26 (s, 1P); **HRMS (ESI-MS)** m/z $[MH]^+$ calcd. for $C_7H_9FNO_3P$: 206.0382, found: 206.0377.

3.2.7. Amino-[(2-chlorophenyl)methyl]phosphonic acid (9)

White solid, m p. 244 °C (lit [27]. m p. 247–249 °C); yield: 38%; **^{1}H NMR (600 MHz, D_2O)** δ, ppm: 4.71 (bs, 1H, NH), 4.95 (d, J = 16.5 Hz, 1H, CHP), 7.37–7.30 (m, 2H, 2xCH_{ar}), 7.46 (d, J = 7.4 Hz, 1H, CH_{ar}), 7.55 (dt, J = 7.6, 1.9 Hz, 1H, CH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 139.87 (d, J = 0.8 Hz, C_{ar}), 133.23 (d, J = 7.4 Hz, C_{ar}), 129.06 (d, J = 1.6 Hz, C_{ar}), 128.68 (d, J = 3.7 Hz, C_{ar}), 127.64 (d, J = 2.3 Hz, C_{ar}), 126.90 (d, J = 2.2 Hz,C_{ar}), 51.01 (d, J = 131.3 Hz, CHP); **^{31}P NMR (162 MHz, D_2O)** δ ppm: 9.56 (s, 1P); **HRMS (ESI-MS)** m/z $[MH]^+$ calcd. for $C_7H_9ClNO_3P$: 222.0087, found: 220.0088.

3.2.8. Amino-[(2-bromophenyl)methyl] phosphonic acid (10)

White solid, m p. 234 °C (lit [27]. m p. 246–248 °C); yield: 38%; **^{1}H NMR (400 MHz, D_2O)** δ, ppm: 4.66 (bs 1H, NH), 4.91 (d, J = 16.4 Hz, 1H, CHP), 7.16–7.23 (m, 1H, CH_{ar}), 7.35 (t, J = 7.6 Hz, 1H, CH_{ar}), 7.52 (dt, J = 7.9 Hz, 1.7 Hz, 1H, CH_{ar}), 7.60 (d, J = 8.1 Hz, 1H, CH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 148.63 (s, C_{ar}), 141.65 (s, C_{ar}), 132.40 (d, J = 1.4 Hz, C_{ar}), 128.82 (d, J = 3.7 Hz, C_{ar}), 127.79 (dd, J = 44.6,2.2 Hz, C_{ar}), 124.32 (d, J = 8.0 Hz, C_{ar}), 53.83 (d, J = 131.2 Hz, CHP); **^{31}P NMR (162 MHz D_2O)** δ, ppm: 10.16 (s, 1P); **HRMS (ESI-MS)** m/z $[MH]^-$ calcd. for $C_7H_9BrNO_3P$: 263.9425, found: 263.9431.

3.2.9. Amino-[(3-fluorophenyl)methyl]phosphonic acid (11)

White solid, m p. 325 °C (lit [27]. m p. 273–275 °C); yield: 76%; **^{1}H NMR (400 MHz, D_2O)** δ, ppm: 4.40 (d, J = 16.0 Hz, 1H, CHP), 4.71 (br s, 1H, NH), 7.09–7.15 (m, 1H, CH_{ar}), 7.15–7.23 (m, 2H, 2xCH_{ar}) 7.40 (td, J = 8.1,6.0 Hz, 1H, CH_{ar}); **^{13}C NMR (101 MHz, D_2O)** δ, ppm: 163.70 (s, C_{ar}), 135.16 (s, C_{ar}), 130.83 (d, J = 8.2 Hz, C_{ar}), 123.64 (d, J = 3.7 Hz, C_{ar}), 115.68 (d,J = 20.1 Hz, C_{ar}), 114.64 (dd, J = 23.0,4.7 Hz, C_{ar}), 53.07 (d, J = 138.5 Hz, CHP); **^{19}F NMR (376 MHz, D_2O)** δ, ppm: −112.58 to −112.68 (m, 1F); **^{31}P NMR (243 MHz, D_2O)** δ, ppm: 10.84 (s, 1P); **HRMS (ESI-MS)** m/z $[MH]^+$ calcd. for $C_7H_9FNO_3P$: 206.0382, found: 206.0377.

3.2.10. Amino-[(3-chlorophenyl)methyl]phosphonic acid (12)

White solid, m p. 260 °C (lit [27]. m p. 271–274 °C); yield: 41%; **^{1}H NMR (600 MHz, D_2O)** δ, ppm: 3.67 (d, J = 15.9 Hz, 1H, CHP), 4.72 (bs, 1H, NH), 7.13–7.22 (m, 3H, 3xCH_{ar}), 7.29 (d, J = 1.5 Hz, 1H, CH_{ar}); **^{31}P NMR (162 MHz, D_2O)** δ ppm: 17.24 (s, 1P); **HRMS (ESI-MS)** m/z $[MH]^-$ calcd. for $C_7H_9ClNO_3P$: 219.9930, found: 219.9929.

