IMMOBILIZED NATIVE AND MUTANT AMMONIA LYASES FOR THE SYNTHESIS OF ALPHA AND BETA PHENYLALANINE ANALOGUES

Substrate specificity of *Pa*PAM was tested with a wide range of aromatic and heteroaromatic (*S*)-α-arylalanines. Electronic and steric effects of substituents at the aromatic ring significantly influenced both catalytic efficiency and the formation of arylacrylates as by-products. It was observed that 3-substituted (*S*)-α-phenylalanines were transformed faster than the 2- or 4-substituted isomers. In order to explain these observations, computational analysis of substrate–*Pa*PAM structural interactions was performed by substrate docking studies.[19] Recently it was shown that recombinant whole cell *E. coli* expressing *Pa*PAM could also produce enantiopure (*S*)-β-arylalanines from (*S*)-α-arylalanines. Worth noting, that *Pa*PAM failed to catalyse the transformation of several 2-substituted (*S*)-α-phenylalanines studied.

The time course of the reaction (Supplementary material) showed that after a relatively short timp period (2-6 hours) an equlibrium state is reached in case of both enzymatic reactions, using *rac*-β-phenylalanines (Fig. 1C) or *rac*-α-phenylalanines (Fig. 1B) as substrates.

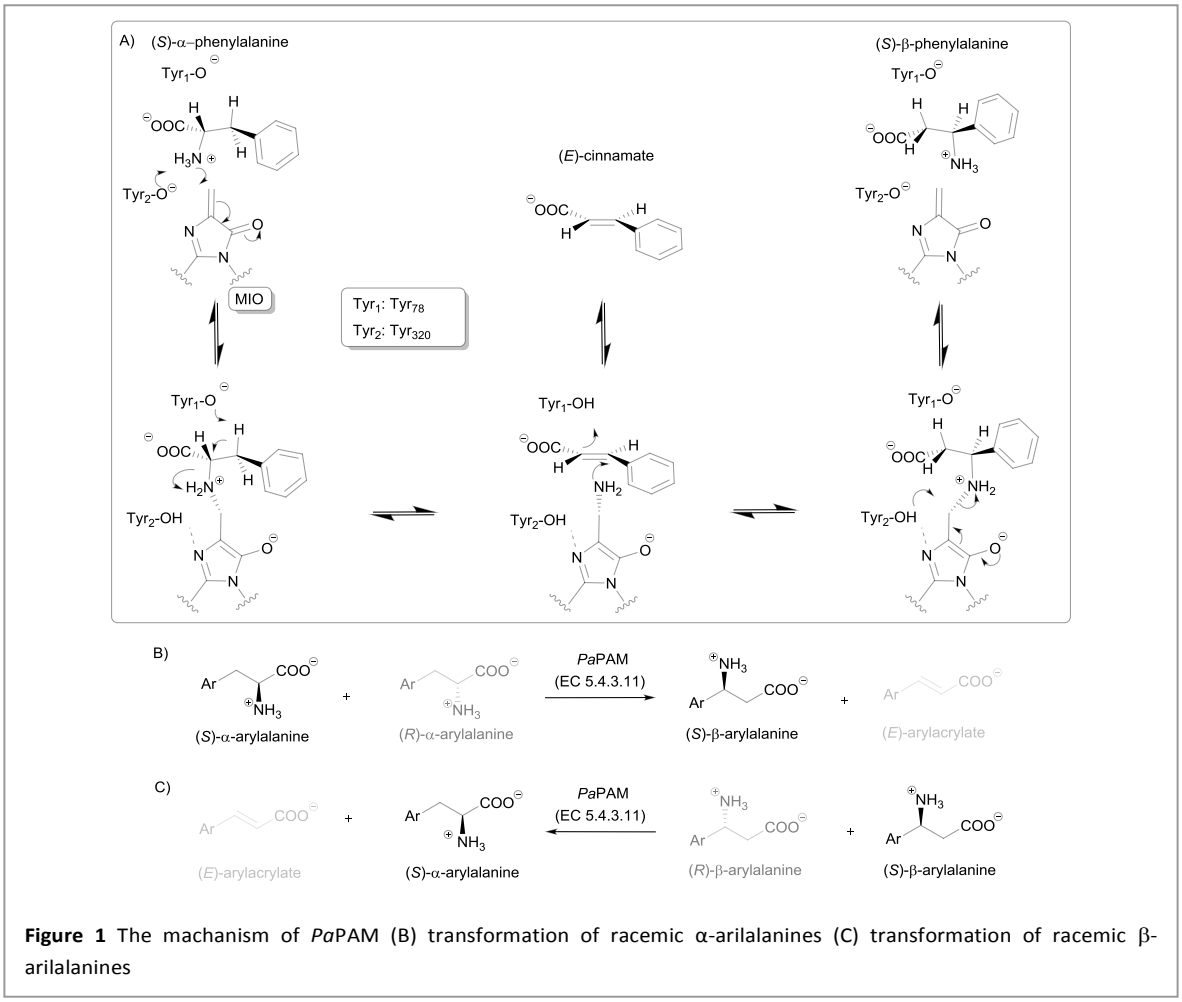


Figure 1 The machanism of *Pa*PAM (B) transformation of racemic α-arilalanines (C) transformation of racemic β-arilalanines

Therefore the results after 20 h show the final product distribution near to the equilibrium state, after longer reaction times further product formation was not observed.

Transformation of (±)-α-arylalanines(*rac*-1a-l) with *Pa*PAM

According to previous results *Pa*PAM failed to catalyse transformation of 2-substituted (*S*)-α-phenylalanines bearing large electron withdrawing substituents. Our study starting from (±)-α-arylalanines *rac*-**1a-l** (Fig. 2A) confirmed the validity of this observation also for the racemic mixtures (Table 1).

In the presence of *Pa*PAM, no product could be detected with (±)-α-(2-chlorophenyl)alanine (*rac*-**1g**) or (±)-α-(2-nitrophenyl)alanine (*rac*-**1j**), while with (±)-α-(2-fluorophenyl)alanine (*rac*-**1d**) being substituted with the smallest halogen atom moderate mutase-activity was observed resulting in the formation of enantiopure (*S*)-β-(2-fluorophenyl)alanine [(*S*)-**2d**].

Conversion of the (±)-3-substituted-α-phenylalanines (*rac*-**1e,h,k**) with *Pa*PAM within 20 h was moderately faster than that of the 2-substituted substrate *rac*-**1d**, but still slower than with (±)-α-phenylalanine (*rac*-**1a**).

Comparison of the conversions of the (±)-4-substituted-α-phenylalanines (*rac*-**1c,f,i,l**) with *Pa*PAM for 20 h indicated that with (±)-4-fluoro- (*rac*-**1f**) and (±)-4-bromo-α-phenylalanine (*rac*-**1c**) about 1.2 times higher conversions were attained than with (±)-4-chloro-(*rac*-**1i**) or (±)-4-nitro-α-phenylalanine (*rac*-**1l**).

Reactions of (±)-α-arylalanine bearing thiophen-2-yl group as a small heteroaromatic moiety (*rac*-**1b**) with *Pa*PAM were also investigated. It was found that the conversion of (±)-α-(thiophen-2-yl)alanine (*rac*-**1b**) was similar to (±)-α-phenylalanine (*rac*-**1a**).

Tabel 1 Composition of the mixture obtained from (±)-α-arylalanines *rac*-**2a-l** with *Pa*PAM (after 20 hours)

Substrate(Ar)	x_1	$x_{(S)\text{-}2}$[a]	x_3
rac-**1a**(phenyl)	0.66	0.28	0.04
rac-**1b** (tiophen-2-yl)	0.74	0.15	0.11
rac-**1c**(4-bromophenyl)	0.82	0.18	[c]
rac-**1d** (2-fluorophenyl)	0.8	0.07	0.13
rac-**1e**(3-fluorophenyl)	0.77	0.19	0.04
rac-**1f** (4-fluorophenyl)	0.77	0.20	0.03
rac-**1g**(2-chlorophenyl)	1.00[b]	[b]	[b]
rac-**1h** (3-chlorophenyl)	0.79	0.2	0.02
rac-**1i** (4-chlorophenyl)	0.94	0.06	[c]
rac-**1j** (2-nitrophenyl)	1.00[b]	[b]	[b]
rac-**1k** (3-nitrophenyl)	0.78	0.22	[c]
rac-**1l** (4-nitrophenyl)	0.94	0.06	[c]

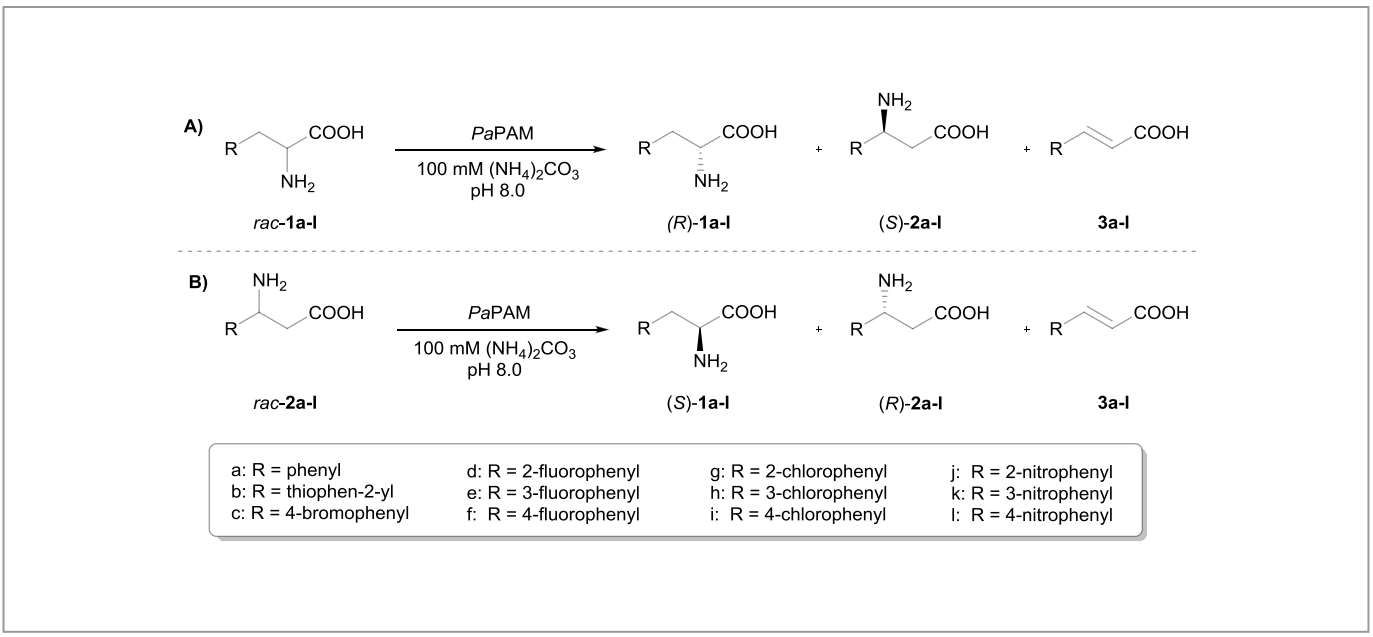


Figure 2. Transformation of (±)-α arilalanines *rac*-**1a-l(A) and** (±)-β-arilalanines *rac*-**2a-l (B)** mediated by *Pa*PAM

In an earlier study with (*S*)-**1a**, negligible ammonia-lyase activity was predicted for *Pa*PAM at low temperatures (under 30 °C).7 In another study[19] and also in our present one, however, formation of significant amount of (*E*)-arylacrylates was observed in many cases (**3a,b,d,e,f,h,l** in Table 1) indicating substantial lyase-activity of *Pa*PAM. Note that with
(±)-4-bromo- (*rac*-**1c**), (±)-4-chloro (*rac*-**1i**) and (±)-3-nitro-α-phenylalanine (*rac*-**1k**) as substrates no such activity was observed. Relatively high amounts of arylacrylates were formed in reactions with substrates bearing the smallest aromatic moieties i.e. with (±)-α-phenylalanine (*rac*-**1a**), (±)-α-(thiophen-2-yl)alanine (*rac*-**1b**) and (±)-2-fluoro-α-phenylalanine (*rac*-**1d**).

Results with the (±)-α-arylalanines (*rac*-**1a-l**) demonstrated that mutase and/or lyase activity of *Pa*PAM was much affected by the nature of the aromatic moiety of the substrates. Importantly, high enantiopurity of the products [>98% *ee* for (*S*)-**2a-f,h,i,k,l**] indicated high enantioselectivity of the *Pa*PAM-catalysed isomerization in the α→β-direction. The observed high stereoselectivity of the *Pa*PAM-catalysed isomerization of the (*S*)-α- and (*S*)-β-arylalanines was a major advantage compared to the incomplete stereoselectivity of *Sm*PAM-catalysed isomerizations.

Transformation of (±)-β-arylalanines (*rac*-2a-l) with *Pa*PAM

In order to explore the potential of kinetic resolutions of (±)-α- and β-arylalanines for the preparation of antipodal products, we extended our study to the reactions of (±)-β-arylalanines *rac*-**2a-l** (Fig. 2B and Table 2). The similar time course profiles of the product formation in *Pa*PAM catalyzed reactions from (*S*)-β-phenylalanine and *rac*-β-phenylalanine (Supplementary material) supported that the unreactive (*R*)-β-phenylalanine did not act as a significant inhibitor. In contrast to the (±)-2-substituted α-phenylalanines with large substituents (*rac*-**1g,j**), which were apparently not accepted as substrates by *Pa*PAM, all the (±)-2-substituted β-phenylalanines in the present study (*rac*-**1d,g,j**) were smoothly transformed. On the other hand, sluggish or no conversion was observed with *Pa*PAM using as substrates (±)-3- and (±)-4-substituted β-phenylalanines bearing bulky electron withdrawing substituents (*rac*-**2c,h,i,k,l**).

The conversions of (±)-β-(thiophen-2-yl)alanine (*rac*-**2b**) and (±)-3-fluoro-β-phenylalanine (*rac*-**2e**) with *Pa*PAM were higher than that of (±)-β-phenylalanine (*rac*-

2a). The (±)-4-fluoro-β-phenylalanine (*rac*-**2f**) was converted similarly as (±)-β-phenylalanine (*rac*-**2a**), while (±)-4-bromo-β-phenylalanine (*rac*-**2c**) was transformed to the α-isomer (*S*)-**1c** and a small amount of 4-bromocinnamate (**3c**) at significantly lower conversion than *rac*-**2a**. In all cases, except for (±)-2-nitro-β-phenylalanine (*rac*-**2j**), reactions catalysed by *Pa*PAM proceeded with the formation of the enantiopure (*S*)-α-isomer [(*S*)-**3a-g**] and some arylacrylate (**3a-g**). Transformation of (±)-2-nitro-β-phenylalanine (*rac*-**2j**) was an exception owing to the absence of the by-product (**3j**) and incomplete stereoselectivity ($ee_{(S)\text{-}\mathbf{1j}}$= 92%).

Table 2 Compozition of the mixture obtained from (±)-β-arylalannes *rac*-**2a-l** with *Pa*PAM (after 20 h)

Substrat (Ar)	x_2	$x_{(S)\text{-}1}$[a]	x_3
rac-**2a** (phenyl)	0.70	0.29	0.01
rac-**2b** (tiophen-2-yl)	0.73	0.22	0.04
rac-**2c** (4-bromophenyl)	0.91	0.08	0.01
rac-**2d** (2-fluorophenyl)	0.63	0.35	0.02
rac-**2e** (3-fluorophenyl)	0.67	0.25	0.08
rac-**2f** (4-fluorophenyl)	0.76	0.23	0.01
rac-**2g** (2-chlorophenyl)	0.54	0.40	0.06
rac-**2h** (3- chlorophenyl)	1.00[b]	[b]	[b]
rac-**2i** (4- chlorophenyl)	1.00[b]	[b]	[b]
rac-**2j** (2-nitrophenyl)	0.85	0.15[c]	[d]
rac-**2k** (3-nitrophenyl)	1.00[b]	[b]	[b]
rac-**2l** (4-nitrophenyl)	1.00[b]	[b]	[b]

x_2, $x_{(S)\text{-}1}$ and x_3 represent the relative molar fractions of the reaction components as determined by ^{1}H-, ^{19}F-NMR measurements

Table 3 Composition of the reaction mixtures obtained from (±)-α- or (±)-β-(thiophen-2-yl)alanine (*rac*-**1b** or *rac*-**2b**) with *Pa*PAM at various ammonium carbonate concentrations (after 20 h)

Substrate	$c_{(NH4)2CO3}$ (mM)	x_{1b}	x_{2b}	x_{3b}
rac-**1b**	50	0.68	0.17	0.15
rac-**1b**	100	0.68	0.17	0.15
rac-**1b**	200	0.72	0.16	0.12
rac-**1b**	1000	0.76	0.12	0.12
rac-**2b**	50	0.19	0.76	0.05
rac-**2b**	100	0.27	0.69	0.04
rac-**2b**	200	0.23	0.75	0.02
rac-**2b**	1000	0.13	0.84	0.03

x_{1b}, x_{2b} and x_{3b} represent the relative molar fractions of the reaction components as determined by ^{1}H-NMR measurements

Effects of pH and ammonia concentration on the *Pa*PAM-catalysed isomerization of (±)-α- and β-(thiophen-2-yl)alanine (*rac*-1b and *rac*-2b)c

Prompted by the fact that conversions from (±)-α- and (±)-β-(thiophen-2-yl)alanine (*rac*-**1b** and *rac*-**2b**) with *Pa*PAM were similar to those from the natural substrates i.e. (±)-α- and β-phenylalanine (*rac*-**1a** and *rac*-**2a**) but more of the by-product [(*E*)-3-(thiophen-2-yl)acrylate, **3b**] was formed (Table 1 and Table 2), transformations of these substrates were studied in more detail by varying pH or ammonia concentration. An alteration of pH of the buffer solution in the range of 7–9 [at 100 mM $(NH_4)_2CO_3$] was indifferent to conversion (data not shown), unlike changing the $(NH_4)_2CO_3$ concentration in the range of 50–1000 mM (at pH 8) which significantly influenced product compositions (Table 3).

Analysis of the *Pa*PAM-catalysed reactions of (±)-α-(thiophen-2-yl)alanine (*rac*-**1b**) at various $(NH_4)_2CO_3$ concentrations with 20 h incubation showed that increasing $(NH_4)_2CO_3$ concentration above 100 mM resulted in decreasing conversion to both (*S*)-β-(thiophen-2-yl)alanine (*S*)-**2b** and the elimination product **3b**. At 1000 mM$(NH_4)_2CO_3$ concentration both (*S*)-β-(thiophen-2-yl)alanine (*S*)-**2b** and arylacrylate**3b** formation dropped to 12%. Interestingly, when starting from (±)-β-(thiophen-2-yl)alanine (*rac*-**2b**), the best conversion to (*S*)-α-(thiophen-2-yl)alanine (*S*)-**1b** (27%) was achieved at 100 mM buffer concentration. At higher $(NH_4)_2CO_3$ concentrations smaller conversions were observed.

Study of the dependence of the *Pa*PAM-catalysed reactions of (±)-α- and β-(thiophen-2-yl)alanine (*rac*-**1b** and *rac*-**2b**, respectively) on $(NH_4)_2CO_3$ concentration indicated that elevated ammonia concentrations lowered both the rate of isomerization and ammonia elimination as side reaction in both directions of the reaction.

Computational modelling of *Pa*PAM-catalysed isomerizations

To rationalize the effect of substituents of aromatic and heteroaromatic (±)-α- and β-arylalanines (*rac*-**1a-l** and *rac*-**2a-l**) on *Pa*PAM-catalysed isomerizations computational and statistical analysis was performed based on modelling and comparison of the *N*-MIO states (*S*)-**1a-i,k,l**$_{N\text{-MIO}}$ and (*S*)-**2a-i,k,l**$_{N\text{-MIO}}$ formed from the corresponding (*S*)-α- and (*S*)-β-arylalanines. Because of

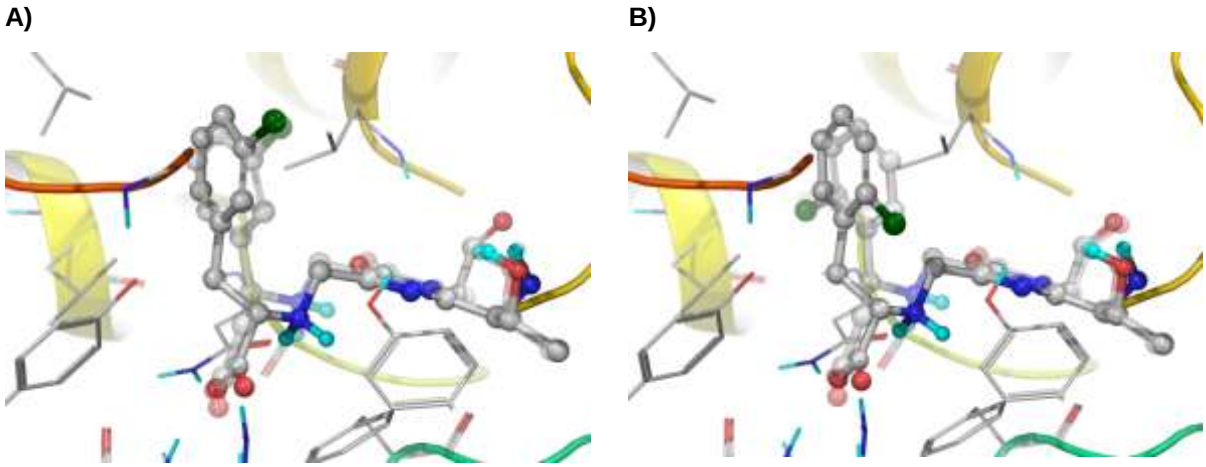


Figure 3 Comparison of the arrangements of regioisomeric *N*-MIO intermediates in their *Pa*PAM-catalysed transformations.
(**A**) *N*-MIO intermediates of (*S*)-α-(3-chlorophenyl)alanine (solid model) and of (*S*)-β-(3-chlorophenyl)alanine (transparent model) [(*S*)-**1h**$_{N\text{-MIO}}$ and (*S*)-**2h**$_{N\text{-MIO}}$]; (**B**) the *N*-MIO intermediates of (*S*)-α-(2-chlorophenyl)alanine (solid model) and (*S*)-β-(2-chlorophenyl)alanine (transparent model) [(*S*)-**1g**$_{N\text{-MIO}}$ and (*S*)-**2g**$_{N\text{-MIO}}$]. Panel **A** depicts a case where the lowest energy conformations of the two regioisomers adopt similar orientations of the aromatic ring. Panel **B** illustrates a case where in the lowest energy conformations of the two regioisomers the aromatic rings occupy different orientations (differing by a flip of 180°). Images were created using PyMOL.[25]

the experimentally observed stereospecificity of isomerizations, in the computations only the (*S*)-enantiomers were considered. Due to the mixed mechanism indicated by the incomplete enantioselectivity in the *Pa*PAM-catalysed reaction of *rac*-**2j**, data calculated for the reactions of (*S*)-α- and (*S*)-β-2-nitrophenylalanine [(*S*)-**1j** and (*S*)-**2j**] were omitted from the comparison of reactivities.

a. Immobilization of *Pa*PAM on MNPs

Into a TRIS buffer solution (3 mL, 0.1 M, pH 8.8) epoxy-MNPs [MagneCat-250GP14 (magnetic nanoparticles functionalized with epoxy groups, diameter 250nm)n 108 mg] and were dispersed by ultrasonication (35 kHz, 20 min). The MNP suspension was added to a solution of *Pa*PAM (3 mg mL^{-1}, în TRIS buffer: 4 mL, 0.1 M, pH 8.8), and the reaction mixture was shaken for 24 h (25 °C, 450 rpm). The enzyme-coated nanoparticles were colected with a magnet and the solution was removed. The MNP preparation was washed with TRIS buffer 3 × 4 mL, 0.1 M, pH 8.8) and ethanol (4 mL). The obtained biocatalyst was dried at room temperature for 2 h. After immobilization a negligible quantity of protein was detected, determind by the Bradford method.

b. Covalent immobilization of *Pa*PAM on $SwCNT_{COOH}$ via GDE based linker

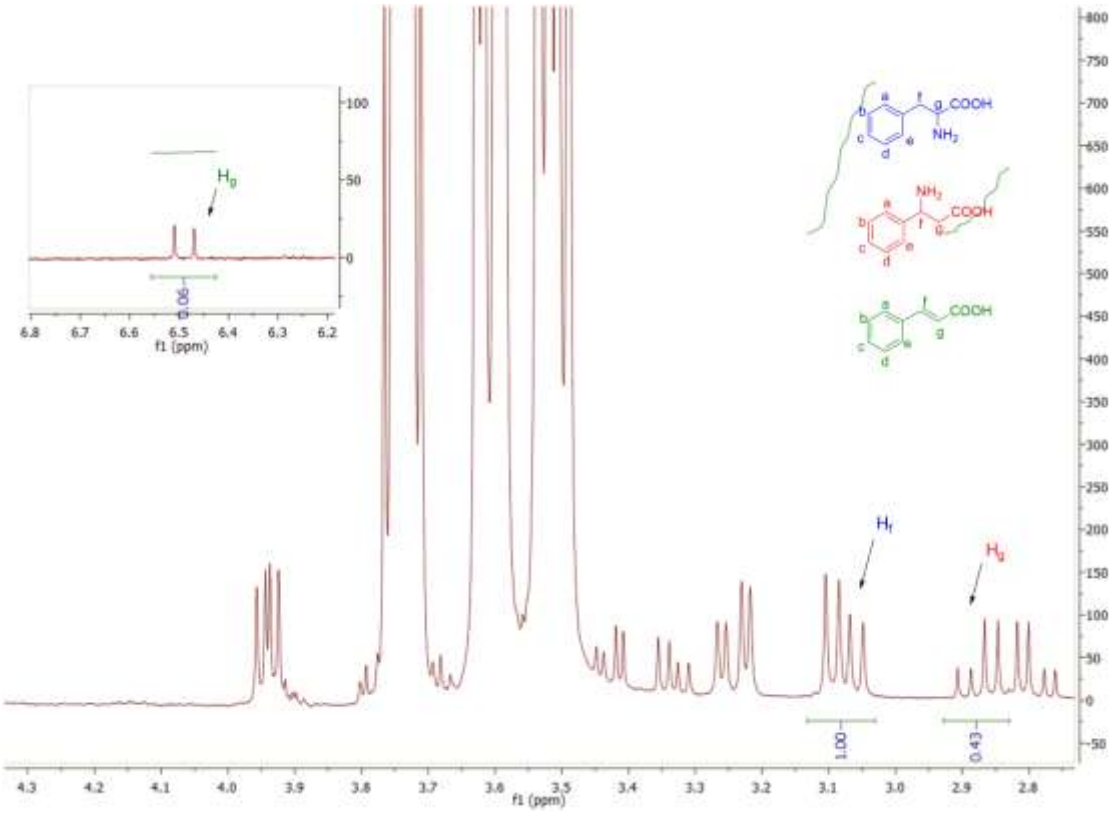
$SwCNT_{COOH}$ (carboxy single walled carbon nanotubes) was incubated with carbonyldiimidazole (CDI) with shaking (at 1350 rpm at room temperature overnight), with occasional sonication to avoid bundled SwCNT formation. After CDI activation, the sample was filtered on membrane filter and then washed with CH_2Cl_2.

To the CDI-activated $SwCNT_{COOH}$, propane-1,3-diamine was added in distilled water and the reaction mixture was shaken (at 1350 rpm at room temperature overnight), with occasional sonication to avoid bundled SwCNT formation. After the propane-1,3-diamine coupling, the sample was filtered on membrane filter and then washed with distilled water.

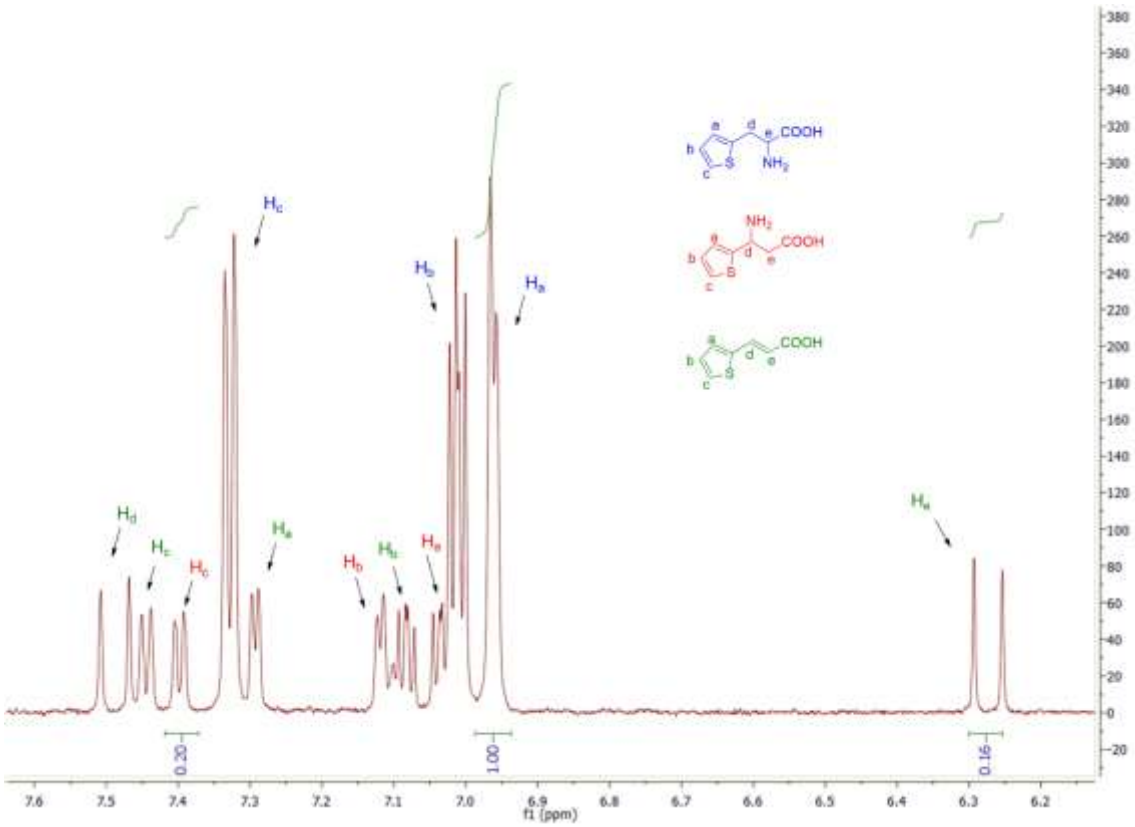
A solution of glycerol diglycidyl ether (GDE)) was added to the propane-1,3-diamine-coupled $SwCNT_{COOH}$, and the reaction mixture was shaken (at 1350 rpm at room temperature overnight), with occasional sonication to avoid bundled SwCNT formation. After incubation the sample was filtered on membrane filter and then washed with CH_2Cl_2.

To the resulted bisepoxide-activated $SwCNT_{COOH}$, PAM was added and the mixture was shaken at room temperature at 1350 rpm, overnight. After the PAM-immobilization, the resulted biocatalyst ($SwCNT_{COOH}$-PAL^{II}) was filtered off on a membrane filter and washed with distilled water. The amount of PAM immobilized on the bisepoxide-activated $SwCNT_{COOH}$ was determined from the total mass of the enzyme in the solution before the immobilization and in the unified filtrates after the immobilization (measured spectrophotometrically using the Bradford method).

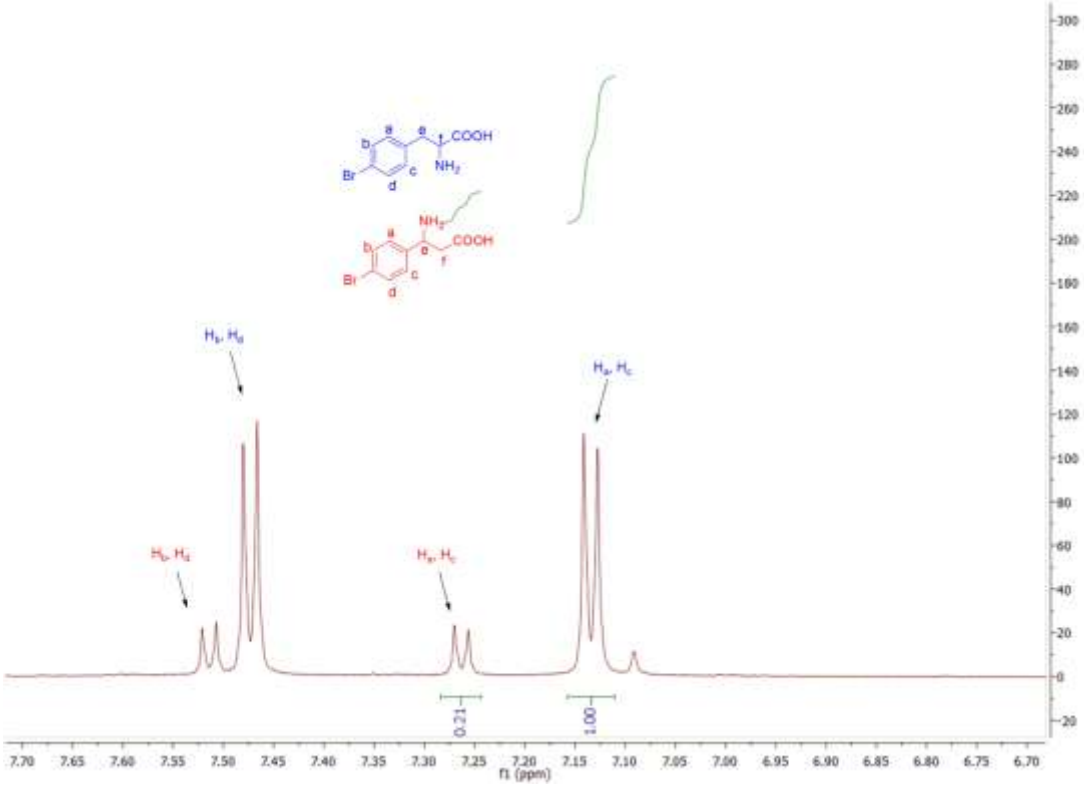
^{1}H- and ^{19}F-NMR spectra of the enzymatic transformations



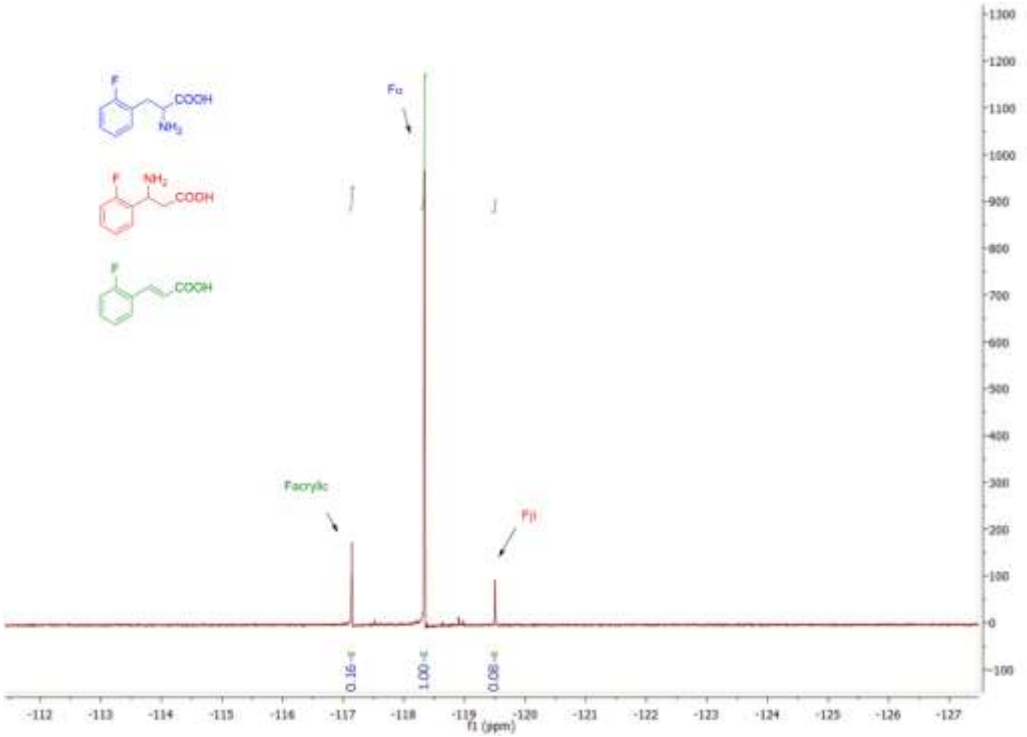
^{1}H-NMR of the products of *Pa*PAM-catalysed transformation of (±)-α-phenylalanine *rac*-**1a**



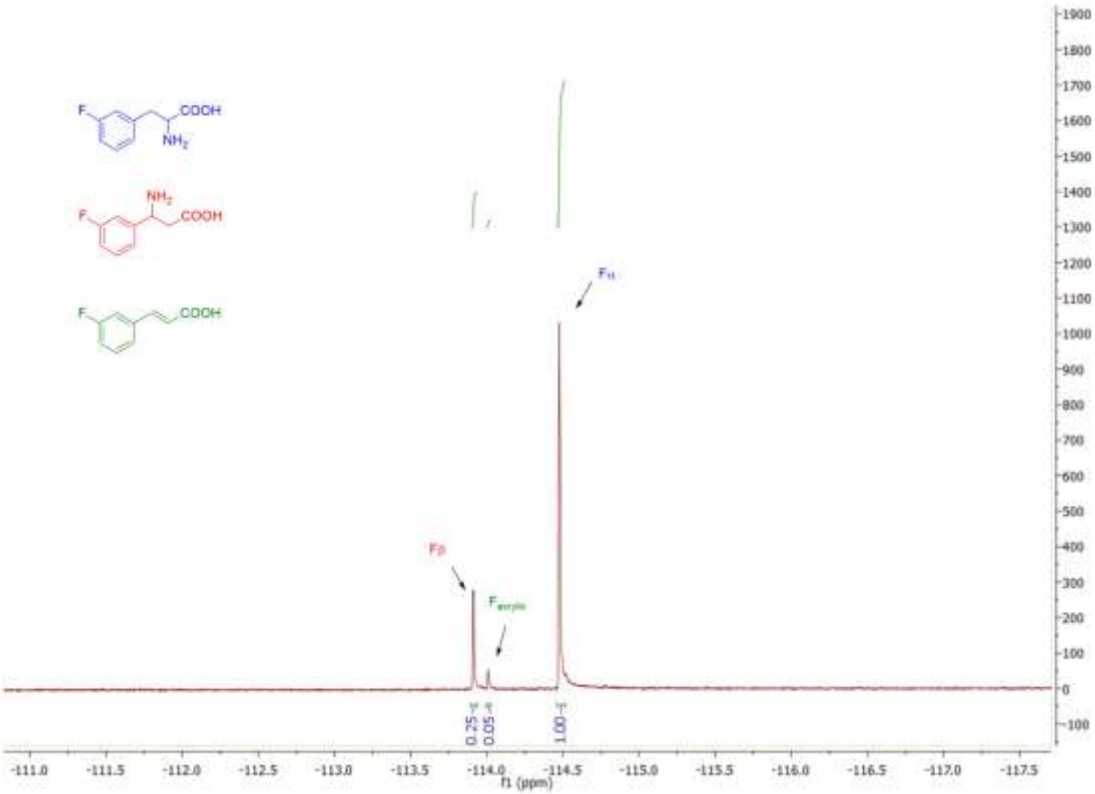
[1]H-NMR of the products of *Pa*PAM-catalysed transformation of (±)-α-(thiophen-2-yl)alanine *rac*-**1b**



[1]H-NMR of the products of *Pa*PAM-catalysed transformation of(±)-4-bromo-α-phenylalanine *rac*-**1c**



^{19}F-NMR of the products of *Pa*PAM-catalysed transformation of (±)-2-fluoro-α-phenylalanine *rac*-**1d**



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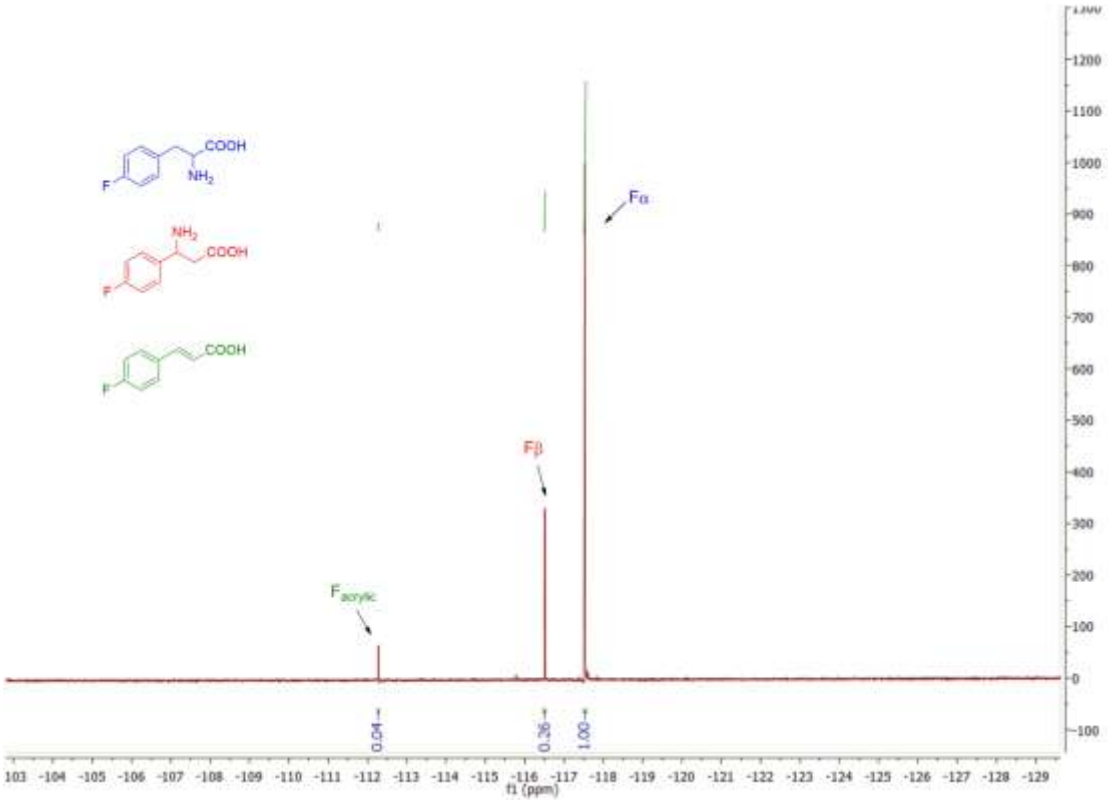

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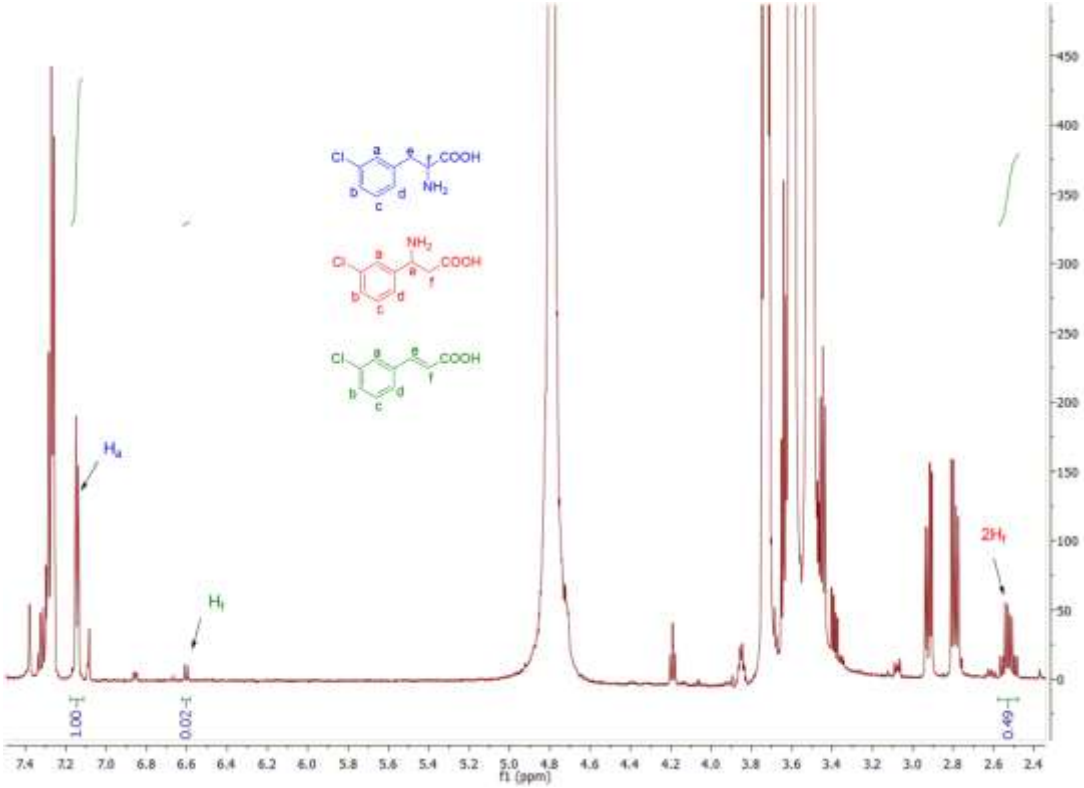

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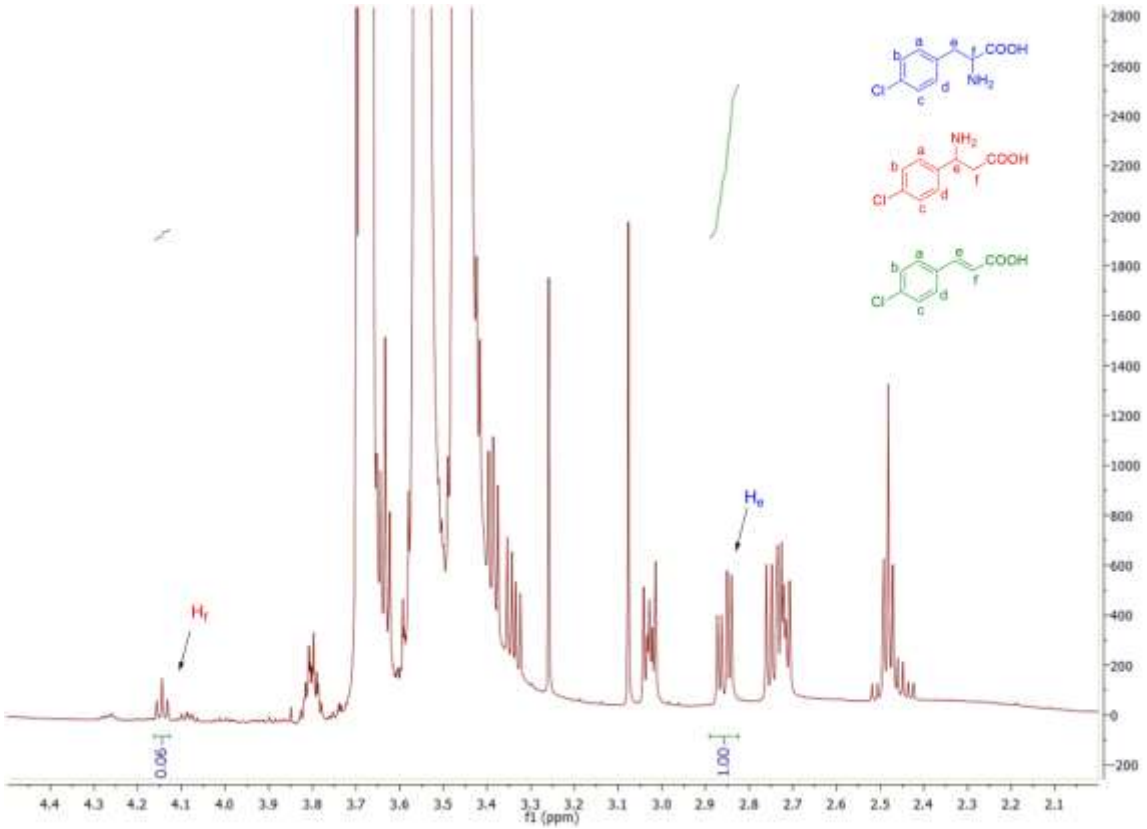