3.2.11. Amino-[(2,4-difluorophenyl)methyl]phosphonic acid (13)

White solid, m p. 270 °C; yield: 71%; **^{1}H NMR (600 MHz, D_2O)** δ, ppm: 4.58 (d, J = 16.6 Hz, 1H, CHP), 4.72 (bs, 1H, NH), 6.95–7.03 (m, 2H, 2xCH_{ar}), 7.50–7.54 (m, 2H, 2xCH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 161.76 (dd, J = 18.6,12.5,4.3 Hz, Car), 159.32 (dd, J = 18.6,12.4,4.2 Hz, Car), 129.65 (dd, J = 9.9,6.0,3.9 Hz, Car), 125.09 (dd, J = 14.9,3.6,1.7 Hz, Car), 110.88 (dd, J = 21.0,3.6,2.1 Hz, Car), 103.18 (dd, J = 27.3,25.4,1.8 Hz, Car), 47.82 (dd, J = 132.9,1.2 Hz, CHP); **^{19}F NMR (565 MHz, D_2O)** δ, ppm: −109.15 (q, J = 9.0,5.6,3.7 Hz, 1F), −112.84 (q, J = 8.8,4.5,3.4 Hz, 1F); **^{31}P NMR (243 MHz, D_2O)** δ, ppm: 9.12 (t, ^4J = 3.4 Hz, 1P); **HRMS (ESI-MS)** m/z $[MH]^-$ calcd. for $C_7H_8F_2NO_3P$: 222.0132, found: 222.0139.

3.2.12. Amino-[(4-chloro-2-fluorophenyl)methyl]phosphonic acid (14)

White solid, m p. 263 °C; yield: 71%; **^{1}H NMR (600 MHz, D_2O)** δ ppm: 4.65 (d, J = 16.7 Hz, 1H, CHP), 4.71 (bs, 1H, NH), 7.27 (d, J = 8.9 Hz, 2H, 2xCH_{ar}), 7.46 (dt, J = 8.4,4.3 Hz, 1H, CH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 159.85 (dd, J = 246.6,5.7 Hz, C_{ar}), 131.88 (dd, J = 10.7,2.9 Hz, C_{ar}), 129.83 (dd, J = 4.8,4.3 Hz, C_{ar}), 127.81 (d, J = 14.2 Hz, C_{ar}), 124.26 (t, J = 3.0 Hz, C_{ar}), 115.67 (dd, J = 26.8,1.7 Hz, C_{ar}), 48.06 (d, J = 131.5 Hz, CHP); **^{19}F NMR (376 MHz, D_2O)** δ, ppm: −114.39 to −114.51 (m, 1F); **^{31}P NMR (162 MHz, D_2O)** δ, ppm: 9.64 (d, ^7J = 3.5 Hz, 1P); **HRMS (ESI-MS)** m/z $[MH]^+$ calcd. for $C_7H_8ClFNO_3P$: 239.9993, found: 239.9990.

3.2.13. Amino-[(2,4-dichlorophenyl)methyl]phosphonic acid (15)

White solid, m p. 245 °C; yield: 72%; **^{1}H NMR (400 MHz, D_2O + D_2SO_4)** δ, ppm: 4.13 (d, J = 17.4 Hz, 1H, CHP), 5.10 (bs, 1H, NH), 6.49 (dd, J = 59.1,8.5,2.1 Hz, 2H, 2xCH_{ar}), 6.59 (dd, J = 2.1 Hz, 1.0 Hz, 1H, CH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 138.91 (d, J = 0.9 Hz, C_{ar}), 133.88 (d, J = 7.4 Hz, C_{ar}), 131.72 (d, J = 2.8 Hz, C_{ar}), 129.66 (d, J = 3.7 Hz, C_{ar}), 128.52 (d, J = 1.6 Hz, C_{ar}), 127.00 (d, J = 2.3 Hz, C_{ar}), 50.68 (d, J = 131.0 Hz, CHP); **^{31}P NMR (162 MHz, D_2O + D_2SO_4)** δ, ppm: 10 l9 (s, 1P); **HRMS (ESI-MS)** m/z $[MH]^+$ calcd. for $C_7H_8Cl_2NO_3P$: 255.9697, found: 255.9706.

3.2.14. Amino-[(3,4-difluorophenyl)methyl]phosphonic acid (16)

White solid, m p. 294 °C (lit [27]. m p. 286–289 °C); yield: 78.5%; **^{1}H NMR (400 MHz, D_2O)** δ, ppm: 4.40 (d, J = 15.7 Hz, 1H, CHP), 4.83 (bs, 1H, NH), 7.38–7.23 (m, 2H, 2xCH_{ar}), 7.42 (ddt, J = 11.5,7.5,1.9 Hz, 1H, CH_{ar}); **^{13}C NMR (101 MHz, D_2O)** δ, ppm: 155.76 (s, C_{ar}), 155.15 (s, C_{ar}), 129.81 (s, C_{ar}), 124.50 (s, C_{ar}), 117.81 (d, J = 17.6 Hz, C_{ar}), 116.90 (d, J = 15.2 Hz, C_{ar}), 52.38 (d, J = 140.2 Hz, CHP); **^{19}F NMR (565 MHz, D_2O)** δ, ppm: −137.34 (d, J = 21.4 Hz, 1F), 138.15 (dd, J = 21.4 Hz, 4.0 Hz, 1F); **^{31}P NMR (162 MHz, D_2O)** δ, ppm: 10.53 (d, J = 2.7 Hz, 1P); **HRMS (ESI-MS)** m/z $[MH]^-$ calcd. for $C_7H_8F_2NO_3P$: 222.0132, found: 222.0131.

3.2.15. Amino-[(4-chloro-3-fluorophenyl)methyl]phosphonic acid (17)

White solid, m p. 251 °C; yield: 52.5%; **^{1}H NMR (600 MHz, D_2O)** δ, ppm: 4.39 (d, J = 16.1 Hz, 1H, CHP), 4.71 (bs, 1H, NH), 7.19 (d, J = 8.3 Hz, 1H, CH_{ar}), 7.30 (dt, J = 10.2,1.9 Hz, 1H, CH_{ar}), 7.50 (t, J = 8.0 Hz, 1H, CH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 157.28 (dd, J = 244.2,2.2 Hz, C_{ar}), 143.53 (dd, J = 6.6,2.6 Hz, C_{ar}), 129.68(d, J = 2.0 Hz, C_{ar}), 124.25 (dd, J = 4.9,3.4 Hz, C_{ar}), 117.37 (dd, J = 17.6,3.1 Hz, C_{ar}), 115.48 (dd, J = 21.5,4.8 Hz, C_{ar}), 54.95 (dd, J = 129.4,1.2 Hz, CHP); **^{19}F NMR (376 MHz, D_2O)** δ, ppm: 114.99 (t, ^5J = 9.0 Hz, 1F); **^{31}P NMR (162 MHz, D_2O)** δ, ppm: 9.89 (d, ^5J = 1.6 Hz, 1P); HRMS (ESI-MS) m/z $[MH]^+$ calcd. for $C_7H_8ClFNO_3P$: 239.9993, found: 240.0314.

3.2.16. Amino-[(3-chloro-4-fluorophenyl)methyl]phosphonic acid (18)

White solid, m p. 260 °C; yield: 84%; ^{31}P NMR (162 MHz, D_2O + D_2SO_4) δ, ppm: 12.33; ^{1}H NMR (400 MHz, D_2O + D_2SO_4) δ, ppm: 4.13 (d, J = 16.8 Hz, 1H, CHP), 4.90 (bs, 1H, NH), 6.84 (t, J = 8.9 Hz, 1H, CH_{ar}), 6.93–6.96 (m, 1H, CH_{ar}), 7.13 (dt, J = 6.8,2.2 Hz, 1H, CH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 156.30 (dd, J = 243.4,2.8 Hz, C_{ar}), 139.42 (dd, J = 3.7,2.5 Hz, C_{ar}), 129.31 (d, J = 4.9 Hz, C_{ar}), 127.50 (dd, J = 7.3,5.0 Hz, C_{ar}), 119.25 (dd, J = 17.6,2.3 Hz, C_{ar}), 115.82 (dd, J = 20.9,2.0 Hz, C_{ar}), 54.64 (d, J = 130.5 Hz, CHP); **^{19}F NMR (565 MHz, D_2O)** δ, ppm: −115.75 (dd, J = 11.1,8.9,4.4 Hz, 1F); **HRMS (ESI-MS)** m/z $[MH]^+$ calcd. for $C_7H_8ClFNO_3P$: 239.9993, found: 239.9994.

3.2.17. Amino-[(2,4,6-trifluorophenyl)methyl] phosphonic acid (19)

White solid, m p. 248 °C; yield: 48%; **^{1}H NMR (400 MHz, $D_2O + D_2SO_4$)** δ, ppm: 4.52 (d, J = 17.6 Hz, 1H, CHP), 6.85 (t, J = 9.0 Hz, 3H, 3xCH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 162.42–161.89 (m, Car), 159.99–159.44 (m, 2xCar), 113.30 (tdd, J = 18.7,4.6,1.1 Hz, Car), 100.26 (t, J = 27.7 Hz, 2xCar), 46.71 (d, J = 132.4 Hz, CHP); **^{19}F NMR (376 MHz $D_2O + D_2SO_4$)** δ, ppm: −110.12 (td, J = 8.5,3.2 Hz, 2F), −106.47 to −106.60 (m, 1F); **^{31}P NMR (162 MHz $D_2O + D_2SO_4$)** δ, ppm: 8.72 (dd, $^{4,6}J$ = 7.5 Hz, 3.6 Hz, 1P); **HRMS (ESI-MS)** m/z $[MH]^+$ calcd. for $C_7H_7F_3NO_3P$: 242.0194, found: 242.0191.

3.2.18. Amino-[(4-methylphenyl)methyl]phosphonic acid (20)

White solid, m p. 280 °C, (lit [27]. mp 254–256 °C); yield: 84%; **^{1}H NMR (600 MHz, D_2O)** δ, ppm: 2.28 (s, 3H, CH_3), 4.33 (d, J = 15.9 Hz, CHP), 4.70 (bs, 1H, NH), 7.23 (d, J = 7.6 Hz, 2H, 2xCH_{ar}), 7.29 (dd, J = 8.3,1.9 Hz, 2H, 2xCH_{ar}); **^{13}C NMR (151 MHz, D_2O) δ, ppm:** 139.19 (d, J = 2.4 Hz, C_{ar}), 129.51 (d, J = 1.6 Hz, C_{ar}), 127.69 (d, J = 5.1 Hz. C_{ar}), 53.25 (d, J = 139.8 Hz, CHP), 20.21 (s, 1C, CH_3); **^{31}P NMR (162 MHz, D_2O)** δ, ppm: 11.25 (s, 1P); **HRMS (ESI-MS)** m/z $[MH]^-$ calcd. for $C_8H_{12}NO_3P$: 200.0447, found: 200.0475.