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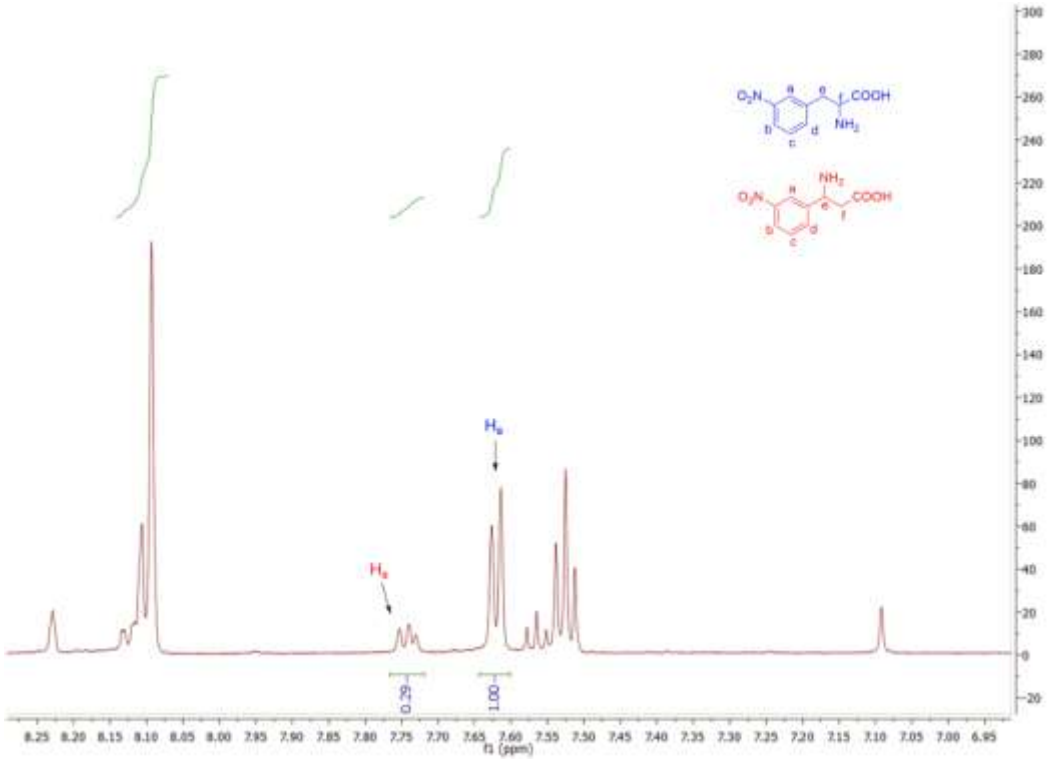
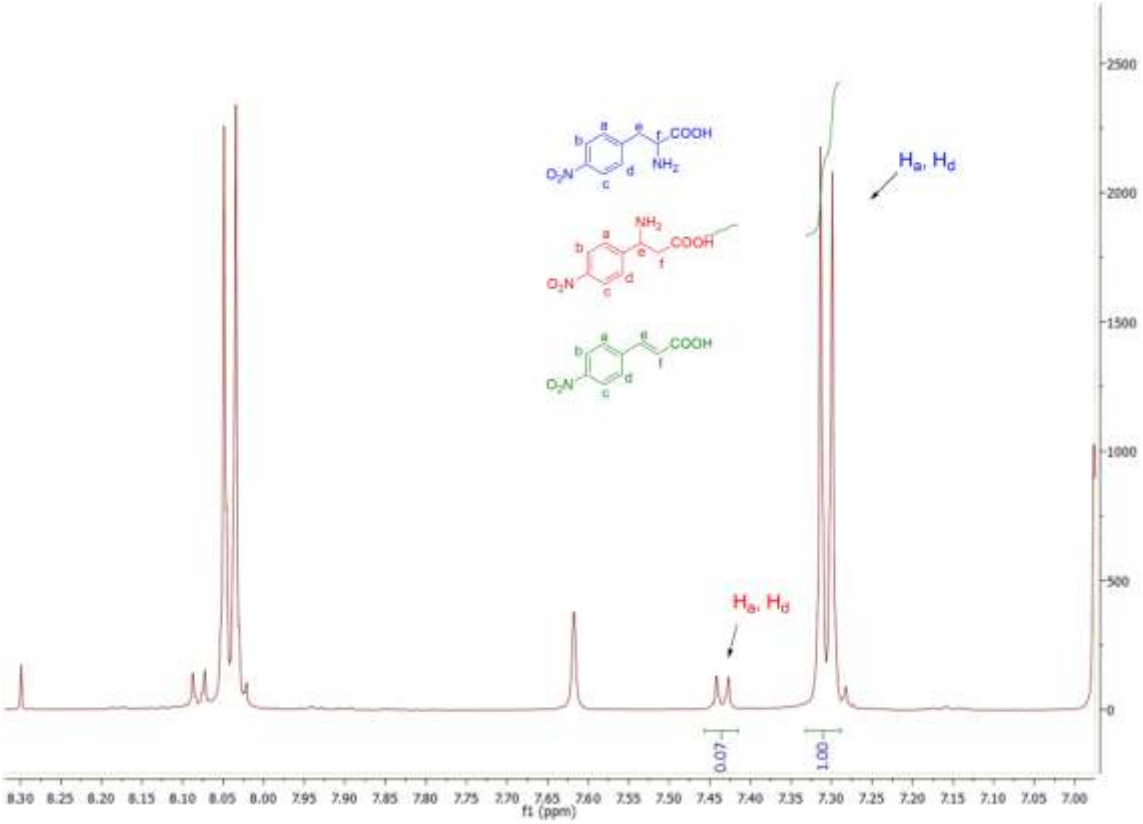
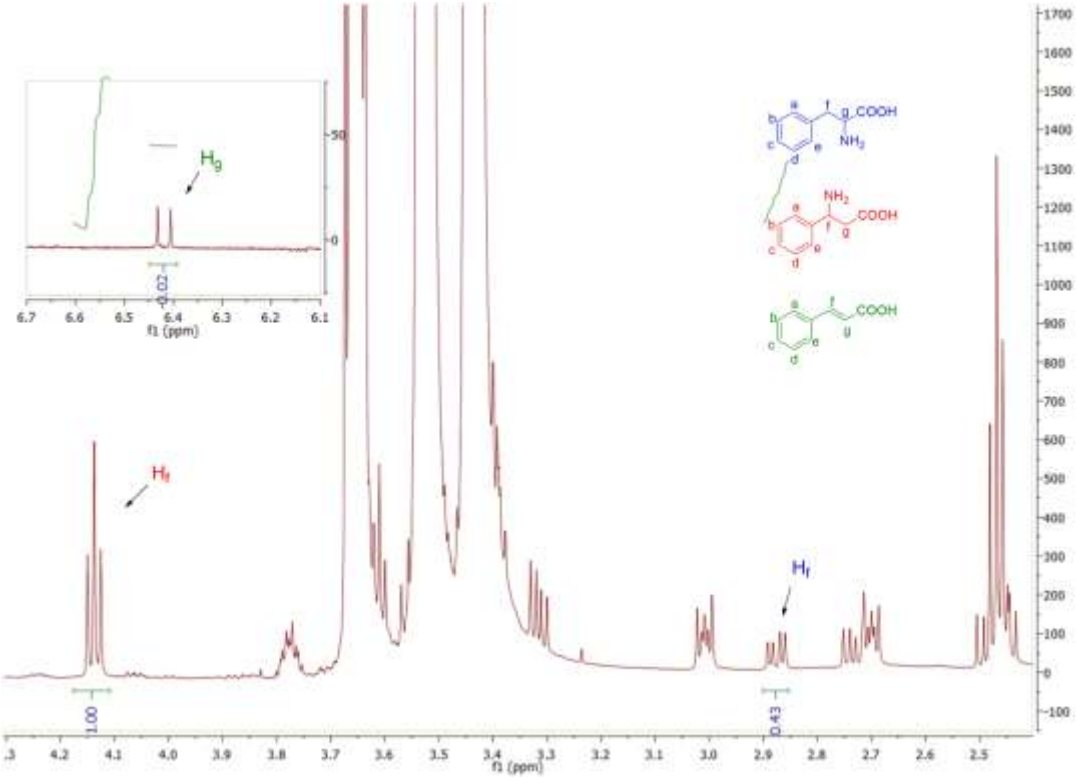


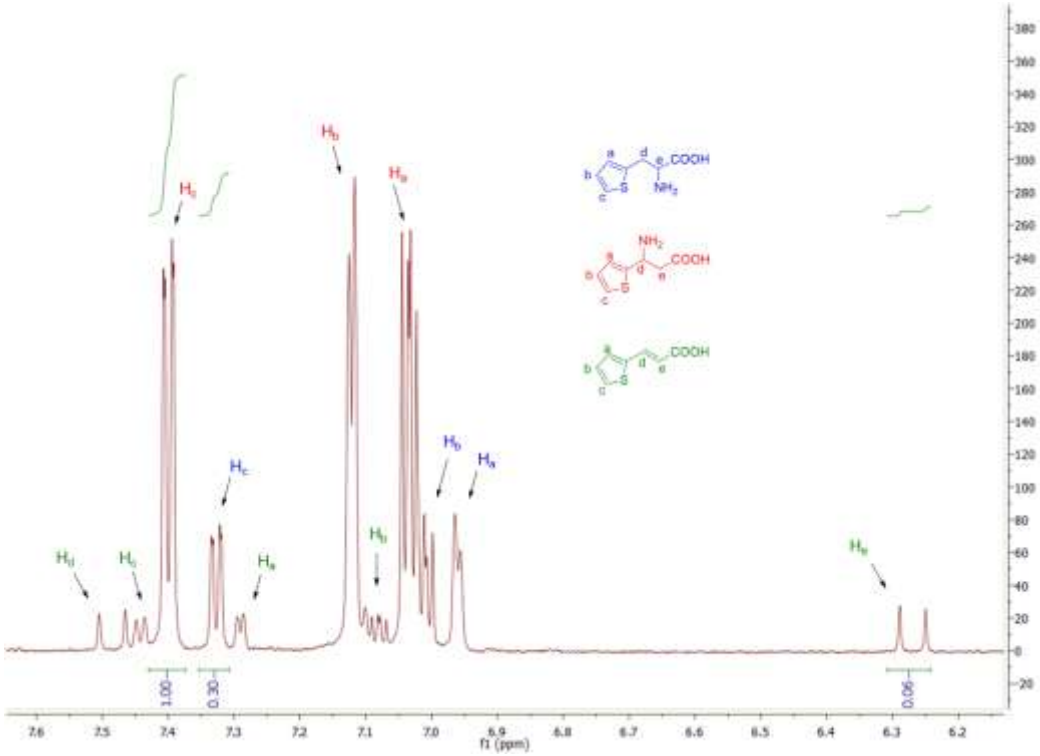
^{1}H-NMR of the products of *Pa*PAM-catalysed transformation of (±)-3-nitro-α-phenylalanine *rac*-**1k**



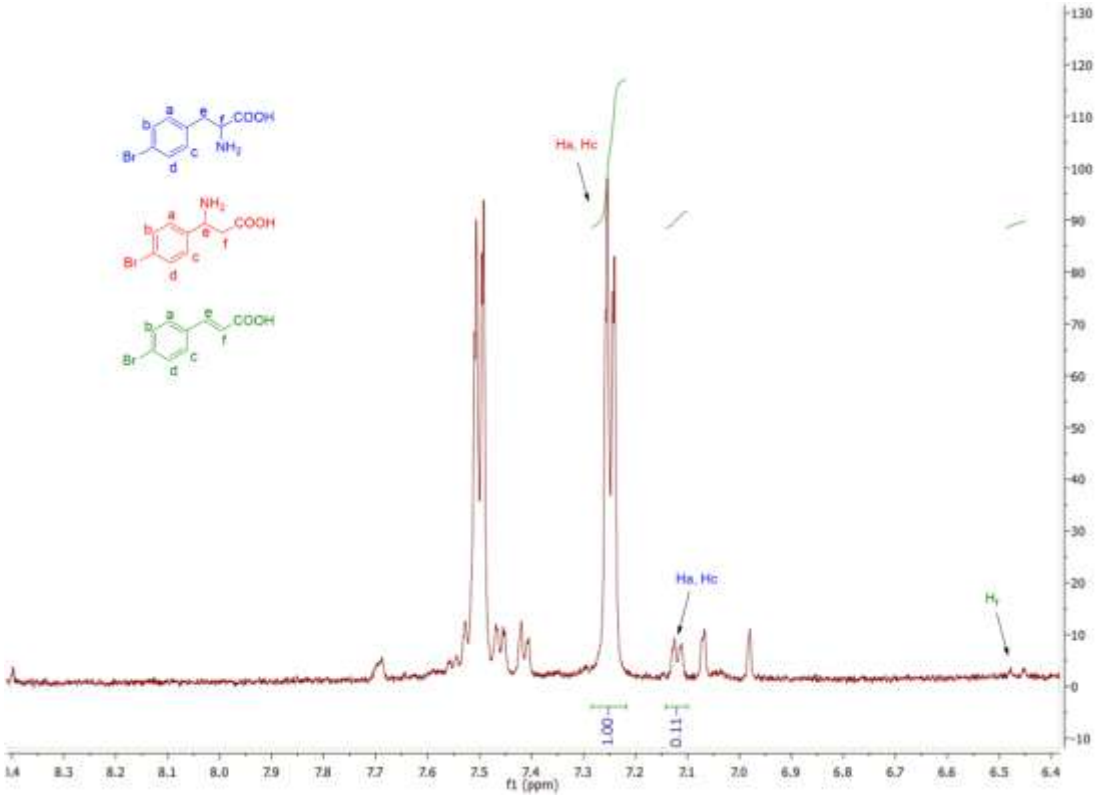
[1]H-NMR of the products of *Pa*PAM-catalysed transformation of (±)-4-nitro-α-phenylalanine *rac*-**1l**



[1]H-NMR of the products of *Pa*PAM-catalysed transformation of (±)-β-phenylalanine *rac*-**2a**



[1]H-NMR of the products of *Pa*PAM-catalysed transformation of (±)-β-(thiophen-2-yl)alanine *rac*-**2b**



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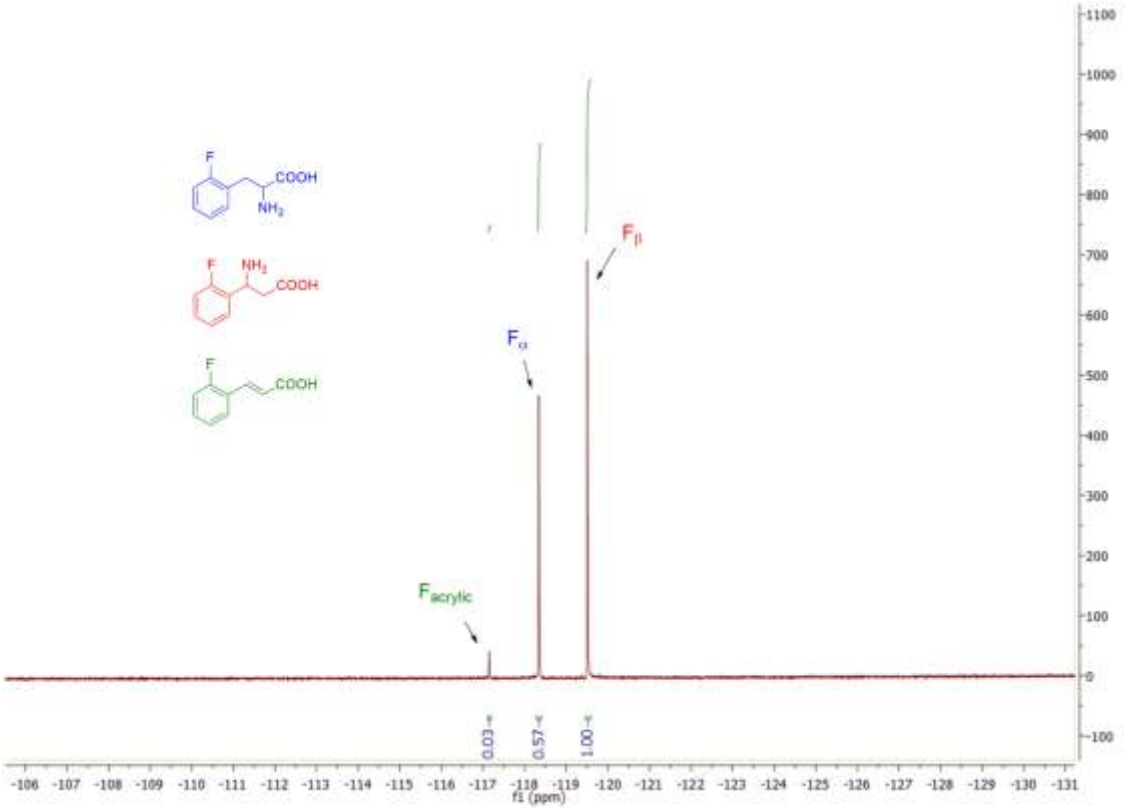

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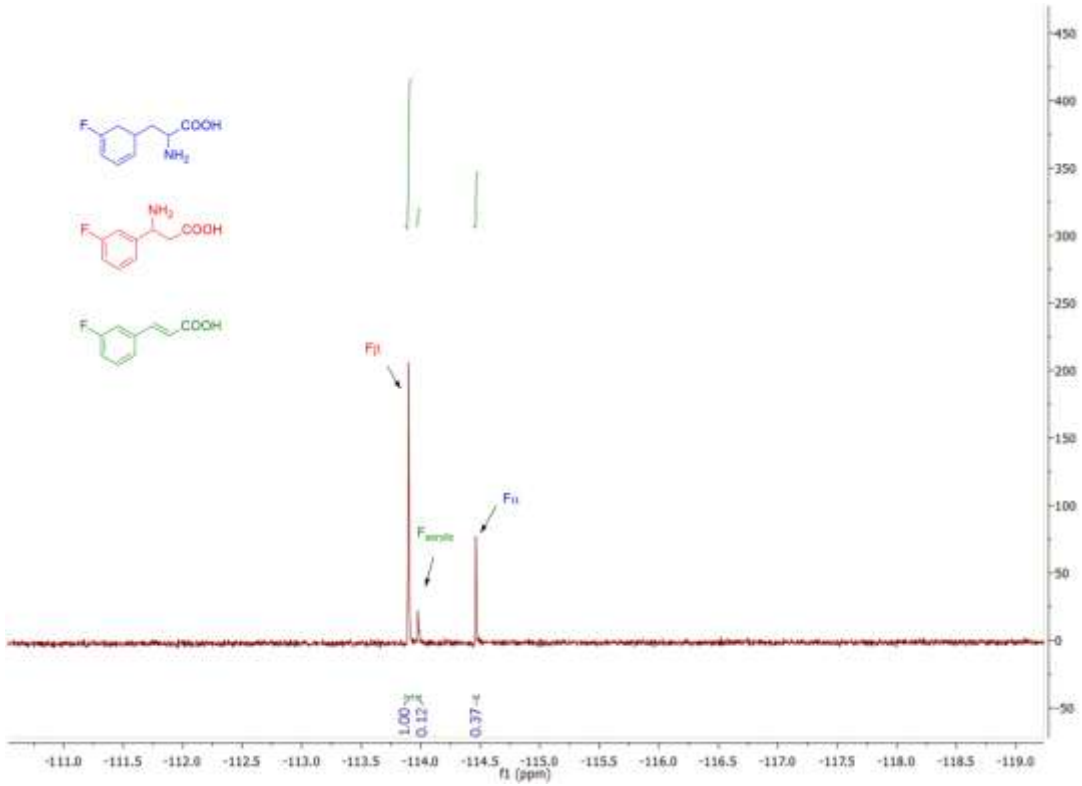
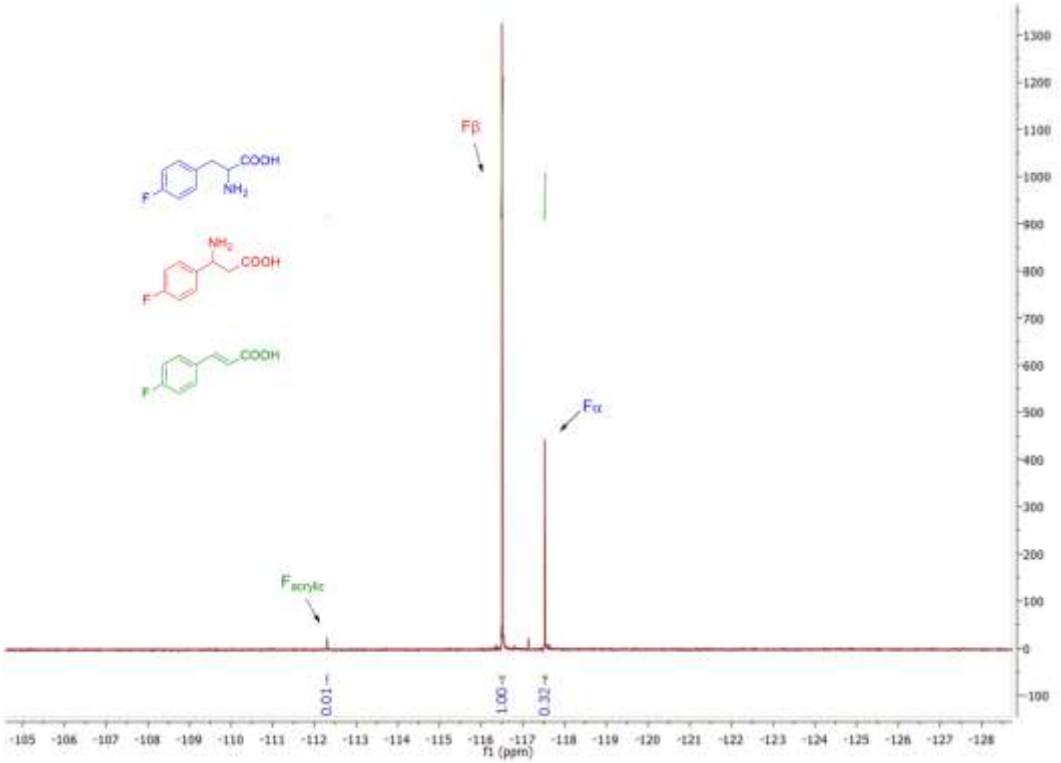
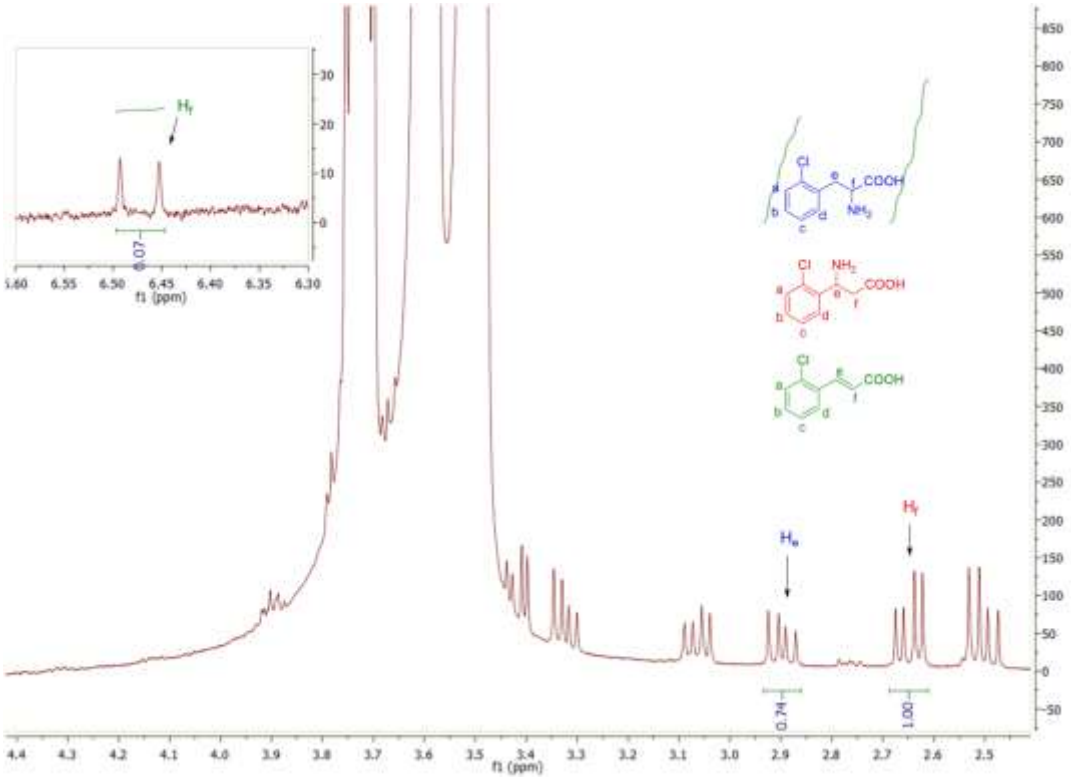