3.2.19. Amino-[(2-fluoro-4-methylphenyl)methyl] phosphonic acid (21)

White solid, m p. 287 °C; yield: 53%; **^{1}H NMR (400 MHz, D_2O)** δ, ppm: 1.56 (s, 3H, CH_3), 4.05 (d, J = 17.2 Hz, 1H, CHP), 4.96 (bs, 1H, NH), 6.23–6.28 (m, 2H, 2xCH_{ar}), 6.67 (dd, J = 9.8, 5.6,2.3 Hz, 1H, CH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD) δ, ppm:** 160.80 (dd, J = 240.5,2.7 Hz, C_{ar}), 138.79 (t, J = 7.2 Hz, C_{ar}), 136.61 (dd, J = 2.5,2.0 Hz, C_{ar}), 128.24 (dd, J = 8.4, 3.6 Hz, C_{ar}), 115.96 (d, J = 20.1 Hz, C_{ar}), 112.15 (dd, J = 0.9,2.1 Hz, C_{ar}), 50.01 (d, J = 132.3 Hz, CHP), 19.41 (s, CH_3); ^{19}F NMR (376 MHz, D_2O) δ, ppm: −114.08 (dd, J = 15.2,10.1,4.5 Hz, 1F); **^{31}P NMR (162 MHz, D_2O)** δ, ppm: 13.12 (d, ^4J = 4.2 Hz, 1P); **HRMS (ESI-MS)** m/z $[MH]^-$ calcd. for $C_8H_{11}FNO_3P$: 218.0382, found: 218.0380.

3.2.20. Amino-[(4-trifluoromethylphenyl)methyl]phosphonic acid (22)

White solid, m p. 295 °C (lit [27]. m p. 277–280 °C); yield: 82%; **^{1}H NMR (400 MHz, D_2O)** δ, ppm: 4.49 (d, J = 16.3 Hz, 1H, CHP), 4.71 (br s, 1H, NH), 7.56 (d, J = 7.6 Hz, 2H, 2xCH_{ar}), 7.72 (d, J = 7.6 Hz, 2H, 2xCH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 168.46 (s, C_{ar}), 146.53 (s, C_{ar}), 127.84 (d, J = 4.7 Hz, C_{ar}), 127.45 (qd, J = 31.9,2.7 Hz, 2xC_{ar}), 124.76 (qd, J = 3.7,2.2 Hz, C_{ar}), 124.58 (qd, J = 271.0,1.0 Hz, CF_3), 55.52 (d, J = 128.6 Hz, CHP); **^{19}F NMR (565 MHz, D_2O)** δ, ppm: −62.47 (s. 3F, CF_3); **^{31}P NMR (243 MHz, D_2O)** δ, ppm: 10.53 (d, ^7J = 1.9 Hz); **HRMS (ESI-MS)** m/z $[MH]^-$ calcd. for $C_8H_9F_3NO_3P$: 254.0194, found: 254.0197.

3.2.21. Amino-[(4-difluorometoxy-2-fluorophenyl)methyl] phosphonic acid (23)

Yellow solid, m p. 255 °C; yield: 26%; **^{1}H NMR (600 MHz, D_2O)** δ, ppm: 2.16 (s, 1H, CHF_2), 4.56 (d, J = 16.0 Hz, 1H, CHP), 4.71 (bs, 1H, NH), 6.30 (dd, J = 14.5,10.4,2.5 Hz, 2H, 2xCH_{ar}), 6,93 (dd, J = 8.7,1.9 Hz, 1H, CH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 165.73 (dd, J = 11.7,1.7 Hz, Car), 161.33 (dd, J = 243.0,6.1 Hz, Car), 129.35 (dd, J = 7.0,3.8 Hz, Car), 114.34 (t, J = 1.8 Hz, CHF_2), 112.65 (dd, J = 15.2,2.3 Hz, 2xCar), 104.45 (dd, J = 20.6,1.3 Hz, Car), 47.77 (dd, J = 135.3,1.0 Hz, CHP); **^{19}F NMR (565 MHz, D_2O)** δ, ppm: −166.58 (s, 2F, F_2CH), −115.36 (dd, J = 12.1,8.7,3.7 Hz, 1F, $C_{ar}F$); **^{31}P NMR (162 MHz, D_2O)** δ, ppm: 12.41 (d, ^4J = 3.6 Hz, 1P).

3.2.22. Amino-[(3-fluoro-5-trifluoromethylphenyl)methyl] phosphonic acid (24)

White solid, m p. 247 °C; yield: 83%; **^{1}H NMR (600 MHz, D_2O + NaOD)** δ, ppm: 3.76 (d, J = 16.5 Hz, 1H, CHP), 4.67 (bs, 1H, NH), 7.21 (dd, J = 19.8,9.5 Hz, 2H, 2xCH_{ar}), 7.39 (s, 1H, CH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 161.93 (dd, J = 244.1,2.4 Hz, C_{ar}), 146.30 (dd, J = 7.6,2.3 Hz, C_{ar}), 130.84 (qdd, J = 32.5,8.6,2.0 Hz, **C_{ar}**CF_3), 123.66 (qd, J = 271.7,3.1 Hz, C_{ar}**C**F_3), 120.28 (dq, J = 7.7,3.8 Hz, C_{ar}), 117.96 (dd, J = 21.9,4.6,1.0 Hz, C_{ar}), 110.19 (dd, J = 25.1,3.9,2.7 Hz, C_{ar}), 55.24 (dd, J = 128.4,1.6 Hz, CHP); **^{19}F NMR (565 MHz, D_2O + NaOD)** δ, ppm: −112.89 (t, J = 9.5 Hz, 1F, FC_{ar}), −62.11 (s, 3F, CF_3); **^{31}P NMR (162 MHz, D_2O + NaOD)** δ, ppm: 16.97 (s, 1P); **HRMS (ESI-MS)** m/z $[MH]^+$ calcd. for $C_8H_8F_4NO_3P$: 274.0256, found: 274.0264.

3.2.23. Amino-[(4-cyanophenyl)methyl]phosphonic acid (25)

White solid, m p. 295 °C; yield: 84%; **^{1}H NMR (600 MHz, $D_2O + D_2SO_4$)** δ, ppm: 3.14 (d, J = 17.6 Hz, 1H, CHP), 5.30 (bs, 1H, NH), 5.91 (dd, J = 8.6,1.8 Hz, 2H, 2xCH_{ar}), 6.39 (d, J = 8.2 Hz, 2H, 2xCH_{ar}); **^{31}P NMR (162 MHz, $D_2O + D_2SO_4$)** δ, ppm: 5.30 (s, 1P); **HRMS (ESI-MS)** m/z $[MH]^-$ calcd. for $C_8H_8N_2O_3P$: 212.0351, found: 212.0408.

3.2.24. Amino-[(4-cyano-2-fluorophenyl)methyl]phosphonic acid (26)

White solid, m p. 265 °C; yield: 100%; **^{1}H NMR (600 MHz, $D_2O + D_2SO_4$)** δ ppm: 3.30 (d, J = 18.1 Hz, 1H, CHP), 5.26 (br s, 1H, NH), 5.89 (td, J = 7.7,2.1 Hz, 1H, CH_{ar}), 6.07 (d, J = 10.8 Hz, 1H, CH_{ar}), 6.15 (dd, J = 8.2,1.3 Hz, 1H, CH_{ar}), **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 159.71 (dd, J = 244.1,5.6 Hz, C_{ar}), 136.36 (dd, J = 6.9,2.5 Hz, C_{ar}), 131.78 (dd, J = 13.9,3.5 Hz, C_{ar}), 128.60 (t, J = 4.1 Hz, C_{ar}), 124.34 (d, J = 2.4 Hz, C_{ar}), 115.40 (d, J = 23.7 Hz, C_{ar}), 48.60 (d, J = 130.4 Hz, CHP); **^{19}F NMR (376 MHz, $D_2O + D_2SO_4$)** δ, ppm: −116.87 (dd, J = 10.9,7.2,3.9 Hz, 1F); **^{31}P NMR (162 MHz, $D_2O + D_2SO_4$)** δ, ppm: 10.71 (t, ^4J = 4.2 Hz, 1P); **HRMS (ESI-MS)** m/z $[MH]^+$ calcd. for $C_8H_8FN_2O_3P$: 231.0335, found: 231.0337.

3.2.25. Amino-[(4-metoksyphenyl)methyl] phosphonic acid (27)

White solid, m p. 272 °C; yield: 54%; **^{1}H NMR (400 MHz, D_2O)** δ, ppm: 3.15 (s, 3H, CH_3O), 3.93 (d, J = 16.7 Hz, 1H, CHP), 6.37 (d, J = 8.8 Hz, 2H, 2xCH_{ar}), 6.75 (dd, J = 8.8, 1.9 Hz, 2H, 2xCH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 157.23 (d, J = 2.5 Hz, C_{ar}), 134.79 (d, J = 2.4 Hz, C_{ar}), 129.12 (d, J = 5.3 Hz, C_{ar}), 128.87 (d, J = 5.1 Hz, C_{ar}), 118.05 (d, J = 1.6 Hz, C_{ar}), 113.54 (d, J = 1.4 Hz, C_{ar}), 55.44 (s, 3H, OCH_3), 54.84 (d, J = 132.4 Hz, CHP); **^{31}P NMR (162 MHz D_2O)** δ, ppm: 13.46 (s, 1P); **HRMS (ESI-MS)** m/z $[MH]^-$ calcd. for $C_8H_{12}NO_4P$: 216.0426, found: 216.0416.