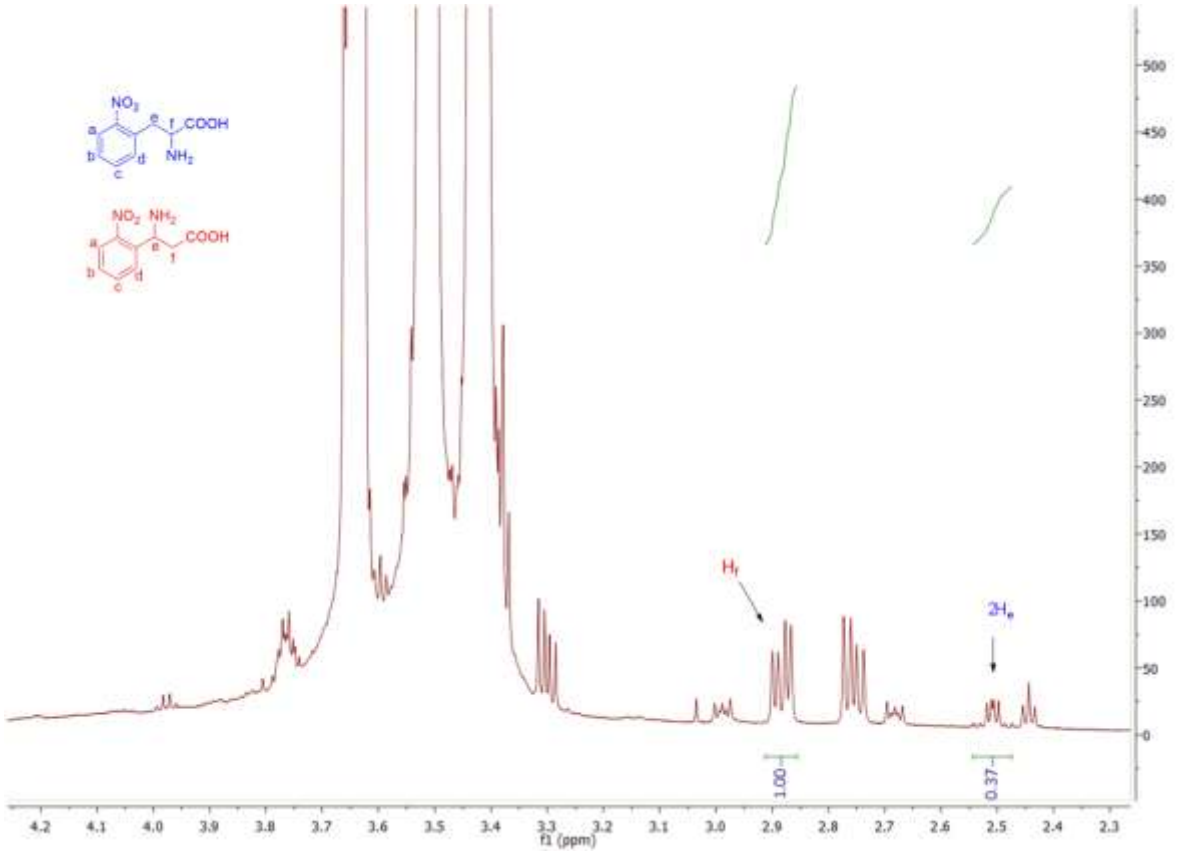
^{19}F-NMR of the products of *Pa*PAM-catalysed transformation of (±)-3-fluoro-β-phenylalanine *rac*-**2e**



[19]F-NMR of the products of *Pa*PAM-catalysed transformation of (±)-4-fluoro-β-phenylalanine *rac*-**2f**



[1]H-NMR of *Pa*PAM-catalysed transformation of (±)-2-chloro-β-phenylalanine *rac*-**2g**



^{1}H-NMR of *Pa*PAM-catalysed transformation of (±)-2-nitro-β-phenylalanine *rac*-**2j**

2. HPLC monitoring of the enzymatic reactions

2.1 Determination of conversion and molar fraction values

In order to determine the conversion and/or molar fractions of the products of *Pa*PAM-catalysed enzymatic transformations, the response factor of each compound was determined by mixtures of known composition of authentic racemic α- and β-amino acids and the corresponding arylacrylate injected onto Gemini NX-C-18 column (150 × 4.6 mm × 5 µm). Mobile phase: A: NH_4OH buffer (0.1 M, pH 9.0) / B: MeOH, flow rate: 0.9 mL/min, measurements performed at 20°C. Reverse phase HPLC analyses were performed on an Agilent 1100 Series system equipped with a G1379A degasser, G1311A quaternary pump, a G1329A autosampler, a G1316A temperature controlled column compartment and a G1315B diode array detector.

Table S1. HPLC conditions and response factors

Aryl moiety in 1,2,3	**Eluent***	**λ**	**Response factor****		
	[% B]	**[nm]**	**2** *vs***1**	**3** *vs***1**	**3***vs***2**
Phenyl	10 to 39 in 12 min	220	1.988	0.161	0.084
Thiophen-2-yl	10 to 39 in 12 min	250	1.146	0.413	0.387

4-Bromophenyl	20 to 54 in 12min	220	1.857	0.372	0.222
2-Fluorophenyl	10 to 39 in 12 min	220	1.249	0.139	0.119
3-Fluorophenyl	10 to 39 in 12min	220	3.039	0.156	0.05
4-Fluorophenyl	10 to 39 in 12 min	220	1.388	0.123	0.095
2-Chlorophenyl	10 to 39 in 12 min	220	–	–	0.386
3-Chlorophenyl	15 to 50 in 15 min	220	0.817	0.265	–
4-Chlorophenyl	15 to 50 in 15 min	220	0.948	0.679	–
2-Nitrophenyl	10 to 50 in 15 min	260	–	–	0.019
3-Nitrophenyl	10 to 50 in 15 min	260	2.238	0.144	–
4-Nitrophenyl	10 to 50 in 15 min	220	1.138	1.228	–

*Eluent **A**: NH_4OH buffer (0.1 M, pH 9.0); **B**: MeOH

** **1**– *rac*-α-arylalanine; **2**– *rac*-β-arylalanine; **3** – arylacrylate

Table S2. Relative molar fractions of the products in the enzymatic reactions of *rac*-β-arylalanines after 20 h

Aryl moiety in 1,2,3	x_2 **[%]**	$x_{(S)\text{-}1}$ **[%]**	x_3 **[%]**
Phenyl	73.9	24.3	1.8
Thiophenyl-2-yl	73.1	24.6	2.3
4-Bromophenyl	92.8	6.2	1
2-Fluorophenyl	54	41.5	4.5
3-Fluorophenyl	74.8	20.3	4.9
4-Fluorophenyl	78.3	20	1.7
2-Chlorophenyl	53.5	44.4	2.5
2-Nitrophenyl	90.8	9.2	0

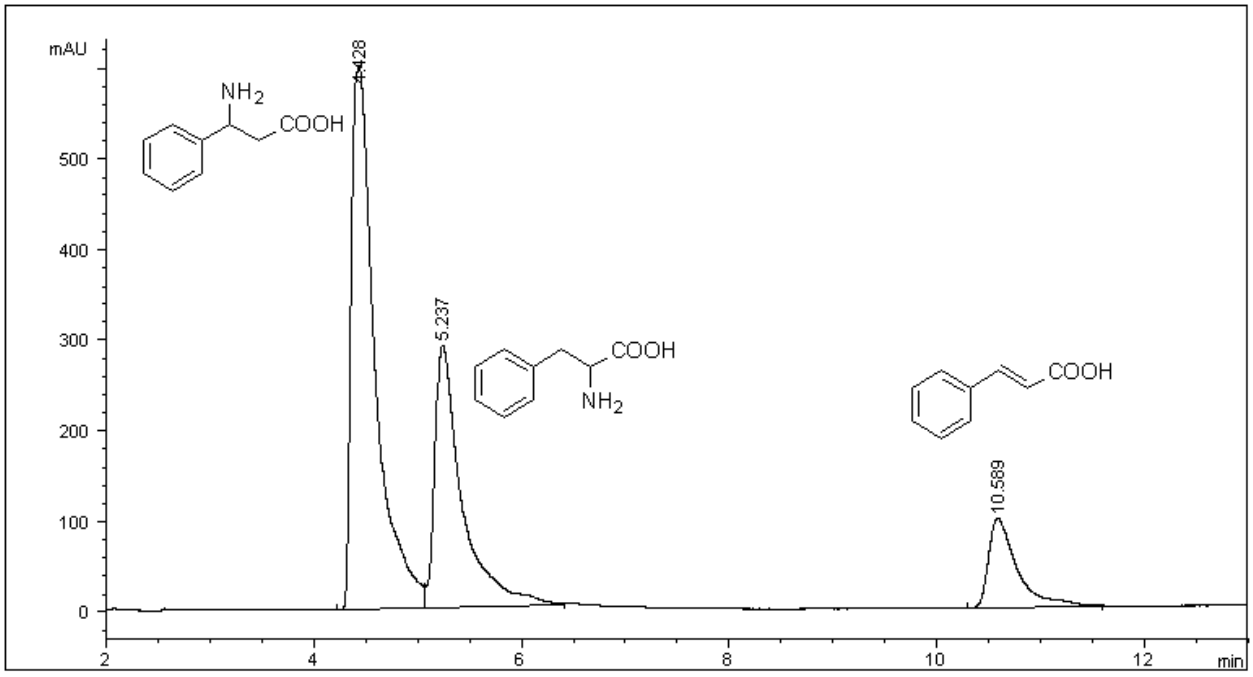
x_2, $x_{(S)\text{-}1}$ and x_3 represent the relative molar fractions of the reaction components as determined by HPLC on Gemini NX-C-18 column

Table S3. Relative molar fractions of the products in the enzymatic reactions of *rac*-α-arylalanines after 20 h

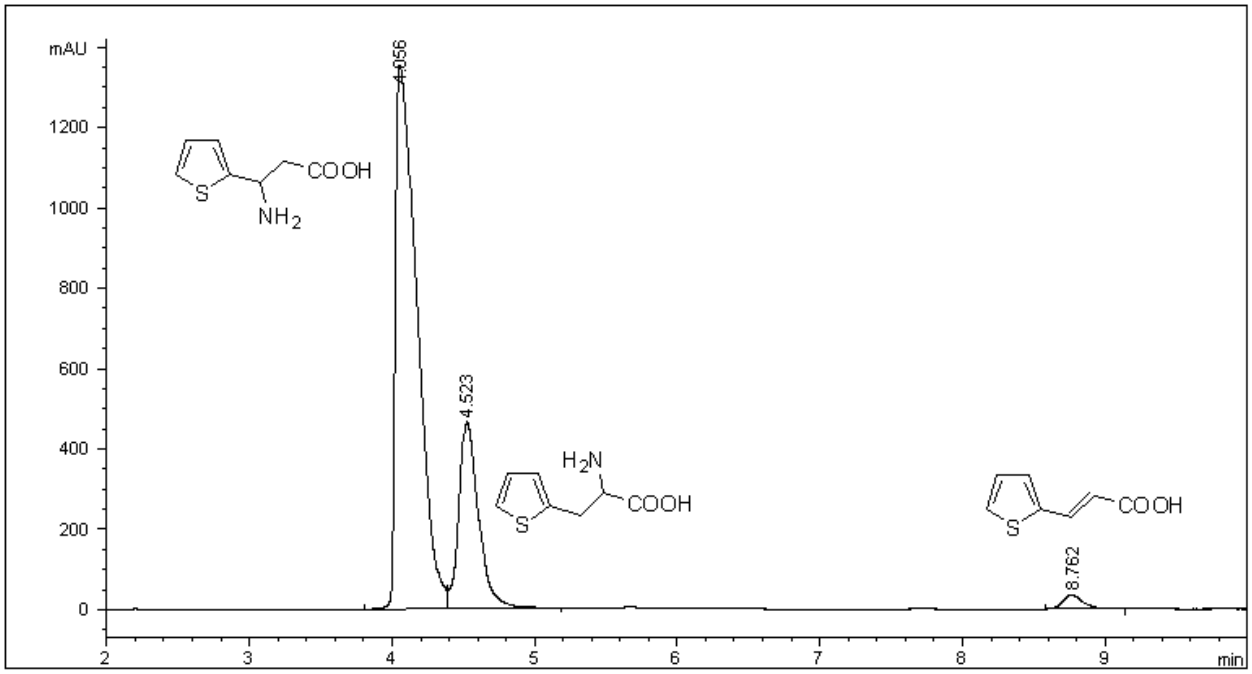
Aryl moiety in 1,2,3	x_2 **[%]**	$x_{(S)\text{-}1}$ **[%]**	x_3 **[%]**
Phenyl	68.3	28.9	2.8
Thiophenyl-2-yl	76.1	17.7	6.2
4-Bromophenyl	82.9	17.1	0
2-Fluorophenyl	88.3	9.3	2.4
3-Fluorophenyl	60.9	37.9	1.2
4-Fluorophenyl	75.5	23.3	1.2
3-Chlorophenyl	82.8	16.0	1.2
4-Chlorophenyl	83.9	16.1	0
3-Nitrophenyl	76.8	23.0	0.2
4-Nitrophenyl	94.3	5.7	0

x_1, $x_{(S)\text{-}2}$ and x_3 represent the relative molar fractions of the reaction components as determined by HPLC on Gemini NX-C-18 column

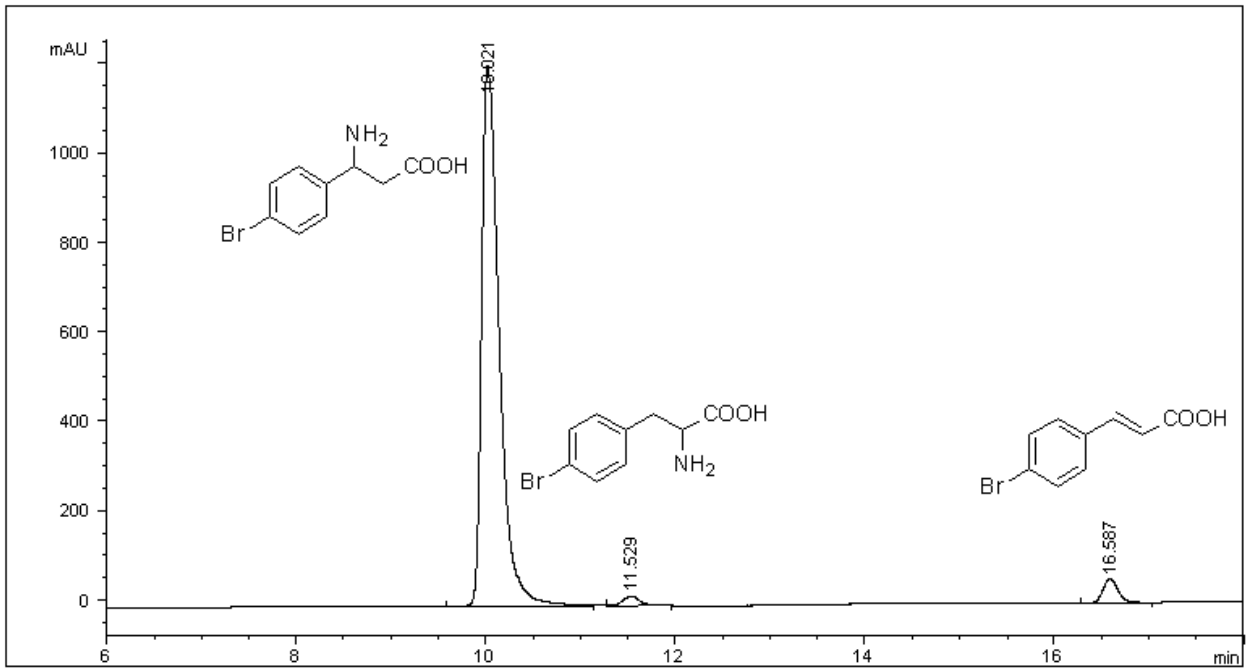
2.2 HPLC analysis of the PaPAM-catalysed reactions after 20 h



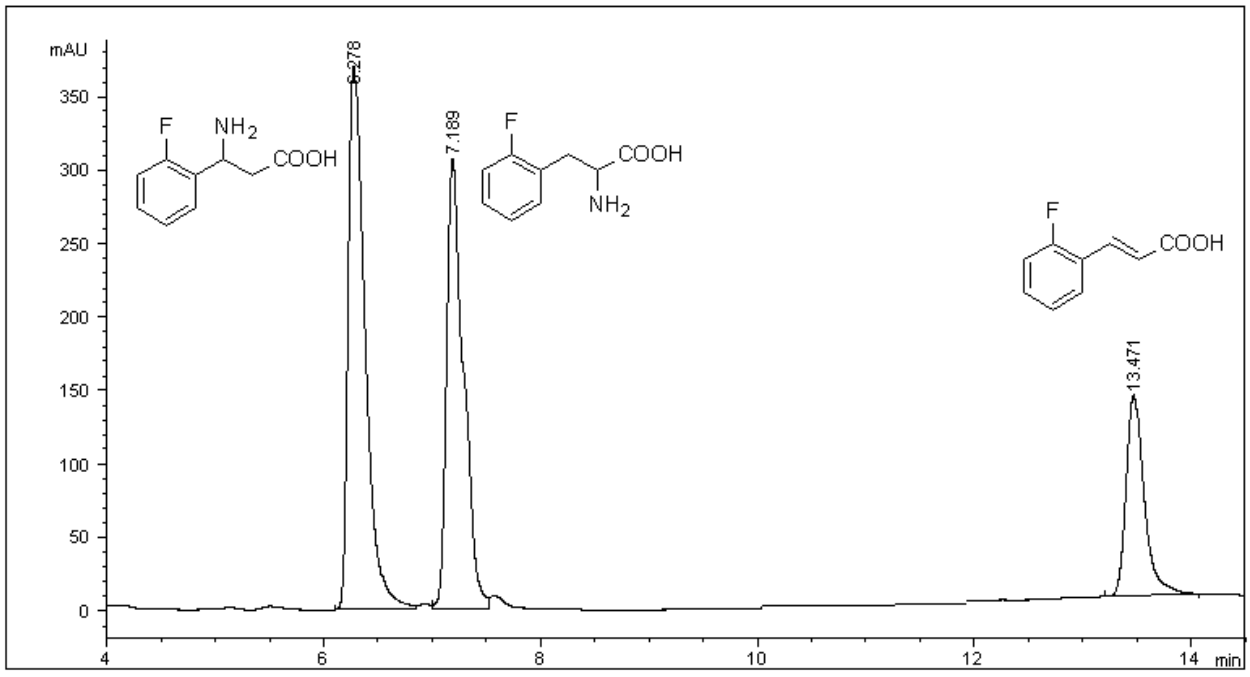
Products from (±)-β-phenylalanine (*rac*-**2a**) by *Pa*PAM after 20 h.



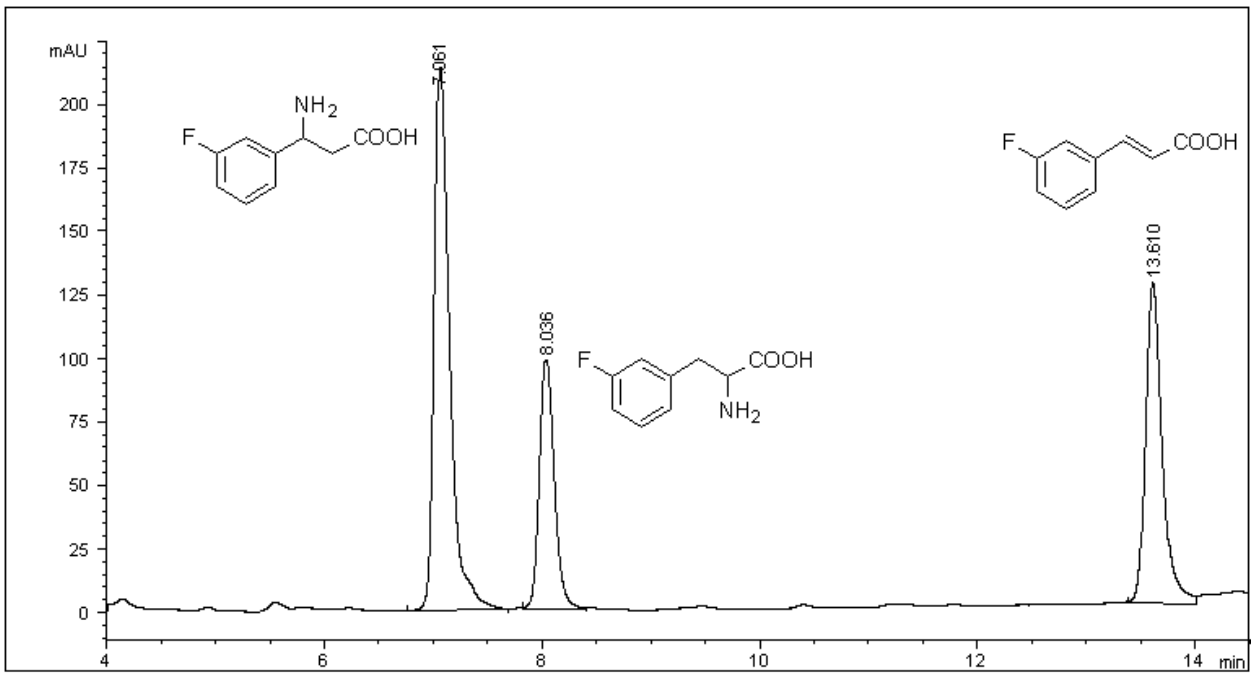
Products from (±)-β-(thiophen-2-yl)alanine (*rac*-**2b**) by *Pa*PAM after 20 h.