3.2.26. Amino-[(4-aminophenyl)methyl]phosphonic acid (28)

White solid, m p. 350 °C; yield: 52%, **^{1}H NMR (400 MHz, D_2O)** δ, ppm: 3.75 (d, J = 17.1 Hz, 1H, CHP), 6.52 (d, J = 8.4 Hz, 2H, 2xCH_{ar}), 6.62 (dd, J = 8.5, 2.0 Hz, 2H, 2xCH_{ar}); **^{13}C NMR (101 MHz, D_2O + NaOD)** δ, ppm: 144.19 (d, J = 2.3 Hz, C_{ar}), 133.10 (d, J = 2.7 Hz, 2xC_{ar}), 128.63 (d, J = 5.2 Hz, C_{ar}), 116.10 (d, J = 1.9 Hz, 2xC_{ar}), 54.92 (d, J = 133.2 Hz, CHP); **^{31}P NMR (162 MHz D_2O)** δ, ppm: 11.16 (s, 1P); **HRMS (ESI-MS)** m/z $[MH]^-$ calcd. for $C_7H_{11}N_2O_3P$: 201.0429, found: 201.0423.

3.3. Isolation of PAL

Phenylalanine ammonia lyase was extracted from 1 mm thick slices of potato tuber (*Solanum tuberosum* L.) basing on the procedure described by Havir & Hanson [38]. Fresh potatoes were purchased from local grocery store. The washed slices were put in the Petri dishes lined with filter paper Whatmann 2 soaked with neomycin sulfate solution (50 mg/l) and exposed to with luminous flux (5220 lumen) at 22 °C for 22 h. The harvested slices were then washed with distilled water, dried with paper towel and weighted.

The prepared plant material (500 g) was homogenized by 1 min (speed 8500 rpm) in 0.1 M borate buffer (pH = 8.7) containing β-mercaptoethanol (80μl/100 ml buffer) and filtered through flip flops layer. The filtrate was brought to pH = 5.5 by using 1 M acetic acid followed by addition of protamine sulfate solution (333 mg + 1.33 ml 1 M acetate buffer, pH = 5.0 and supplemented with distilled water to 16.67 ml volume). The mixture was then centrifuged for 10 min (7000 rpm/min), in 0 °C. The sediment was discarded and to supernatant ammonium sulfate was added to 27% salting, stirring for 30 min at 4 °C in the dark and left for 24 h. After this time, the mixture was centrifuged again under this same condition (10min/7000 rpm/0 °C) and resulting supernatant repeatedly salt out with ammonium sulfate (to 40% of saturation) and centrifuged once more. The precipitate after second fractionation was dissolved in borate buffer (final volume 105 ml). The mixture was divided into aliquots (15 ml) and suspended in ammonium sulfate (to 40% of saturation). Individual portions of the enzyme were directly purified before kinetic tests on G-75 Sephadex with using NGC Medium-Pressure Chromatography controlling the process by UV-VIS (280 nm). Thus, 15 ml of PAL solution was applied to the column and eluted with 0.1 M borate buffer (pH = 8.7) collecting 3 ml fractions. Active PAL fractions was combined and used as enzyme source. The specific activity of the PAL was ranged 2–8mU/mg.

3.4. Kinetic assays

Kinetics of the PAL-catalyzed reaction of L-phenylalanine were determined in 0.1 M borate buffer, pH = 8.7 at 37 °C by following formation of *trans*-cinnamic acid at 290 nm using an enzyme of 3.75mU/mg specific activity. The determined extinction coefficient for *trans*-cinnamic acid is 8940 $M^{-1}cm^{-1}$. The determined Michaelis constant (K_M) was equal of 0.15 mM ± 0.057 and was determined by the Lineweaver-Burk method using weighted regression.

IC_{50} values were determined by using 1 mM concentration of phenylalanine. For the most effective inhibitors inhibition constants were determined by measuring the initial product formation rates at concentration of L-phenylalanine 1 mM and 0.5 mM, respectively and in the presence of varying concentrations of inhibitor, which were dependent on the strength of the inhibitor (in 0.002–0.2 mM range). For each inhibitor, the rate of enzymatic reaction was determined on the basis of reaction progress data (changes in absorbance of *trans*-cinnamic acid over time). Inhibition rates were determined taking into account 2 substrate concentrations and 10 concentrations of individual inhibitors, with each measurement repeated twice.

Kinetic constants K_M, V_{max} and K_i and type of inhibition were determined by Dixon method and modified by us Dixon method for competitive inhibition model (1). For each of the determined parameters a relative error was calculated, with the resultant spread (K_i and IC_{50} values) not exceeding 10% for each of the test inhibitors. The data characterizing the inhibition potency of individual phosphonic phenylglycine analogues K_i and IC_{50} (Table 1.) are the values obtained by method characterized by best statistical parameters.

$$\frac{V_{max}}{V_0} - 1 = \frac{K_M}{[S]} + \frac{K_M}{[S]} \cdot \frac{1}{K_i} \cdot [I] \quad (1)$$

3.5. Molecular modeling

All molecular modeling calculations were made in BIOVIA Discovery Studio 2017 R2 program package. The crystal structure of phenylalanine ammonia lyase (PAL) from *Petroselinum crispum* was obtained from the RCSB Protein Data Bank – ID: 1W27 [39]. Structure was protonated by the pH of experimental method (pH = 8.7) and minimalized by the Smart minimizer algorithm with CHARMm force field. The partial charges were calculated by the Momany-Rone algorithm. The ligand and the water molecules were removed from the structure of the enzyme. New ligands were considered taken into account stereochemistry, protonated and minimized with molecular dynamics calculations. Compounds were minimized by Steepest descent and conjugate gradient algorithms, then the heating step was provided. Target temperature was set to 300 K and simulation time to 10 ps. Equilibration's step time was set at 100 ps and the production step with NVT type to 1000 ps. The lowest potential energy structures were selected and minimized by the DFT calculations with the use of B3LYP functional. The long minimization calculations were used to obtain partial charges on aromatic ring of the compounds for the other studies. Dockings in the active center of the enzyme were made by the usage of the GOLD Algorithm (5.5 version, CCDC, Cambridge, United Kingdom).

Acknowledgements

This work was supported by 2017/26/M/ST5/00437 grant. The Biovia Discovery Studio package was used under a Polish countrywide license. The use of software resources (Biovia Discovery Studio program package) of the Wrocław Center for Networking and Supercomputing is also kindly acknowledged.

References

[1] M.J. MacDonald, D.B. D'Cunha, A modern view of phenylalanine ammonia lyase, Biochem. Cell. Biol. 85 (2007) 273–282.
[2] M.W. Hyun, Y.H. Yun, J.Y. Kim, S.H. Kim, Fungal and plant phenylalanine ammonia-lyase, MYCOBIOLOGY 30 (2011) 257–265.
[3] J.Q. Kong, Phenylalanine ammonia-lyase, a key component used for phenylpropanoids production by metabolic engineering, RSC Adv. 5 (2015) 62587–62603.
[4] B. Schuster, J. Rétey, The mechanism of action of phenylalanine ammonia lyase: the role of prosthetic dehydroalanine, Proc. Natl. Acad. Sci. Unit. States Am. 92 (1995) 8433–8437.
[5] X. Zhang, C.-J. Liu, Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phen[1] B. Schuster, J. Rétey, the mechanism of action of phenylalanine ammonia lyase: the role of prosthetic dehydroalanine, Proc. Natl. Acad. Sci. Unit. States Am. 92 (1995) 8433–8437.
[6] X. Zhang, C.-J. Liu, Multifaceted regulations of gateway enzyme phenylalanine Amylpropanoids, Mol. Plant 8 (2015) 17–25.
[7] J.C. Calabrese, D.B. Jordan, A. Boodhoo, S. Sariaslani, T. Vannelli, Crystal structure of phenylalanine ammonia lyase: multiple helix dipoles implicated in catalysis, Biochemistry 43 (2004) 11403–11416.
[8] J. Barros, J.C. Serrani-Yarce, F. Chen, D. Baxter, B.J. Venables, R.A. Dixon, Role of bifunctional ammonia-lyase in grass cell wall biosynthesis, Nature Plants 2 (2016), 16050.
[9] L. Labeeuw, P.T. Martone, Y. Boucher, R.J. Case, Ancient origin of the biosynthesis of lignin precursors, Biol. Direct. 10 (2015), 23.
[10] L. Nybakken, S. Keski-Saari, M.A. Falck, R. Julkunen-Tiitto, Restoration of secondary metabolism in birch seedlings relieved from PAL-inhibitor, Trees (Berl.) 21 (2007) 273–281.
[11] F.J. van Spronsen, T.G.J. Derks, Recombinant phenylalanine ammonia lyase in phenylketonuria, Lancet 384 (2014) 6–8.
[12] N. Al Hafiz, J. Cristodolou, Phenylketonuria: a review of current and future treatments, Transl. Pediatr. 4 (2015) 304–317.
[13] F. Parmeggiani, N.J. Weise, S.T. Ahmed, N.J. Turner, Synthetic and therapeutic applications of ammonia-lyases and aminomutases, Chem. Rev. 118 (2018) 73–118.
[14] F. Parmeggiani, S.L. Lovelock, N.J. Weise, S.T. Ahmed, N.J. Turner, Synthesis of D- and l-phenylalanine derivatives by phenylalanine ammonia lyases: a multienzymatic cascade process, Angew. Chem. Int. Ed. 54 (2015) 4608–4611.
[15] J.D. Cui, J.Q. Qiu, X.W. Fan, S.R. Jia, Z.L. Tan, Biotechnological production and applications of microbial phenylalanine ammonia lyase: a recent review, Crit. Rev. Biotechnol. 34 (2014) 258–268.
[16] V.P. Kukhar, V.A. Soloshonok, V.A. Solodenko, Asymmetric synthesis of phosphorus analogs of amino acids, Phosphorus, Sulfur Silicon Relat. Elem. 92 (1994) 239–264.
[17] A. Mucha, P. Kafarski, Ł. Berlickir, Remarkable potential of the α-