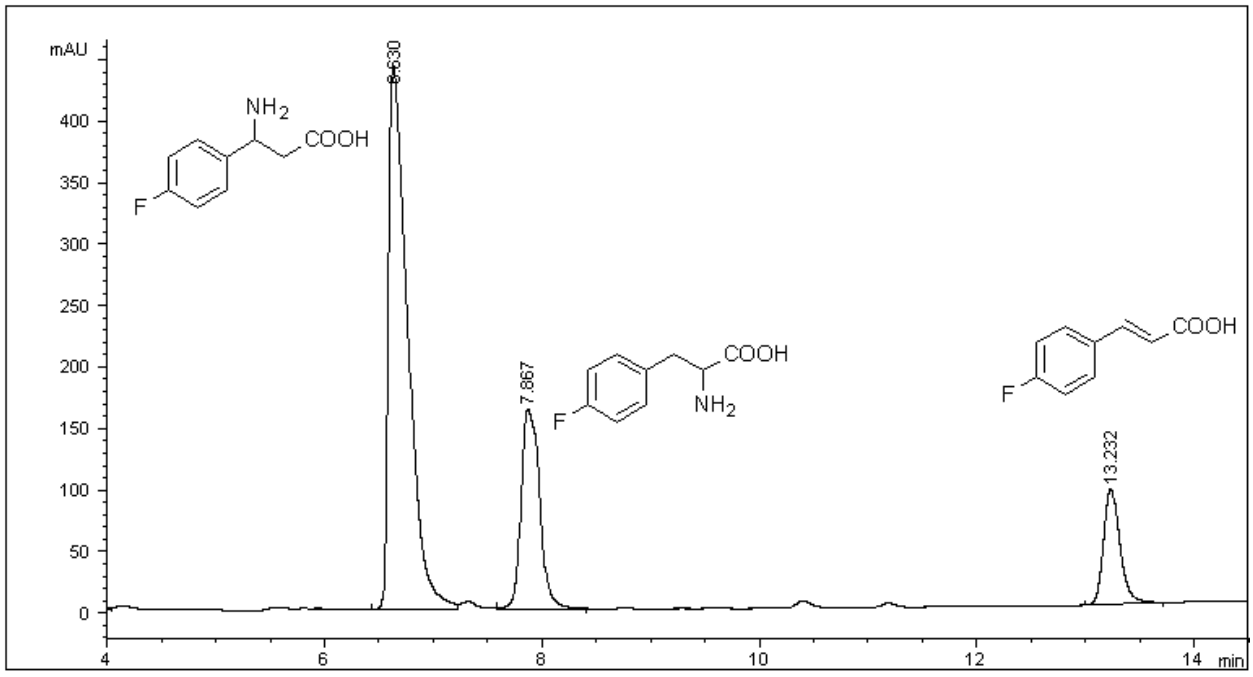
Products from (±)-4-bromo-β-phenylalanine (*rac*-**2c**) by *Pa*PAM after 20 h.



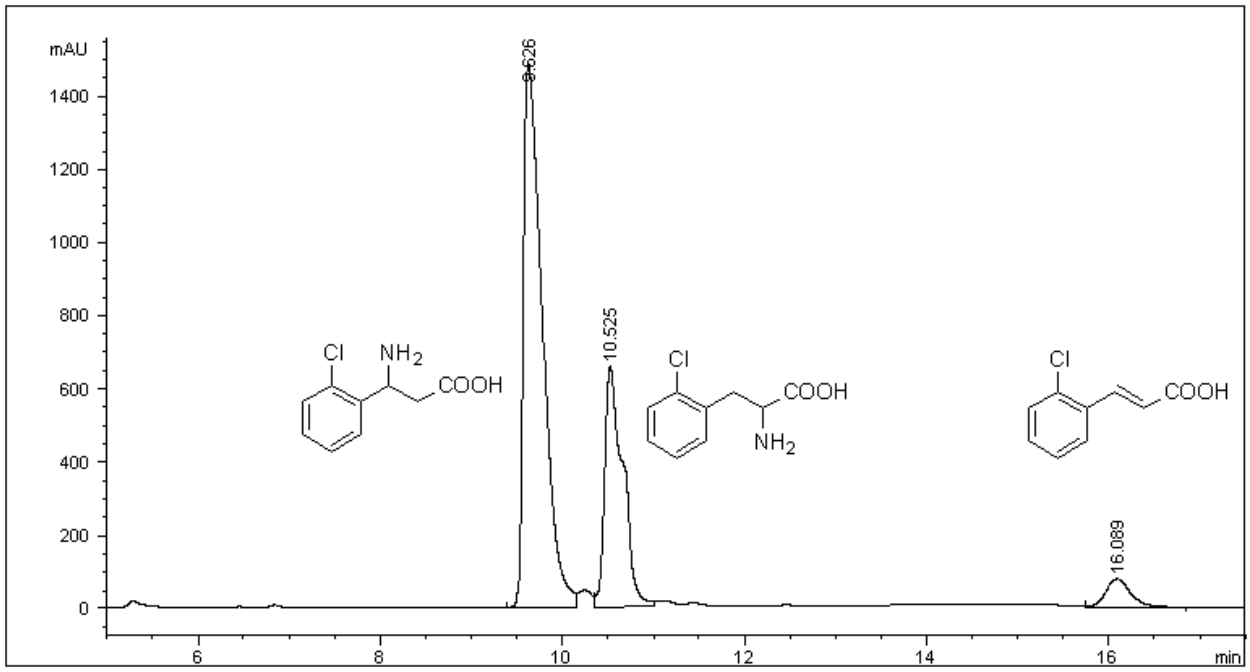
Products from (±)-2-fluoro-β-phenylalanine (*rac*-**2d**) by *Pa*PAM after 20 h.



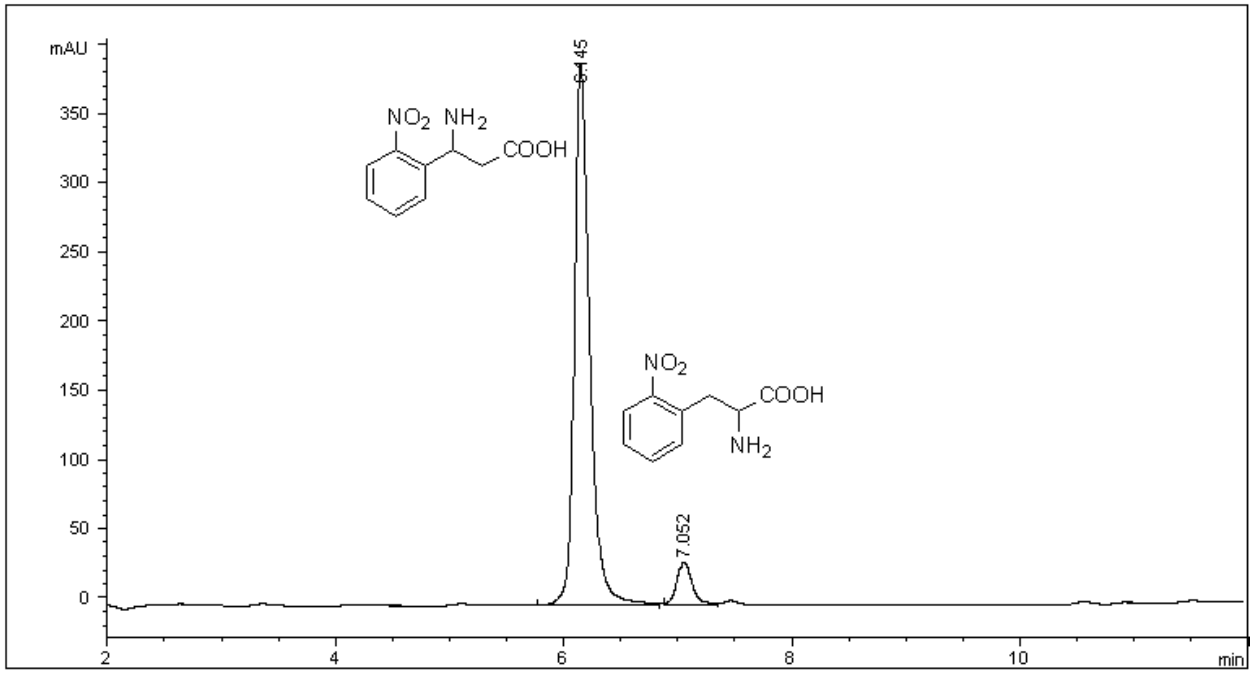
Products from (±)-3-fluoro-β-phenylalanine (*rac*-**2e**) by *Pa*PAM after 20 h.



Products from (±)-4-fluoro-β-phenylalanine (*rac*-**2f**) by *Pa*PAM after 20 h.



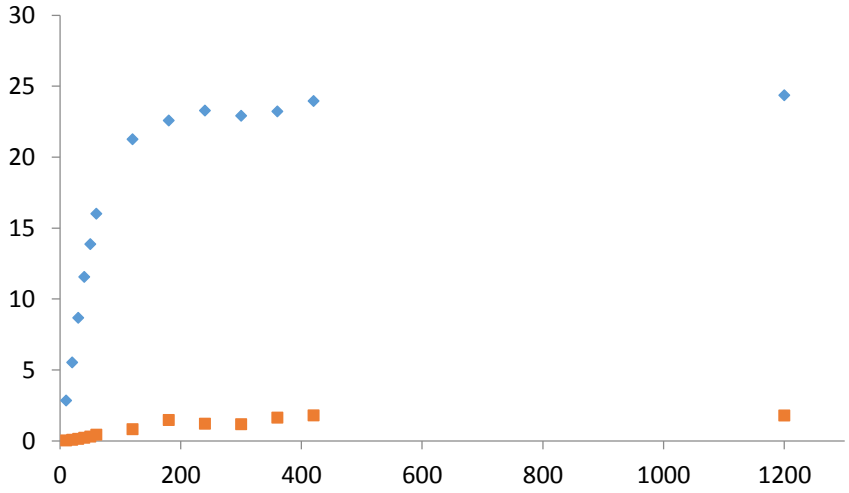
Products from (±)-2-chloro-β-phenylalanine (*rac*-**2g**) by *Pa*PAM after 20 h.



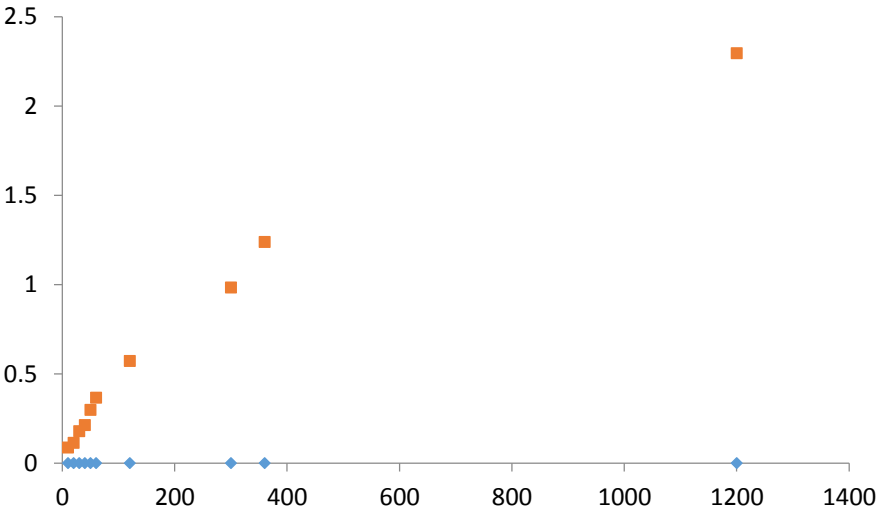
Products from (±)-2-nitro-β-phenylalanine (*rac*-**2j**) by *Pa*PAM after 20 h.

3. Time-course profiles of the PaPAM-catalysed reactions

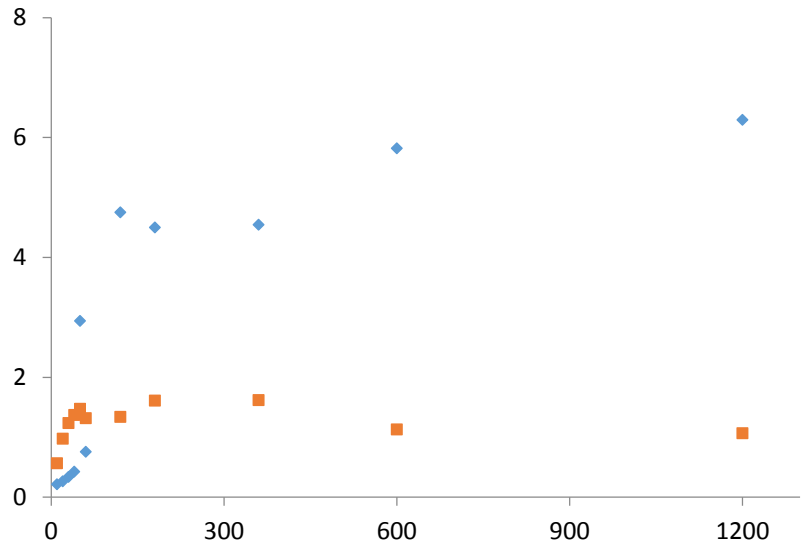
Into the solution of the substrate (*rac*-**1a-f,h,i,k,l**or*rac*-**2a-g,j,** 4 mg) in $(NH_4)_2CO_3$ buffer (100 mM, pH 8.0, 2 mL), *Pa*PAM (1.6 mg) was added and the reaction mixture was stirred at room temperature.



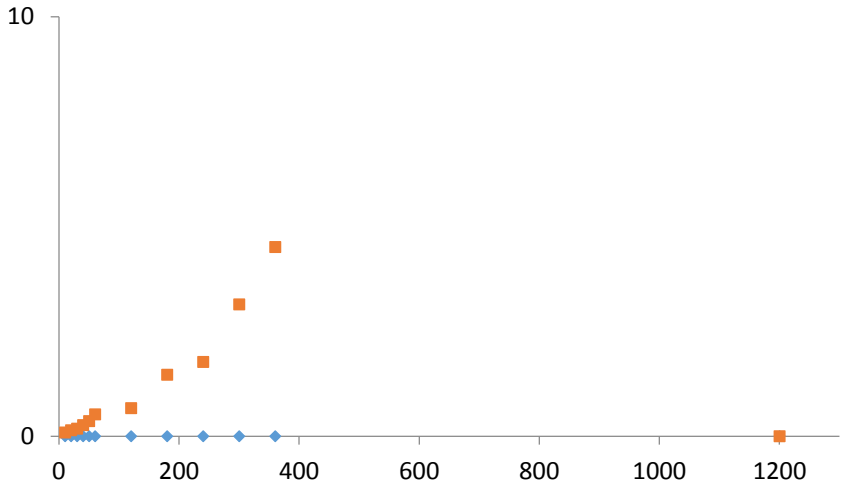
Time-course profile of the conversion of (±)-β-phenylalanine (*rac*-**2a**, ◆) to (*S*)-α-phenylalanine [(*S*)-**1a**] and (*E*)-cinnamic acid (**3a**, ■)



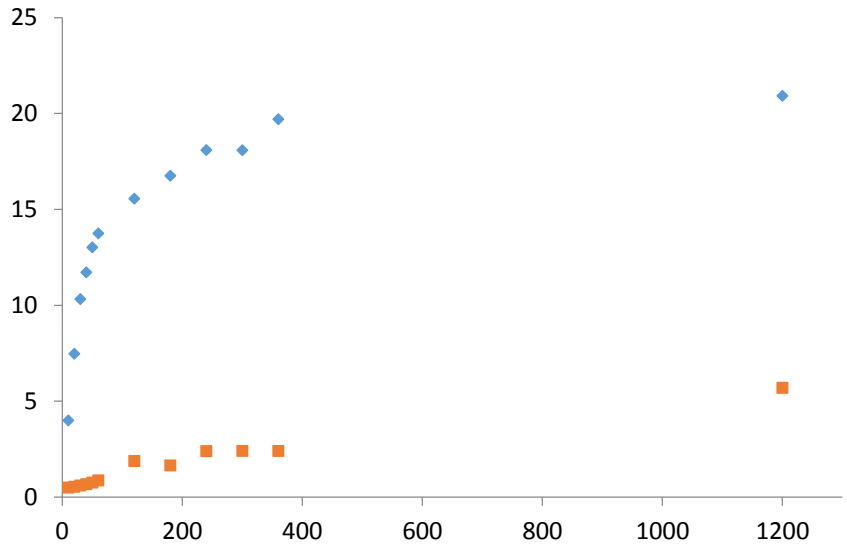
Time-course profile of the conversion of (±)-β-(thiophen-2-yl)alanine (*rac*-**2b**, ◆) to (*S*)-α-(thiophen-2-yl)alanine [(*S*)-**1b**)] and (*E*)-3-(thiophen-2-yl)acrylic acid (**3b**, ■)



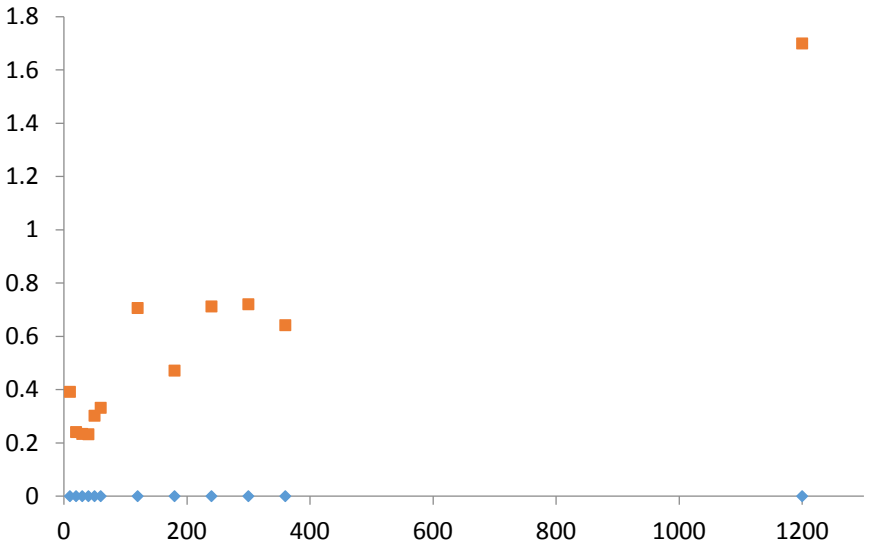
Time-course profile of the conversion of (±)-4-bromo-β-phenylalanine (*rac*-**2c**, ◆) to (*S*)-4-bromo-α-phenylalanine [(*S*)-**1c**)] and (*E*)-4-bromocinnamic acid (**3c**, ■)



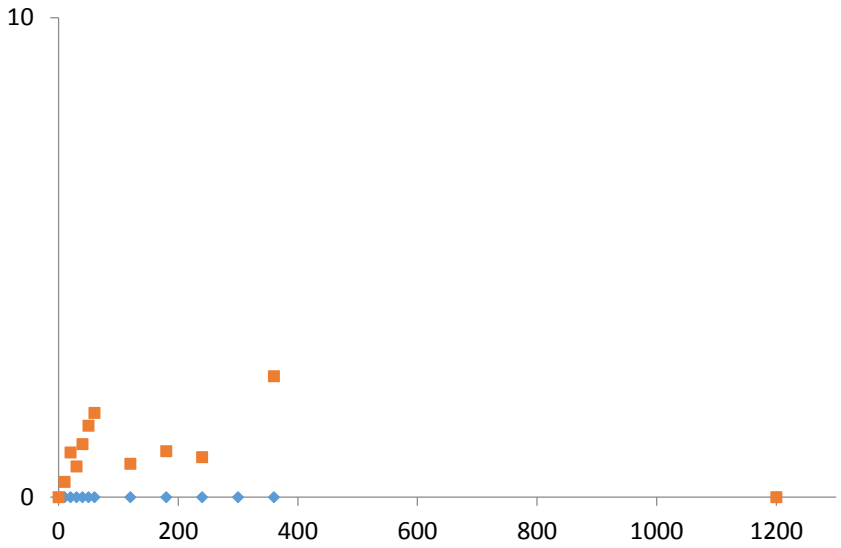
Time-course profile of the conversion of (±)-4-fluoro-β-phenylalanine (*rac*-**2d**, ◆) to (*S*)-4-fluoro-α-phenylalanine [(*S*)-**1d**)] and (*E*)-4-fluorocinnamic acid (**3d**, ■)



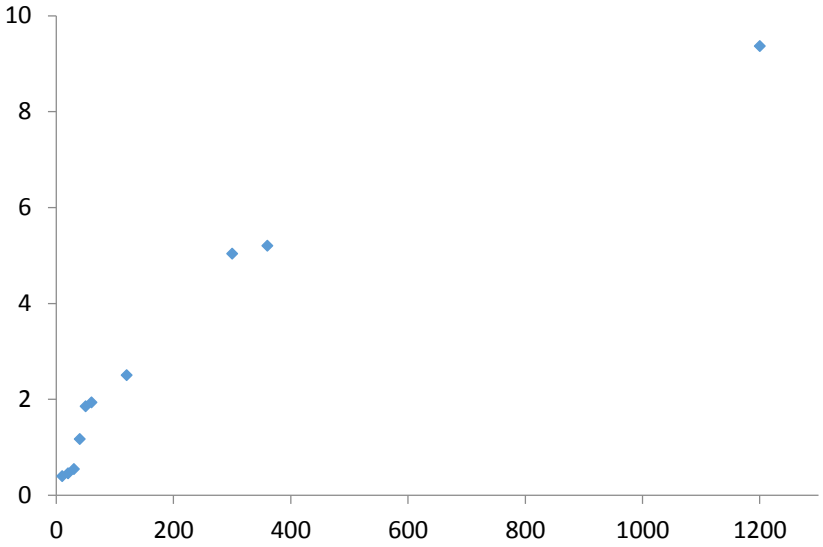
Time-course profile of the conversion of (±)-3-fluoro-β-phenylalanine (*rac*-**2e**, ◆) to (*S*)-3-fluoro-α-phenylalanine [(*S*)-**1e**)] and (*E*)-4-fluorocinnamic acid (**3e**, ■)



Time-course profile of the conversion of (±)-4-fluoro-β-phenylalanine (*rac*-**2f**, ◆) to(*S*)-4-fluoro-α-phenylalanine [(*S*)-**1f**)] and (*E*)-4-fluorocinnamic acid (**3f**, ■)



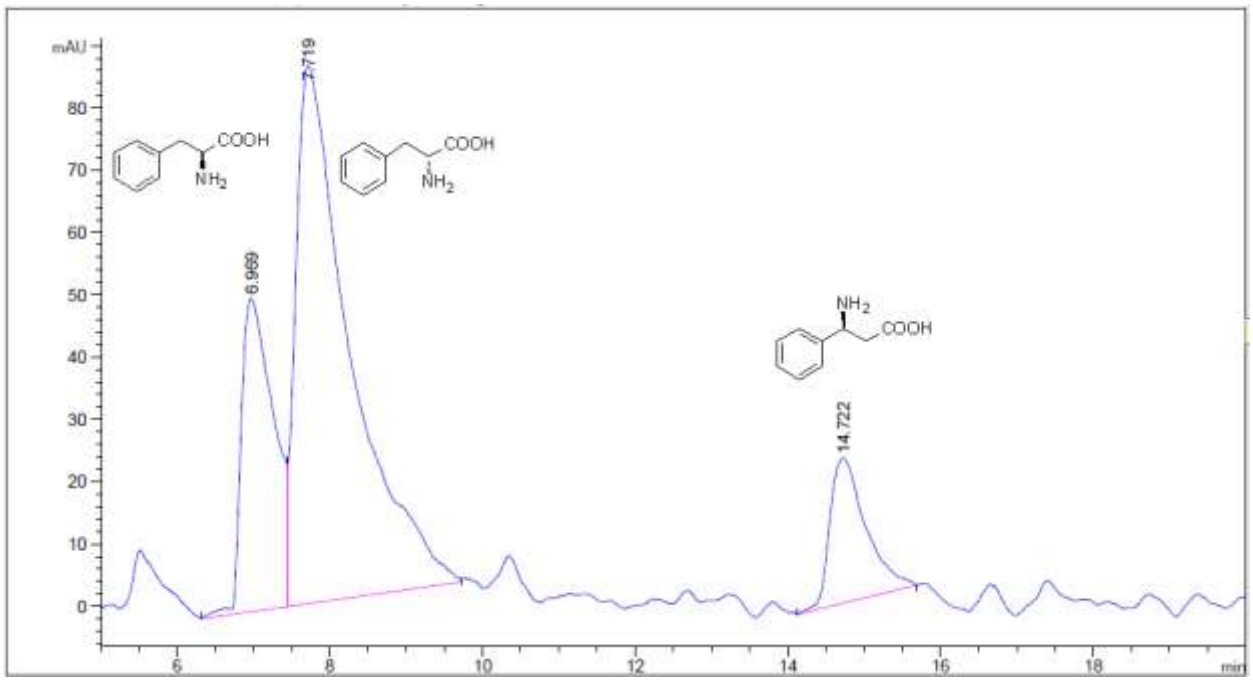
Time-course profile of the conversion of (±)-2-chloro-β-phenylalanine (*rac*-**2g**, ◆) to (*S*)-2-chloro-α-phenylalanine [(*S*)-**1g**)] and (*E*)-2-chlorocinnamic acid (**3g**, ■)



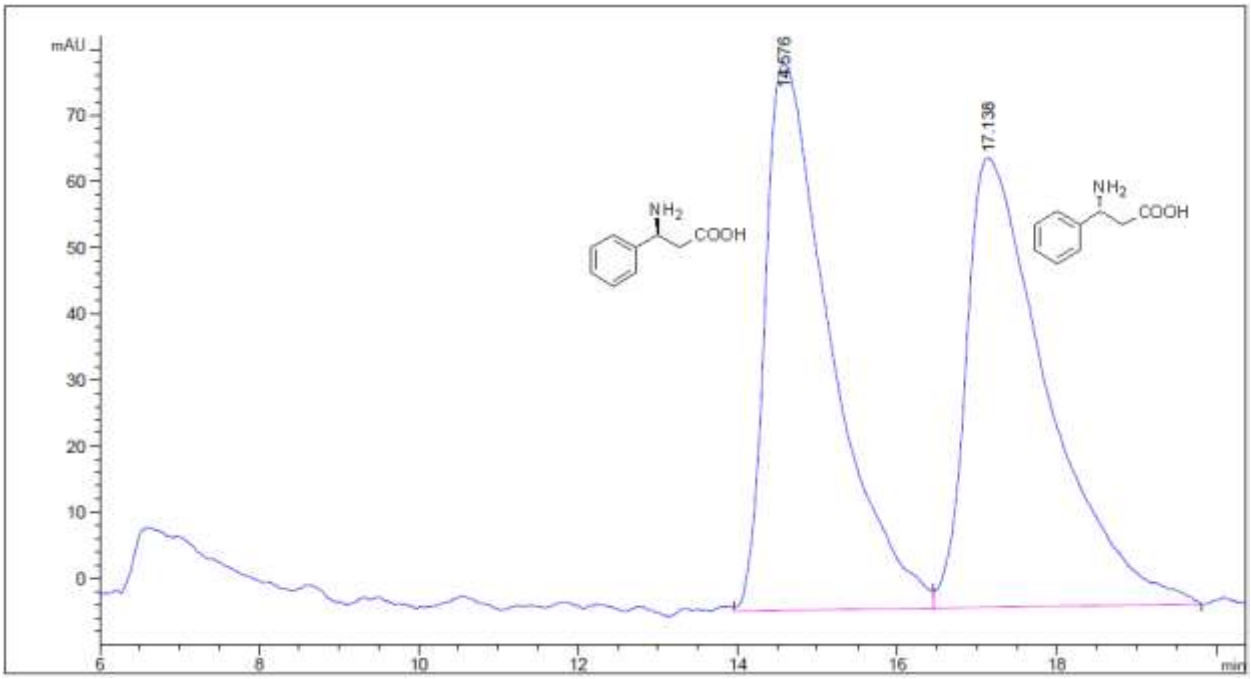
Time-course profile of the conversion of (±)-2-nitro-β-phenylalanine (*rac*-**2j**) to (*S*)-2-nitro-α-phenylalanine [(*S*)-**1j**, ◆]

4. HPLC determination of the enantiomeric compositions

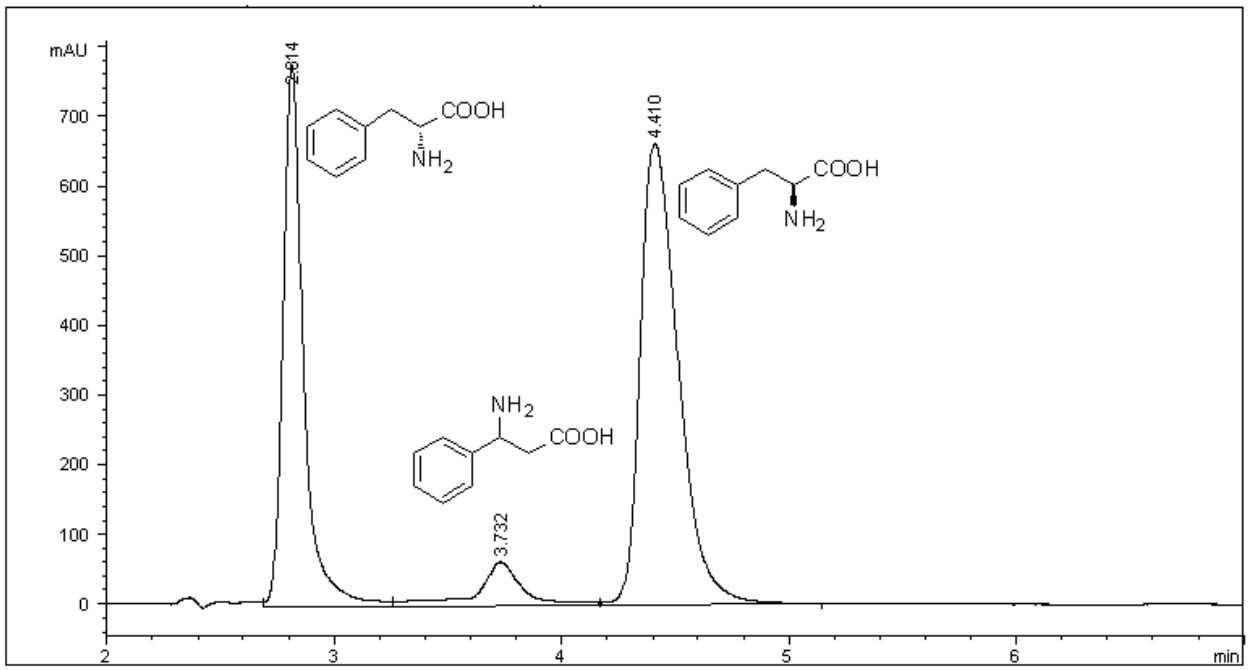
Samples taken from the enzymatic reactions after 20 h as described in section 2 were injected onto Crownpak® CR-I(+)column (150 × 3.0 mm × 5 µm) using $HClO_4$ solution (pH 1.5) : acetonitrile as mobile phase, flow rate: 0.4mLmin^{-1}or onto Chiralpak® ZWIX (+) column (250 × 4.6 mm × 3 µm) using MeOH (50 mM diethylamine, 100 mM formic acid) : acetonitrile : H_2O, 49:49:2 as mobile phase, flow rate: 1 mL/min.



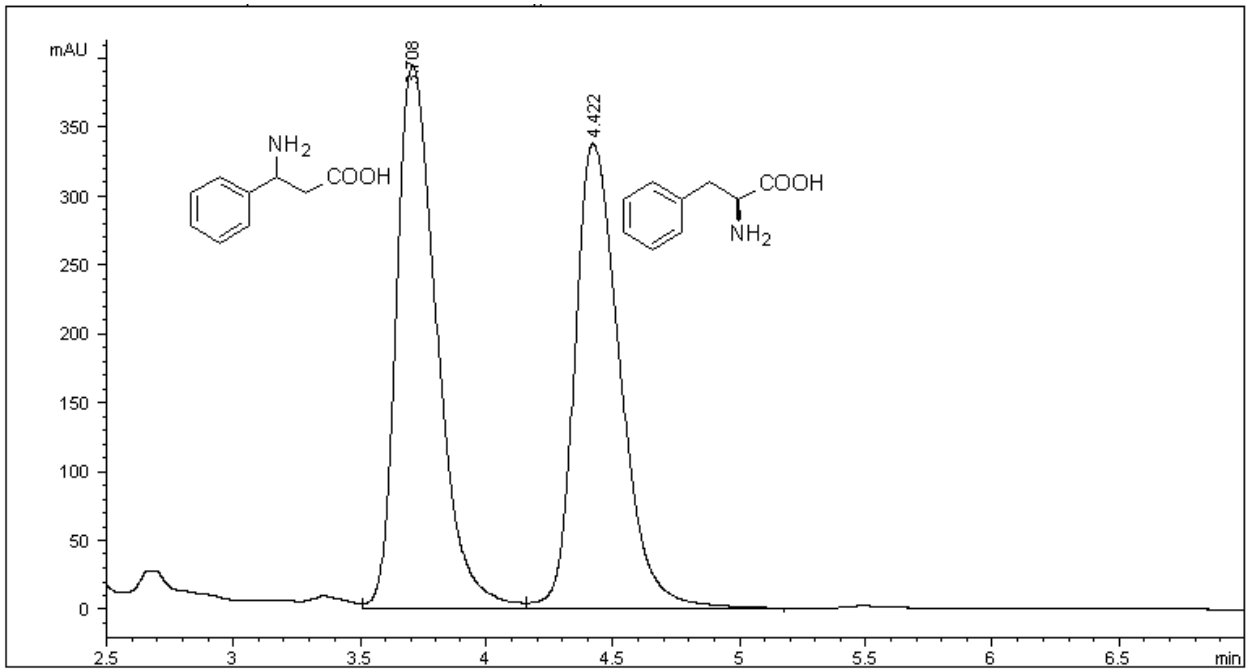
Products from (±)-α-phenylalanine (*rac*-**1a**) by *Pa*PAM after 20 h [Chiralpak ZWIX (+) column]



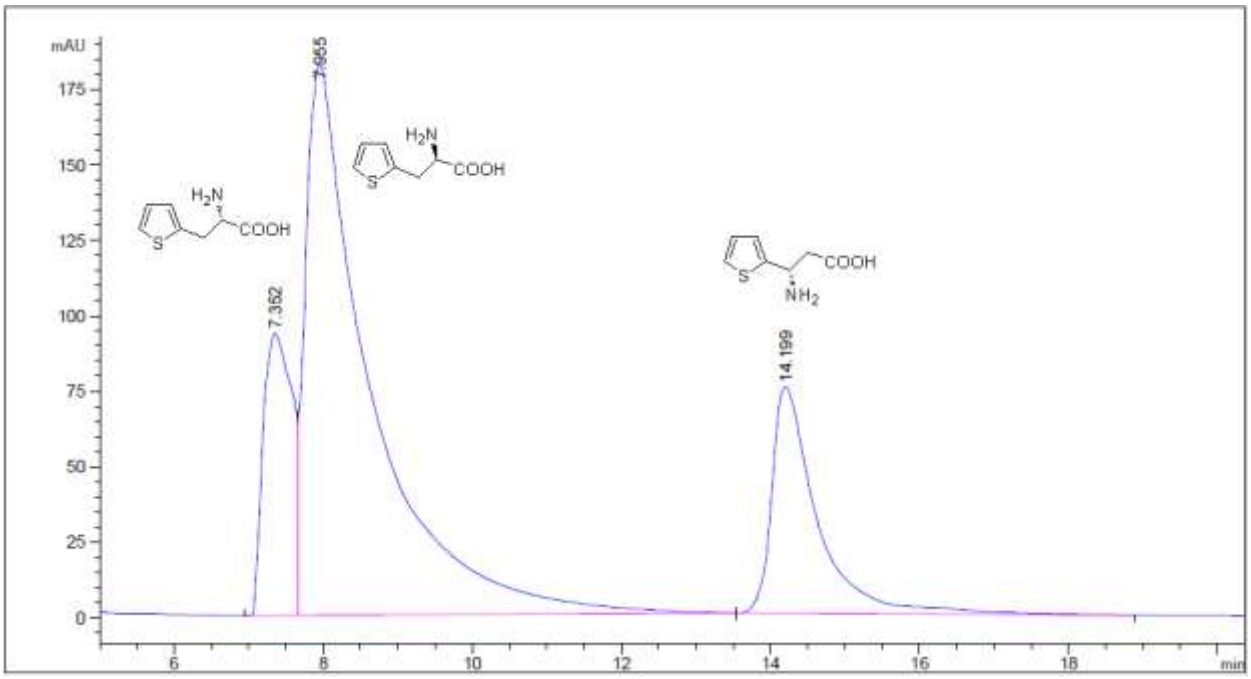
Enantioseparation of authentic (±)-β-phenylalanine (*rac*-**2a**) on Chiralpak ZWIX(+) column



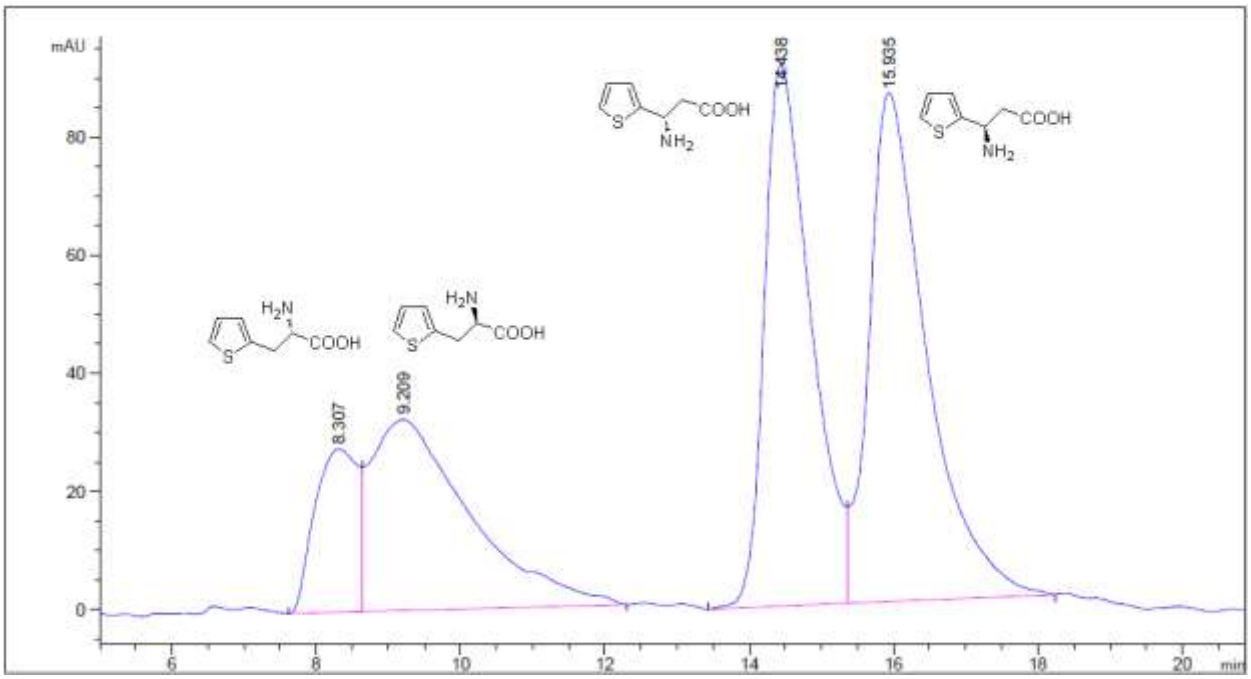
Products from (±)-α-phenylalanine (*rac*-**1a**) by *Pa*PAM after 20 h
[Crownpak CR-I(+) column]



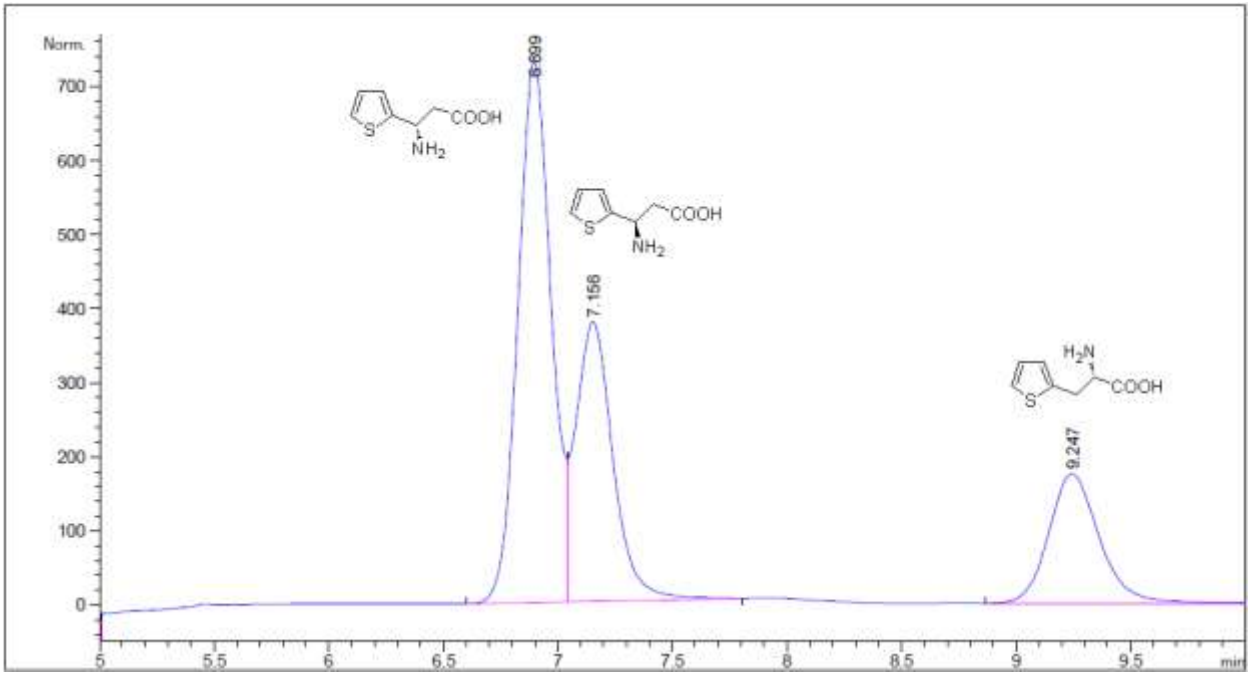
Enantioseparation of authentic (±)-β-phenylalanine (*rac*-**2a**) on Crownpak CR-I(+) column



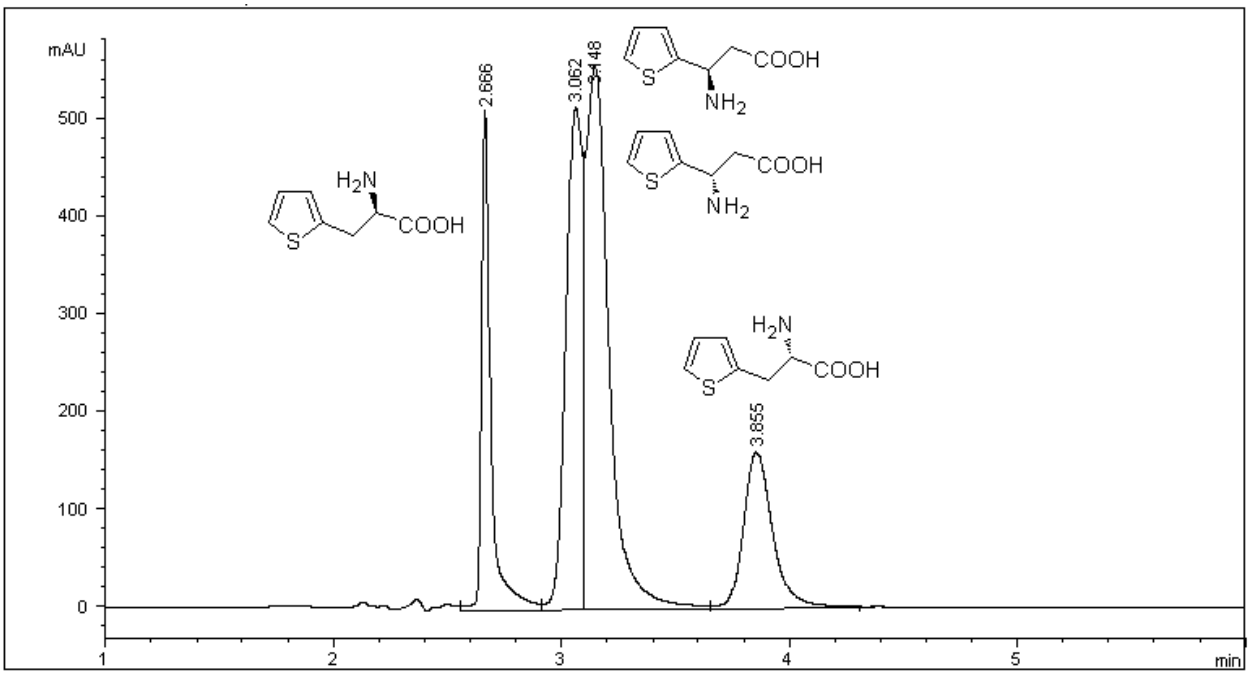
Products from (±)-α-(thiophen-2-yl)alanine (*rac*-**1b**) by *Pa*PAM after 20 h
[Chiralpak ZWIX(+) column]



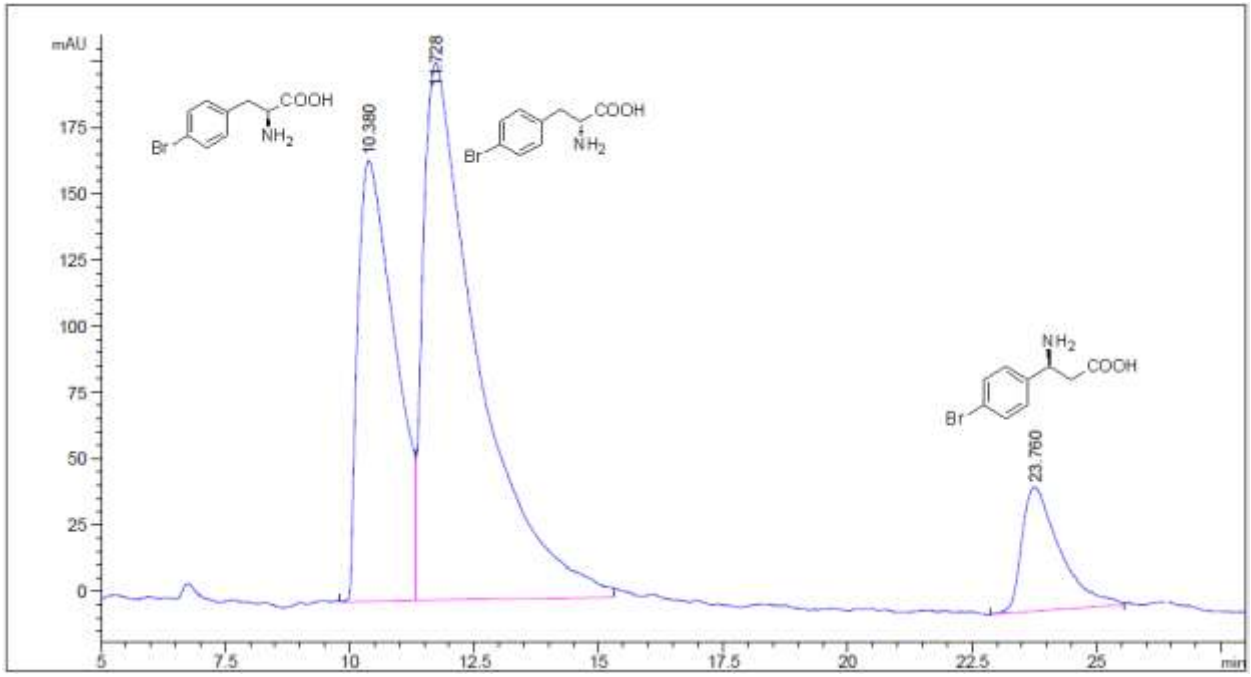
Enantioseparation of authentic (±)-β-(thiophen-2-yl)alanine (*rac*-**2b**) and
(±)-α-(thiophen-2-yl)alanine (*rac*-**1b**) on Chiralpak ZWIX(+) column