Aminophosphonate/phosphinate structural motif in medicinal chemistry, J. Md. Chem. 54 (2011) 5955–5980.
[18] K.V. Turchrniuk, V.P. Kukhar, V. P Röschethaler, J.L. Aceña, V.A. Soloshonok, A.E. Sorochinsky, Recent advances in the synthesis of fluorinated aminophosphonates and aminophosphonic acids, RSC Adv. 19 (2013) 6693–6716.
[19] K.M. Janas, A. Filipiak, J. Kowalik, P. Mastalerz, J.S. Knypl, 1-Amino-2-Phenylethylphosphonic acid: an inhibitor of *l*-phenylalanine ammonia-lyase *in vitro*, Acta Biochim. Pol. 32 (1985) 131–143.
[20] B. Laber, H.H. Kiltz, N. Amrhein, Inhibition of phenylalanine ammonia-lyase *in vitro* and *in vivo* by (1-amino-2-phenylethyl)phosphonic acid, the phosphonic analogue of phenylalanine, Z. Naturforsch. C 41 (1986) 49–55.
[21] P. Miziak, J. Zoń, N. Amrhein, R. Gancarz, Inhibitors of phenylalanine ammonia-lyase: substituted derivatives of 2-Aminoindane-2-phosphonic acid and 1-aminobenzyl phosphonic acid, Phytochemistry 68 (2007) 407–415.
[22] J. Zoń, P. Miziak, 1-Aminobenzocyclobutene-1-phosphonic acid and related compounds as inhibitors of phenylalanine ammonia-lyase, Chem. Biodivers. 14 (2017), e1600488.
[23] Z. Bata, R. Qian, A. Roller, J. Horak, L.C. Bencze, C. Paizs, F. Hammerschmidt, B.G. Vértessy, L. Poppe, A methylidene group in the phosphonic acid analogue of phenylalanine reverses the enantiopreference of binding to phenylalanine ammonia-lyases, Adv. Synth. Catal. 2017 (359) (2017) 2109–2120.
[24] J. Zoń, N. Amrhein, Inhibitors of phenylalanine ammonia-lyase: 2- aminoindan-2-phosphonic acid and related compounds, Liebigs ann. Inside Chem. (1992) 625–628.
[25] C. Appert, J. Zoń, N. Amrhein, Kinetic analysis of the inhibition of phenylalanine ammonia-lyase by 2-aminoindan-2-phosphonic acid and other phenylalanine anologues, Phytochemistry 62 (2003) 415–422.
[26] J. Zoń, P. Miziak, N. Amrhein, R. Gancarz, Inhibitors of phenylalanine ammonia-lyase (PAL): synthesis and biological evaluation of 5-substituted 2-Aminoindan-2-phosphonic acids, Chem. Biodivers. 2 (2005) 1187–1194.
[27] J. Zoń, N. Amrhein, R. Gancarz, Inhibitors of phenylalanine ammonia-lyase: 1-aminobenzylphosphonic acids substituted in the benzene ring, Phytochemistry 59 (2002) 9–21.
[28] H. Zheng, K. Comeforo, J. Gao, Expanding the fluorous arsenal: tetrafluorinated phenylalanines for protein design, J. Am. Chem. Soc. 131 (2009) 18–19.
[29] J. Wang, M. Sánchez-Roselló, L.M. Aceña, C. del Pozo, A.E. Sorochinsky, F. Sanchez, V.A. Soloshonok, H. Liu, Fluorine in pharmaceutical industry: fluorine-containing drugs introduced to the market in the last decade (2001–2011), Chem. Rev. 114 (2014) 2432–2506.
[30] Y. Zhou, J. Wang, Z. Gu, S. Wang, W. Zhu, L.M. Aceña, V.A. Soloshonok, K. Izawa, H. Liu, Next generation of fluorine-containing pharmaceuticals, compounds currently in phase II–III clinical trials of major pharmaceutical companies: new structural trends and therapeutic areas, Chem. Rev. 116 (2016) 422–518.
[31] E.P. Gillis, K.J. Eastman, M.D. Hill, D.J. Donnelly, N.A. Deanwell, Applications of fluorine in medicinal chemistry, J. Med. Chem. 58 (2015) 8315–8359.
[32] S. Swallow, Fluorine in medicinal chemistry, Prog. Med. Chem. 54 (2015) 65–133.
[33] B.C. Buer, J.L. Meagher, J.A. Stuckey, E.N.G. Marsh, Structural basis for the enhanced stability of highly fluorinated proteins, Proc. Natl. Acad. Sci. Unit. States Am. 109 (2012) 4810–4815.
[34] J. Oleksyszyn, R. Tyka, P. Mastalerz, P. Direct synthesis of 1- aminoalkanephosphonic and 1-aminoalkanephosphinic acids from phosphorus trichloride or dichlorophosphines, Synthesis (1978) 479–480.
[35] J. Oleksyszyn, M. Soroka, J. Rachoń, Phosphorus analogs of amino-acids and pepetides. 2. Phosphoanalogs and phosphinanalogs of ccycloleucin, Chimia 32 (1978) 253–255.
[36] M. Soroka, Comments on the synthesis of aminomethylphosphonic acid, Synthesis (1989) 547–548.
[37] F. Durst, The correlation of phenylalanine ammonia-lyase and cinnamic acid-hydroxylase activity changes in Jerusalem artichoke tuber tissues, Planta 132 (1976) 221–227.
[38] E.A. Havir, K.R. Hanson, L-phenylalanine ammonia-lyase (potato tubers), Meth. Enzymol. 17 (1970) 575–581.
[39] H. Ritter, G.E. Schulz, Structural basis for the entrance into the phenylpropanoid metabolism catalyzed by phenylalanine ammonia-lyase, Plant Cell 16 (2004) 3426–3436.
[40] M. Salwiczek, E.K. Nyakatura, U.I.M. Gerling, S. Ye, B. Koksch, Fluorinated amino acids: compatibility with native protein structures and effects on protein–protein interactions, Chem. Soc. Rev. 41 (2012) 2135–2171.