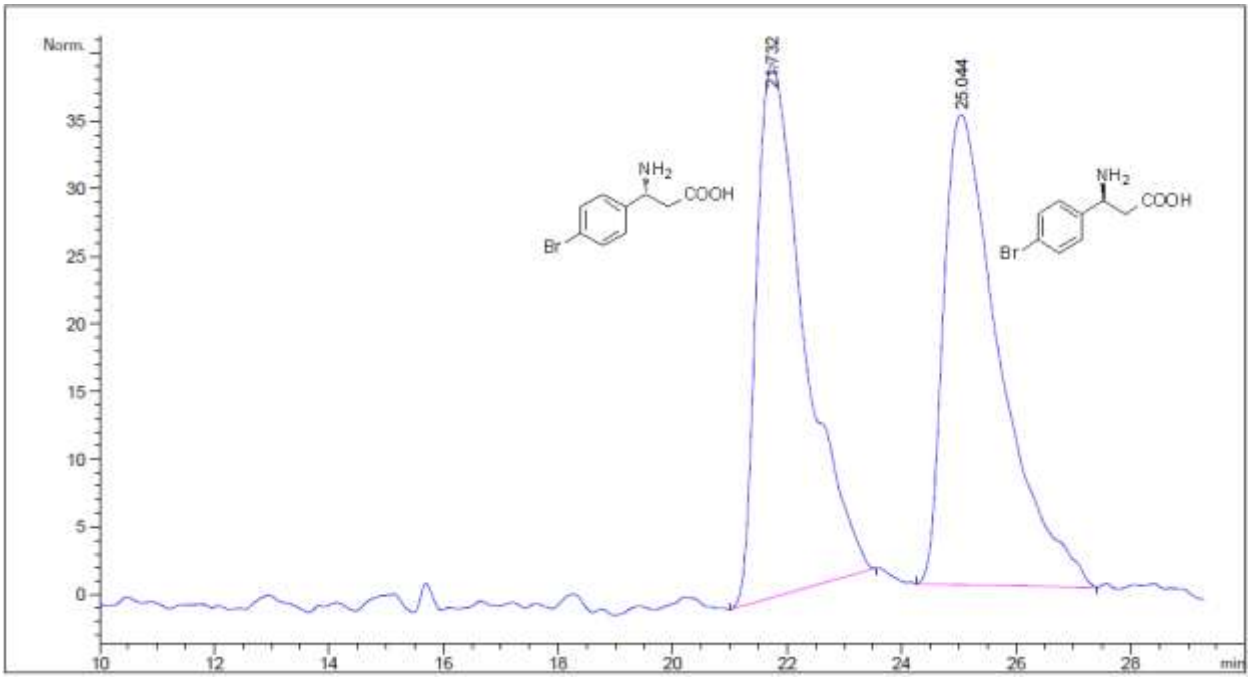
Products from (±)-β-(thiophen-2-yl)alanine (*rac*-**2b**) by *Pa*PAM after 20 h
[Crownpak CR-I(+)column]



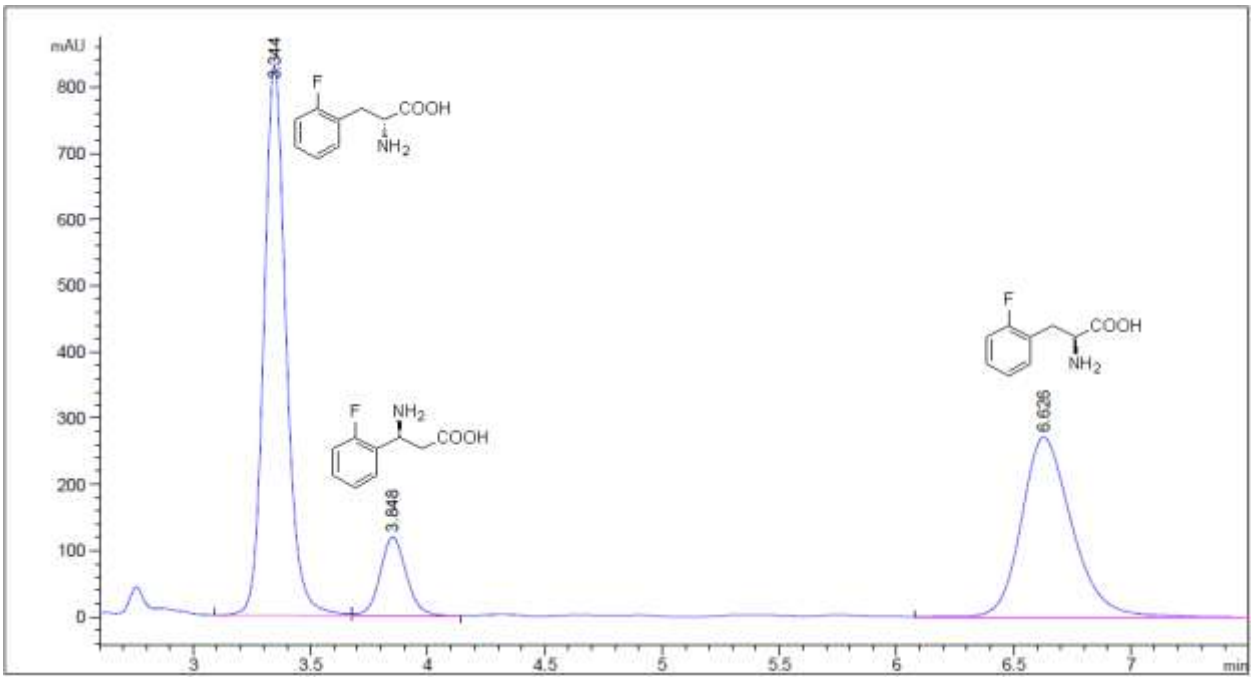
Enantioseparation of authentic (±)-β-(thiophen-2-yl)alanine (*rac*-**2b**) and
(±)-α-(thiophen-2-yl)alanine (*rac*-**1b**) on Crownpak CR-I(+) column



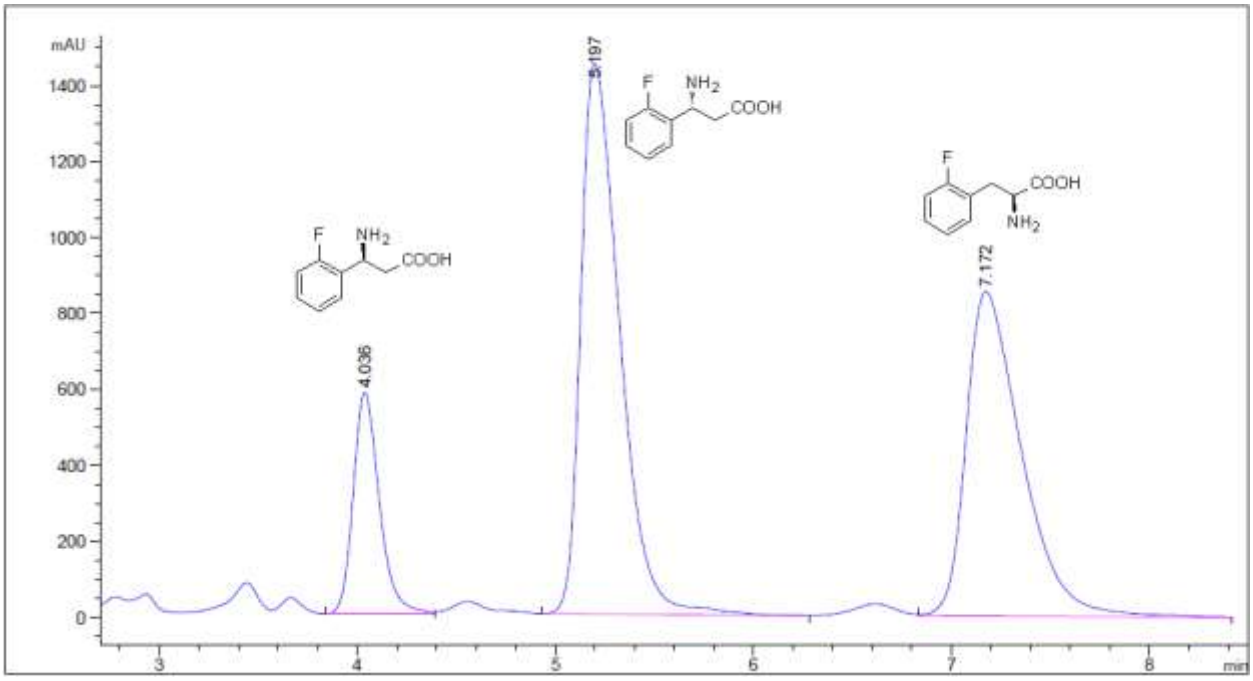
Products from (±)-4-bromo-α-phenylalanine (*rac*-**1c**) by *Pa*PAM after 20 h
[Chiralpak ZWIX(+) column]



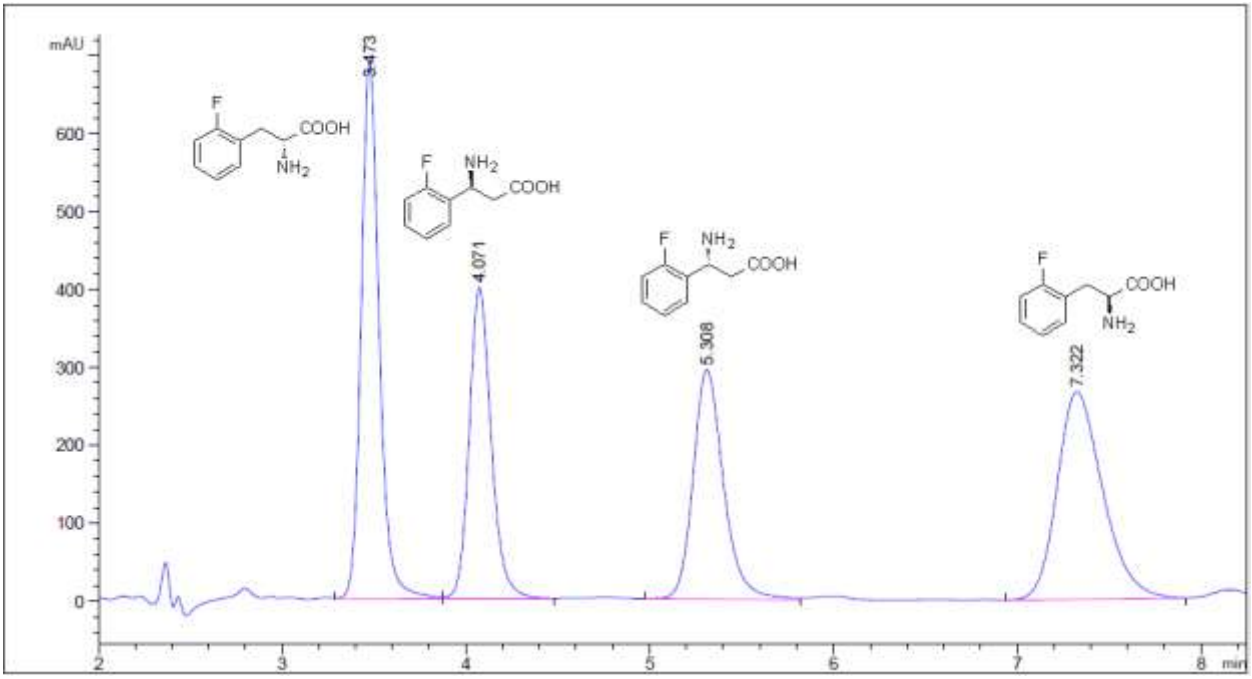
Enantioseparation of authentic (±)-4-bromo-α-phenylalanine (*rac*-**1c**)
on Chiralpak ZWIX(+) column



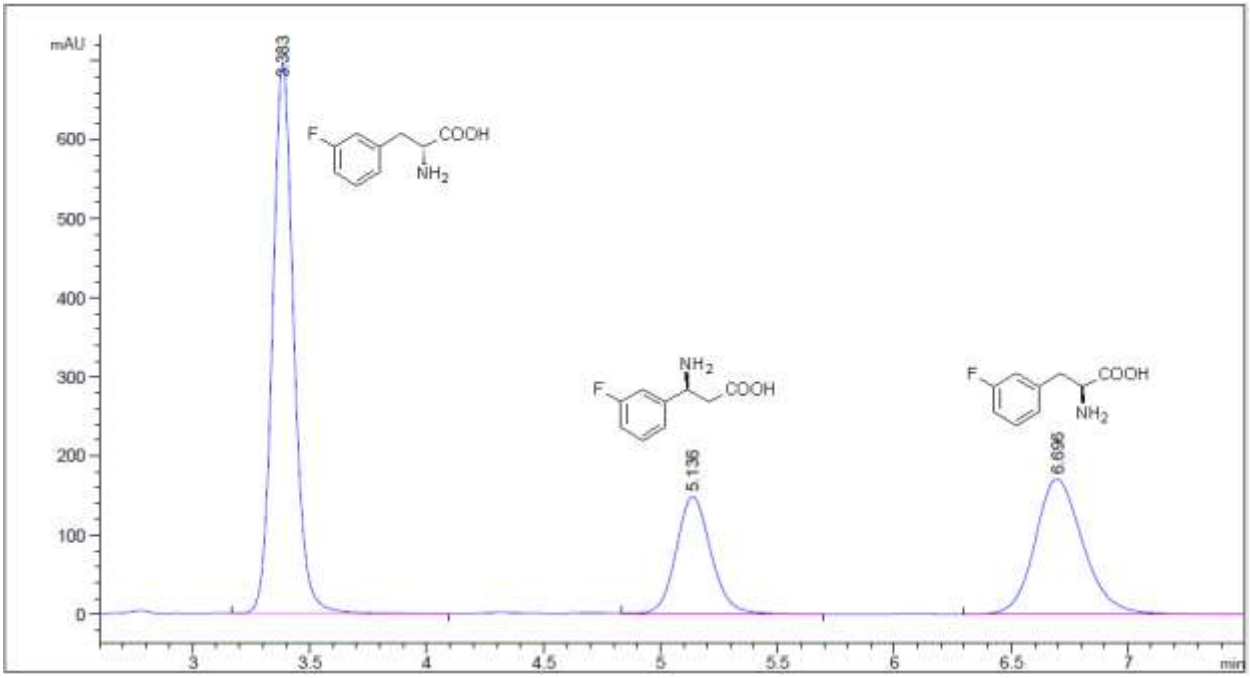
Products from (±)-4-fluoro-α-phenylalanine (*rac*-**1d**) by *Pa*PAM after 20 h
[Crownpak CR-I(+)column]



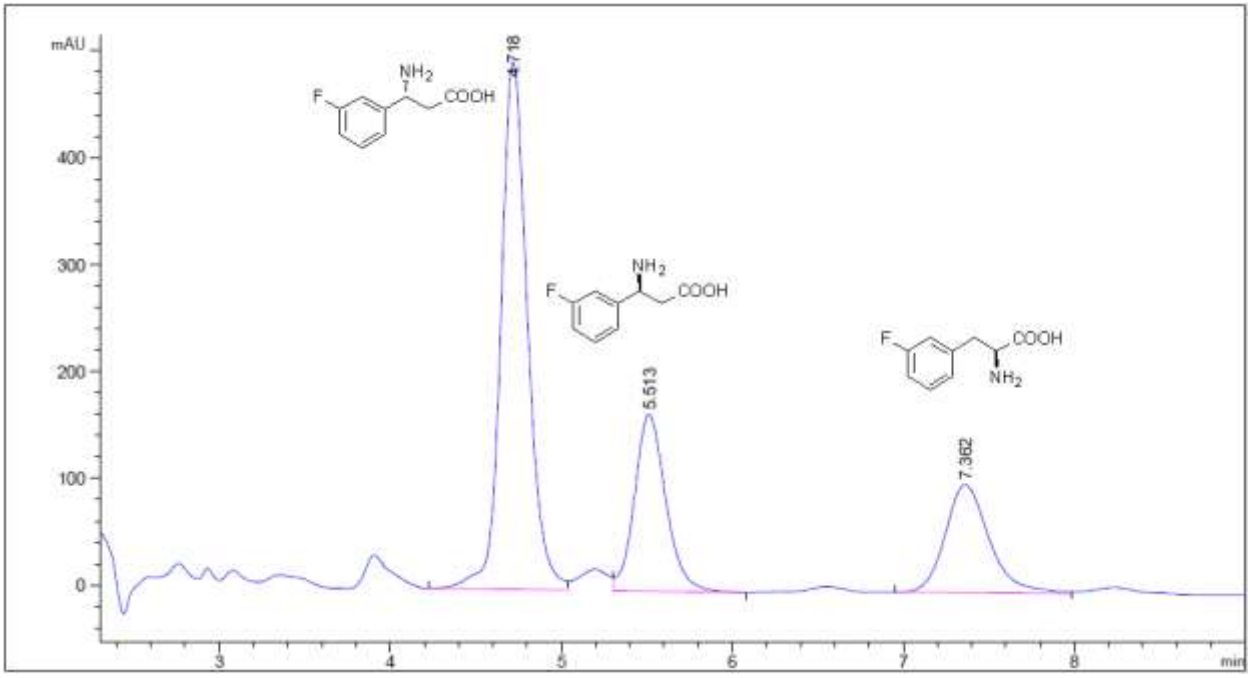
Products from (±)-4-fluoro-β-phenylalanine (*rac*-**2d**) by *Pa*PAM after 20 h
[Crownpak CR-I(+)column]



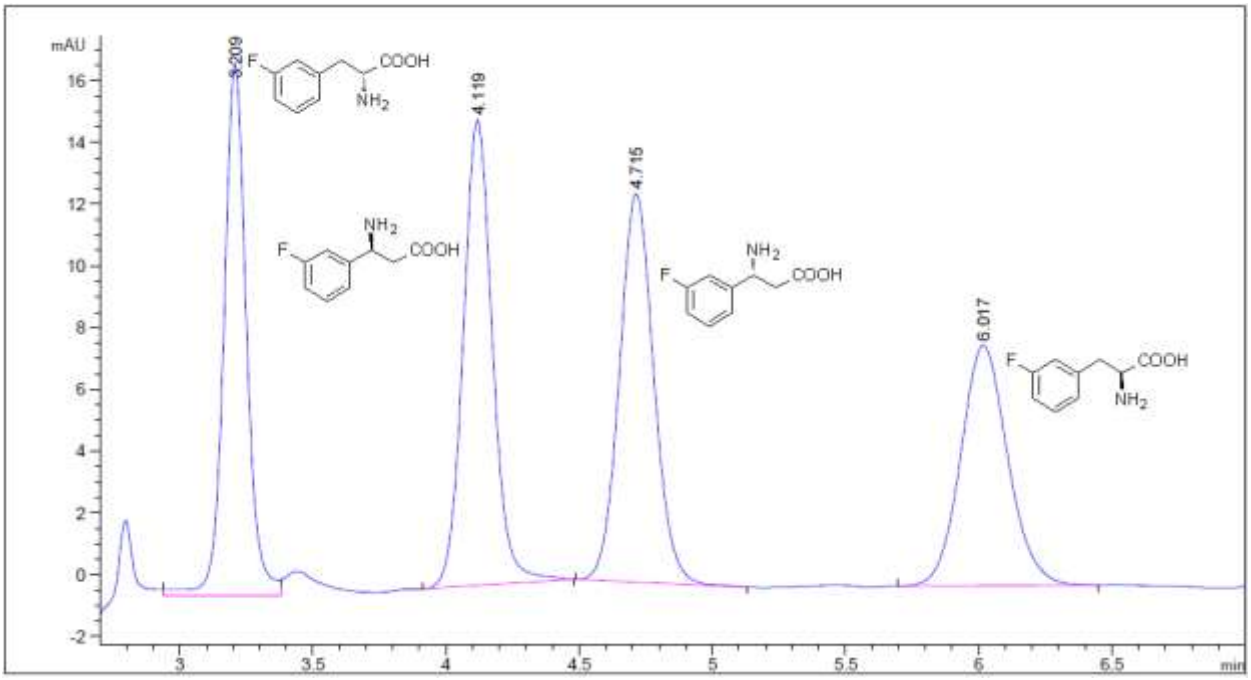
Enantioseparation of authentic (±)-4-fluoro-α-phenylalanine and (±)-4-fluoro-β-phenylalanine on Crownpak CR-I(+)column



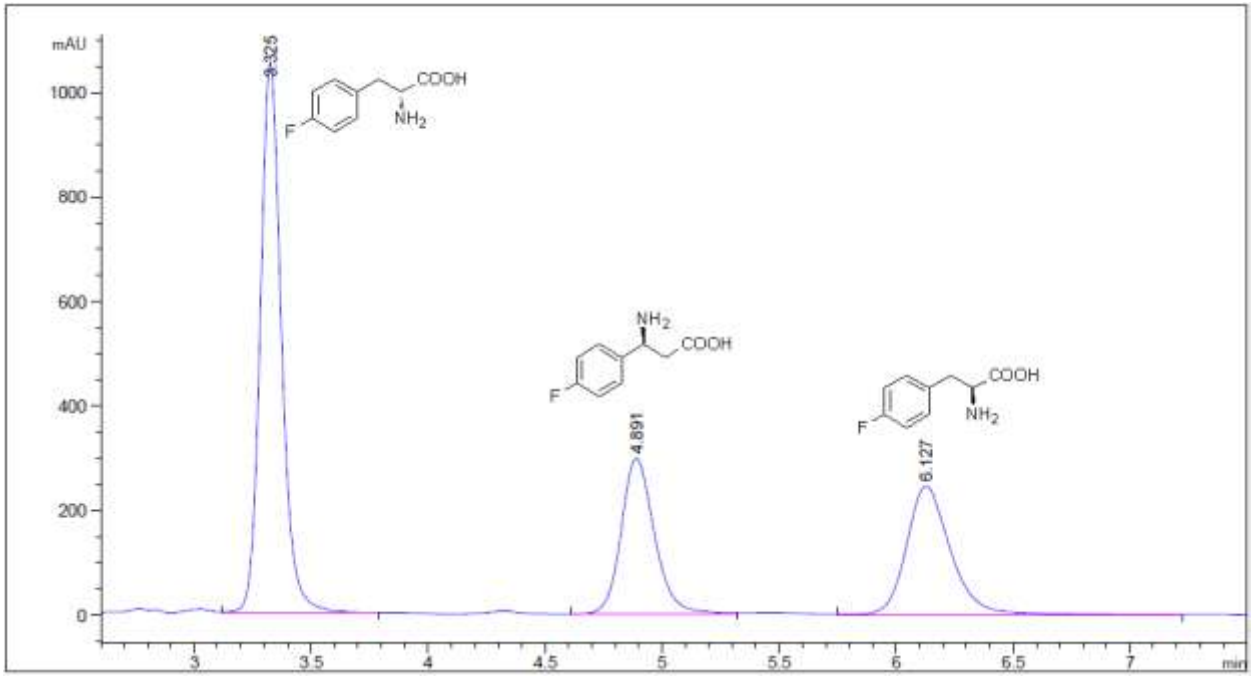
Products from (±)-3-fluoro-α-phenylalanine (*rac*-**1e**) by *Pa*PAM after 20 h [Crownpak CR-I(+)column]



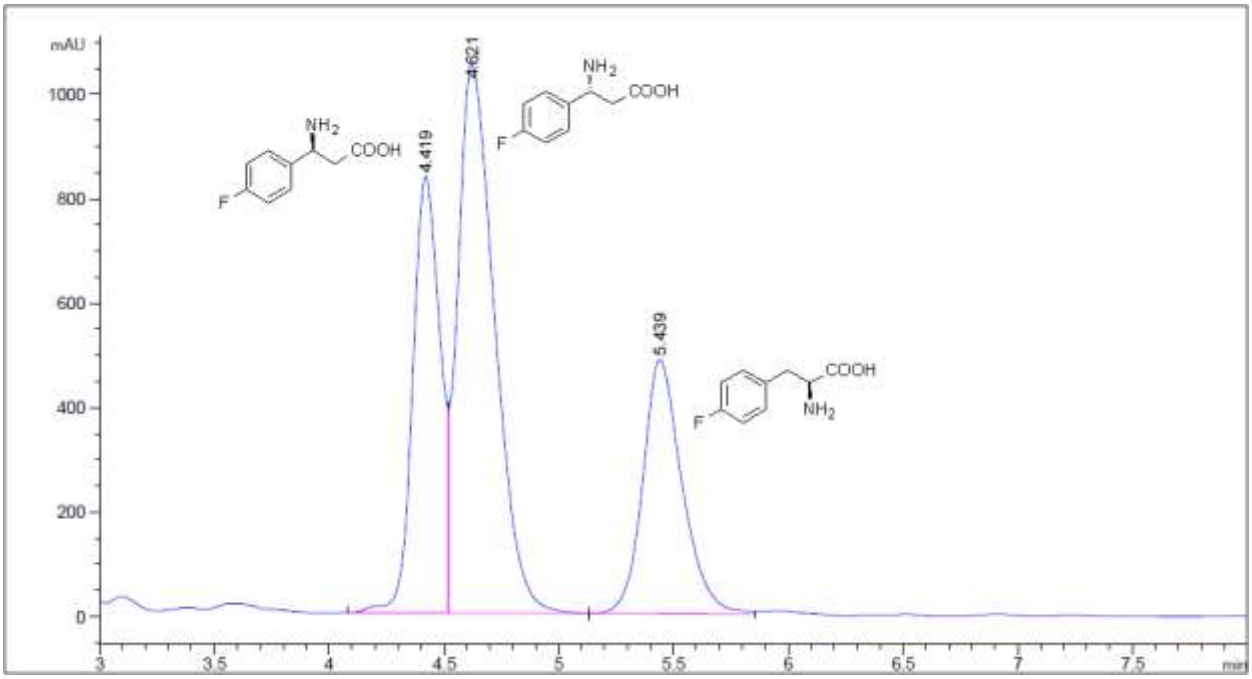
Products from (±)-3-fluoro-β-phenylalanine (*rac*-**2e**) by *Pa*PAM after 20 h
[Crownpak CR-I(+) column]



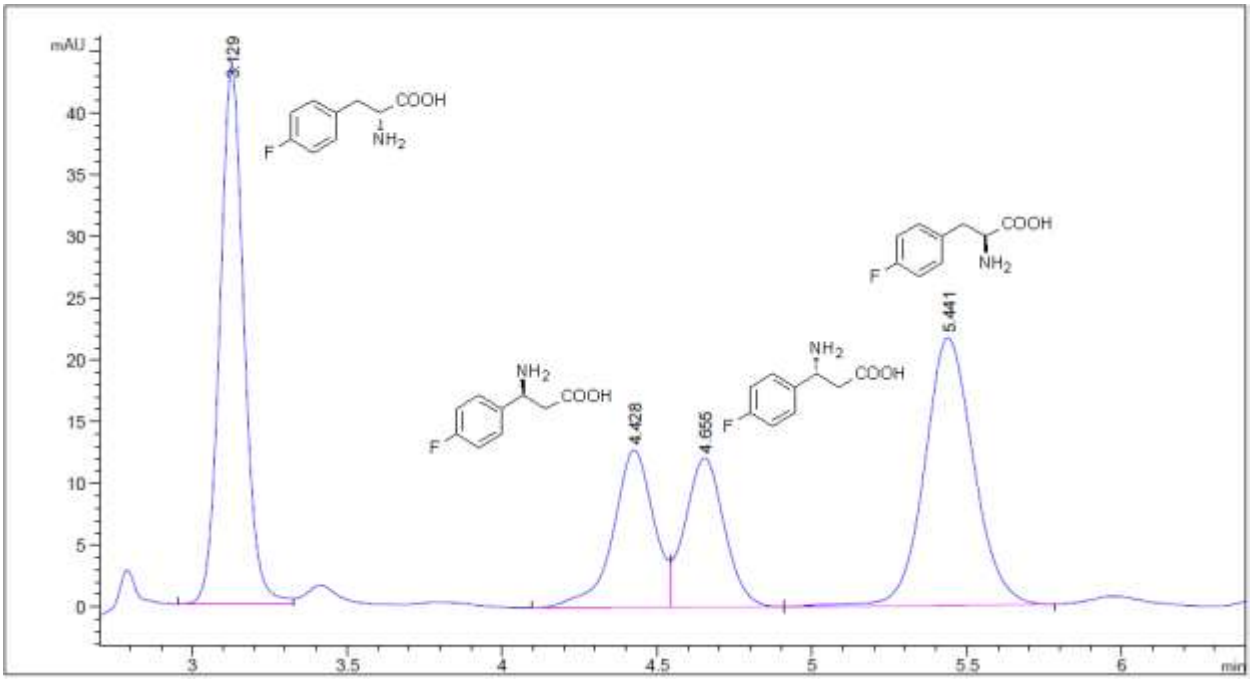
Enantioseparation of authentic (±)-3-fluoro-α-phenylalanine (*rac*-**1e**) and
(±)-3-fluoro-β-phenylalanine (*rac*-**2e**) on Crownpak CR-I(+)column



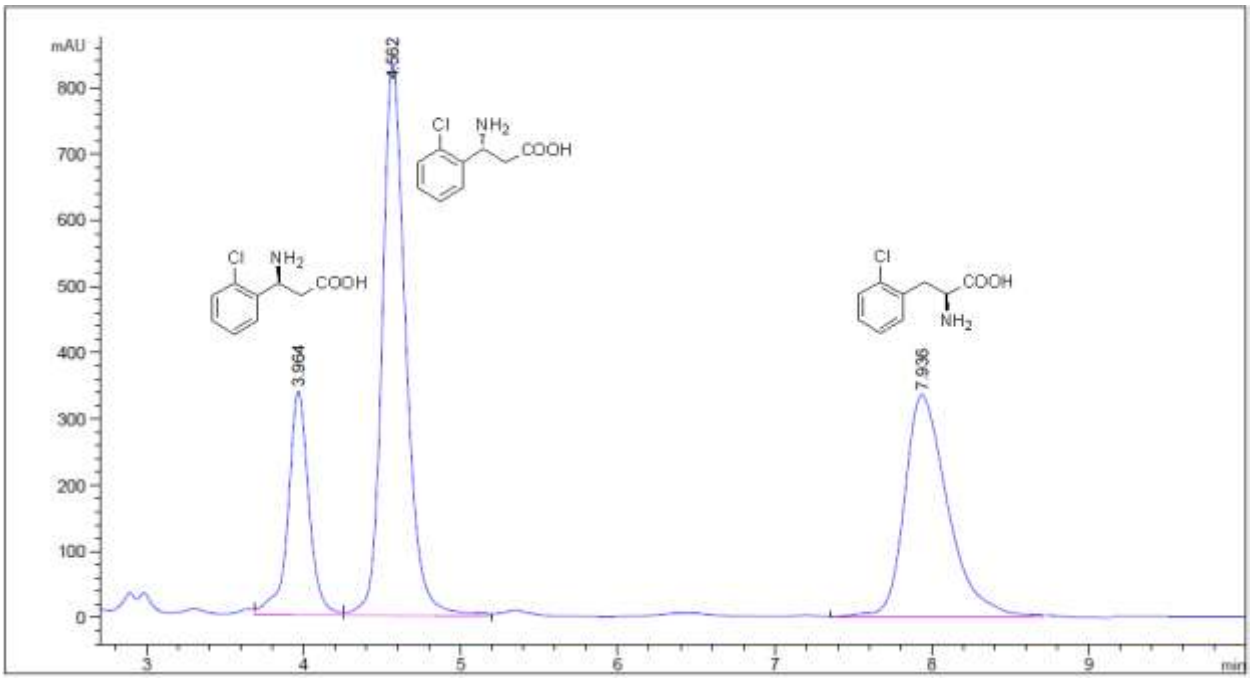
Products from (±)-4-fluoro-α-phenylalanine (*rac*-**1f**) by *Pa*PAM after 20 h
[Crownpak CR-I(+)column]



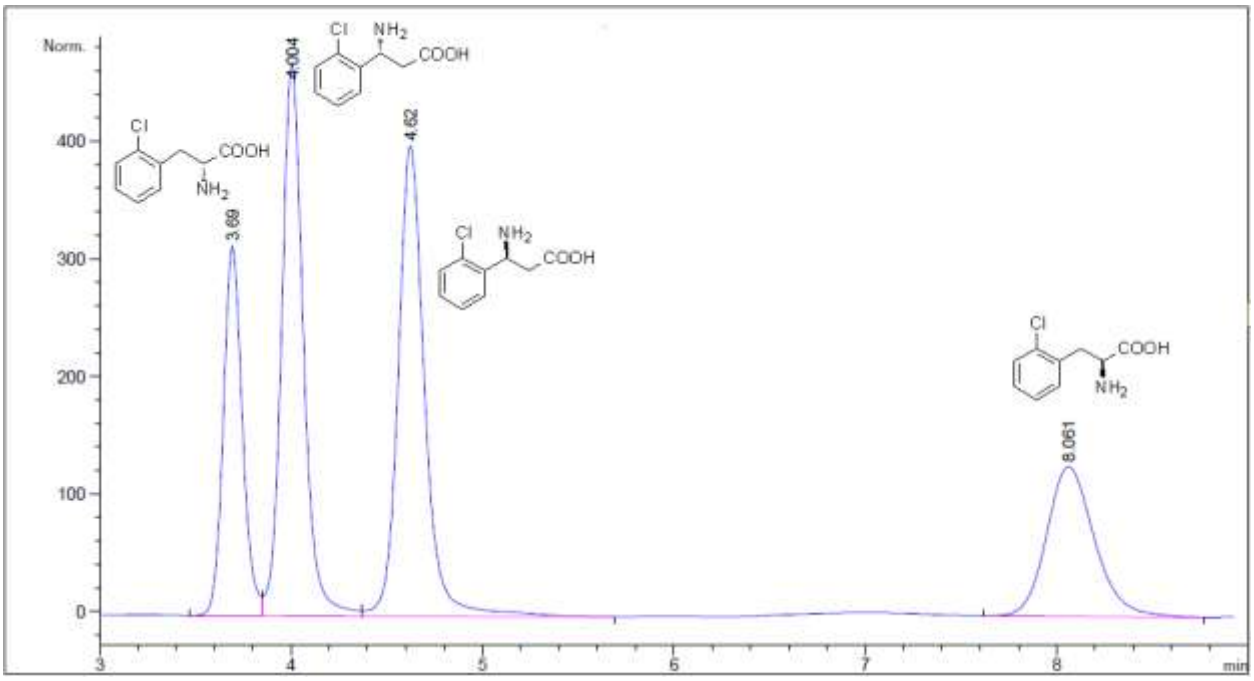
Products from (±)-4-fluoro-β-phenylalanine (*rac*-**2f**) by *Pa*PAM after 20 h
[Crownpak CR-I(+) column]



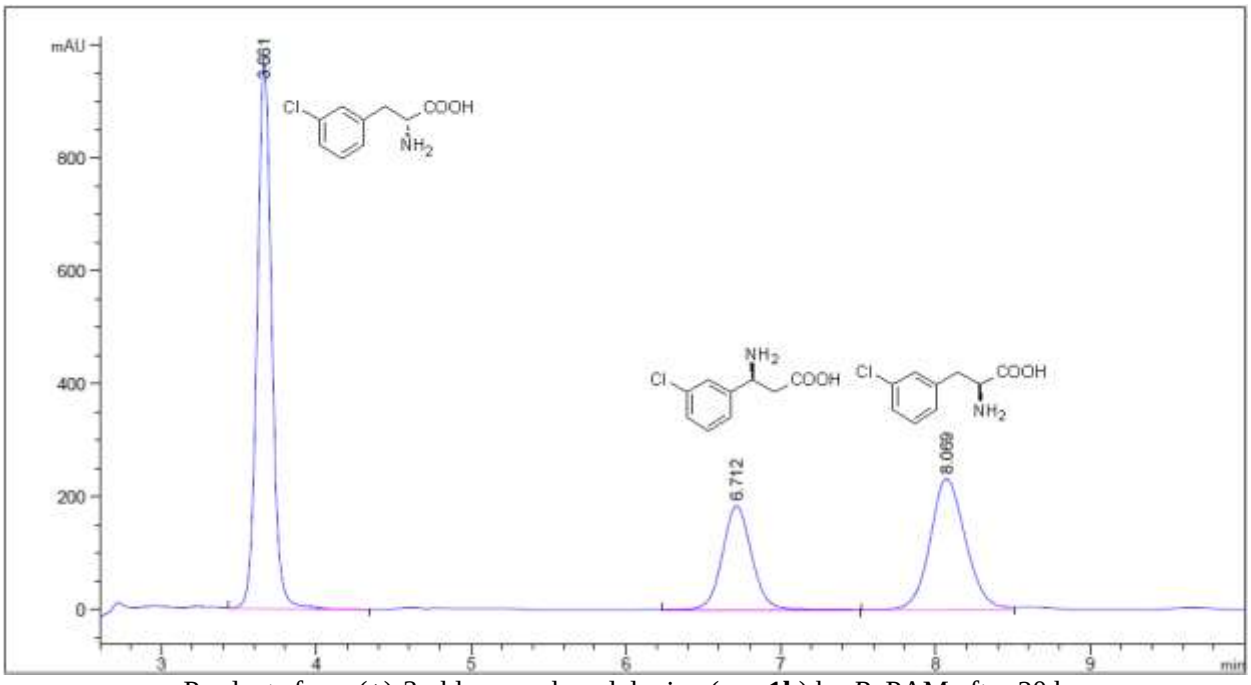
Enantioseparation of authentic (±)-4-fluoro-α-phenylalanine (*rac*-**1f**) and (±)-4-fluoro-β-phenylalanine (*rac*-**2f**) on Crownpak CR-I(+)column



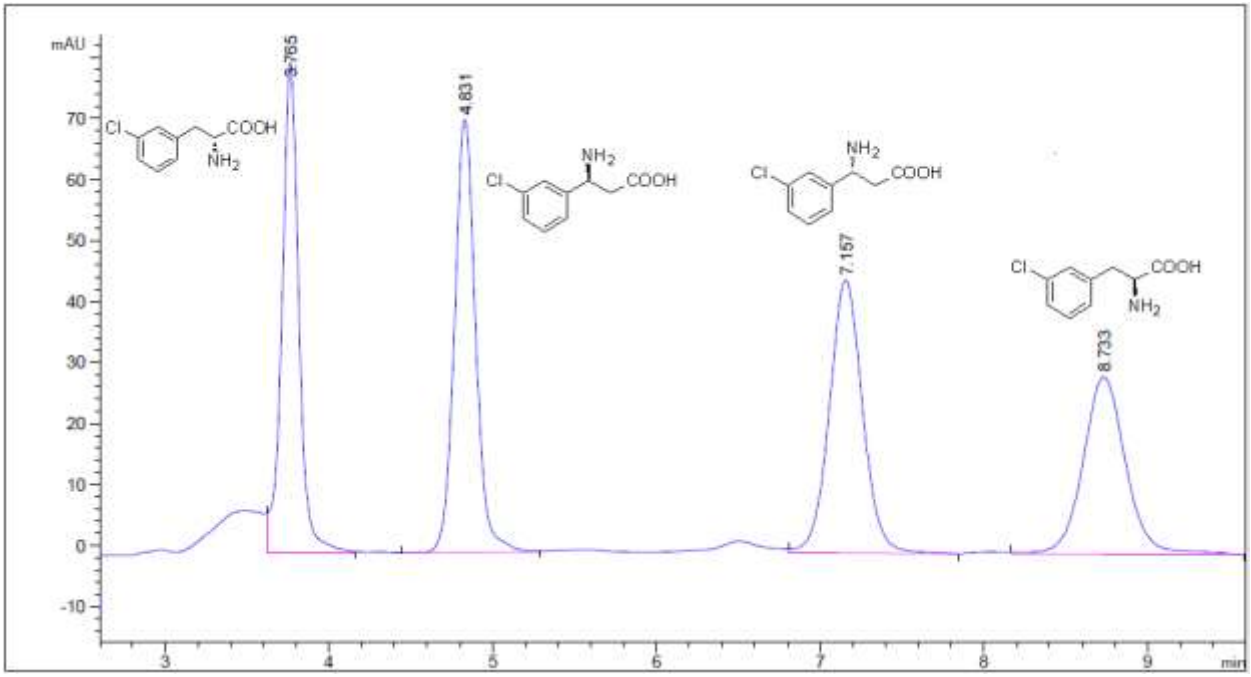
Products from (±)-2-chloro-β-phenylalanine (*rac*-**2g**) by *Pa*PAM after 20 h [Crownpak CR-I(+) column]



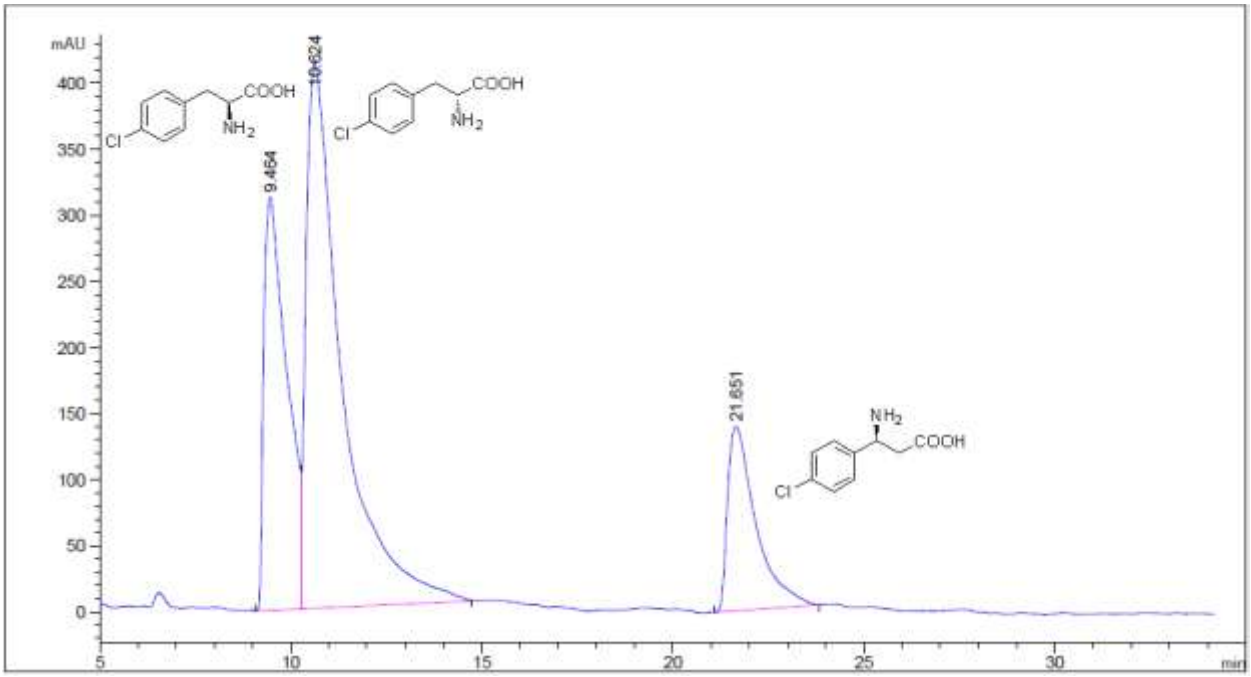
Enantioseparation of authentic (±)-2-chloro-α-phenylalanine (*rac*-**1g**) and (±)-2-chloro-β-phenylalanine (*rac*-**2g**) on Crownpak CR-I(+) column



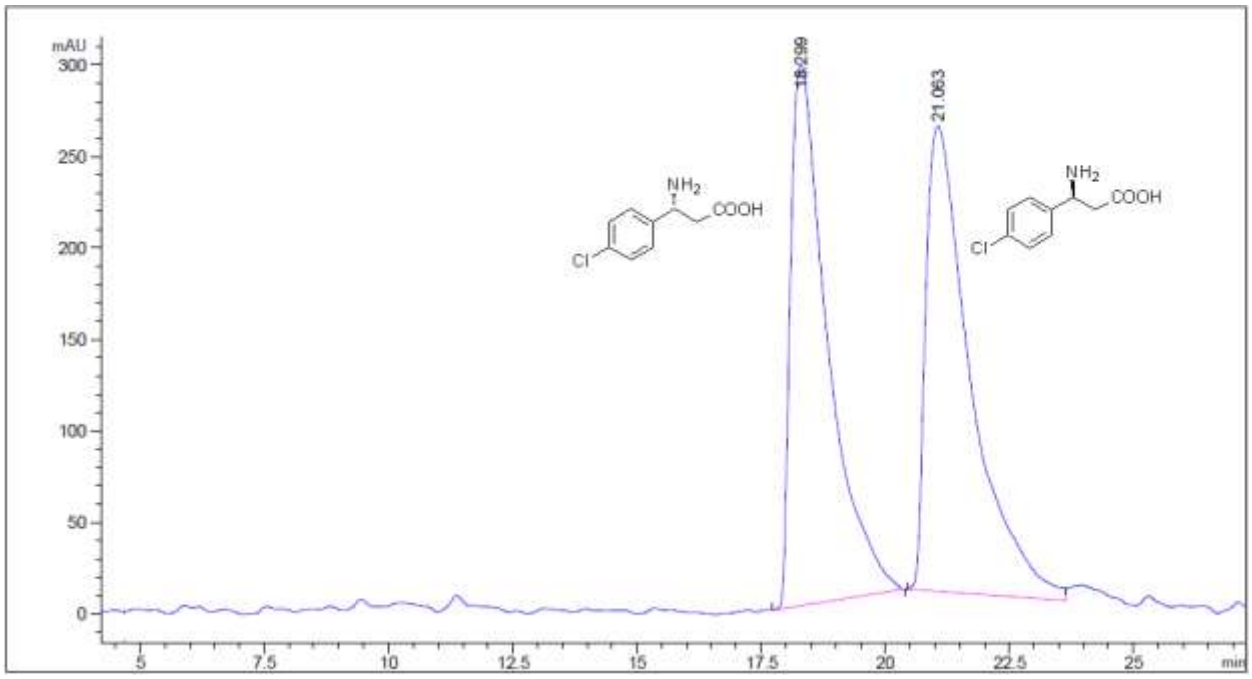
Products from (±)-3-chloro-α-phenylalanine (*rac*-**1h**) by *Pa*PAM after 20 h [Crownpak CR-I(+)column]



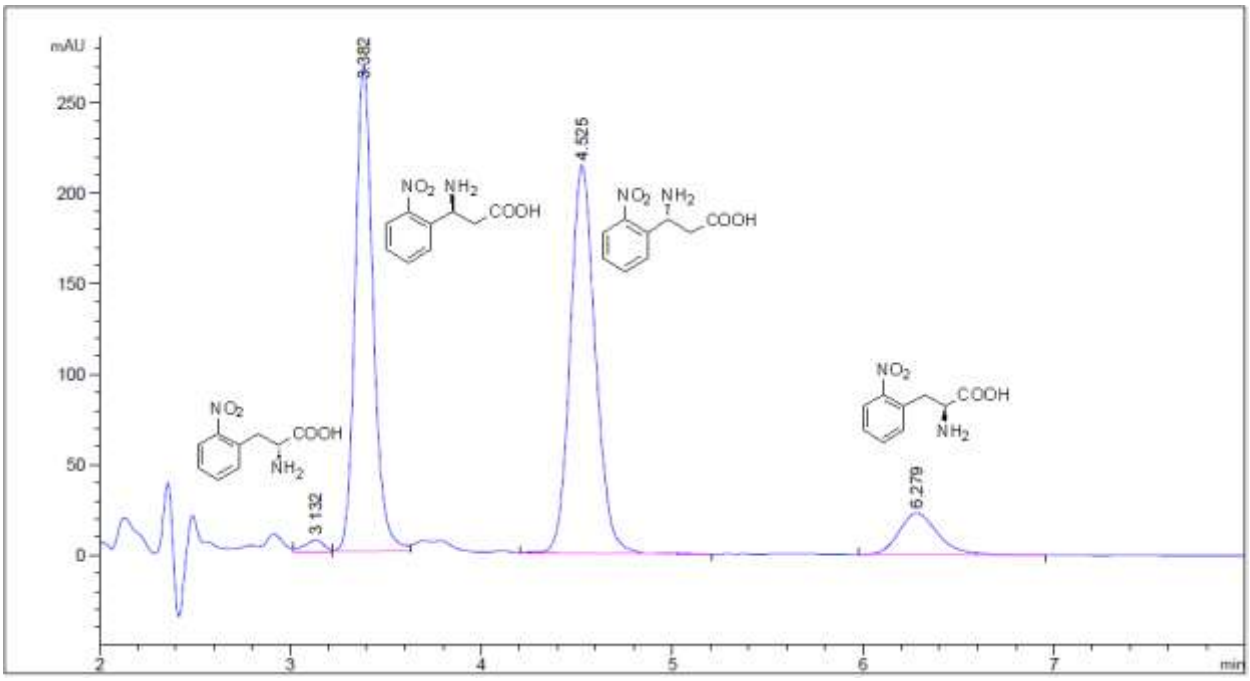
Enantioseparation of authentic (±)-3-chloro-α-phenylalanine (*rac*-**1f**) and (±)-3-chloro-β-phenylalanine (*rac*-**2h**) on Crownpak CR-I(+) column



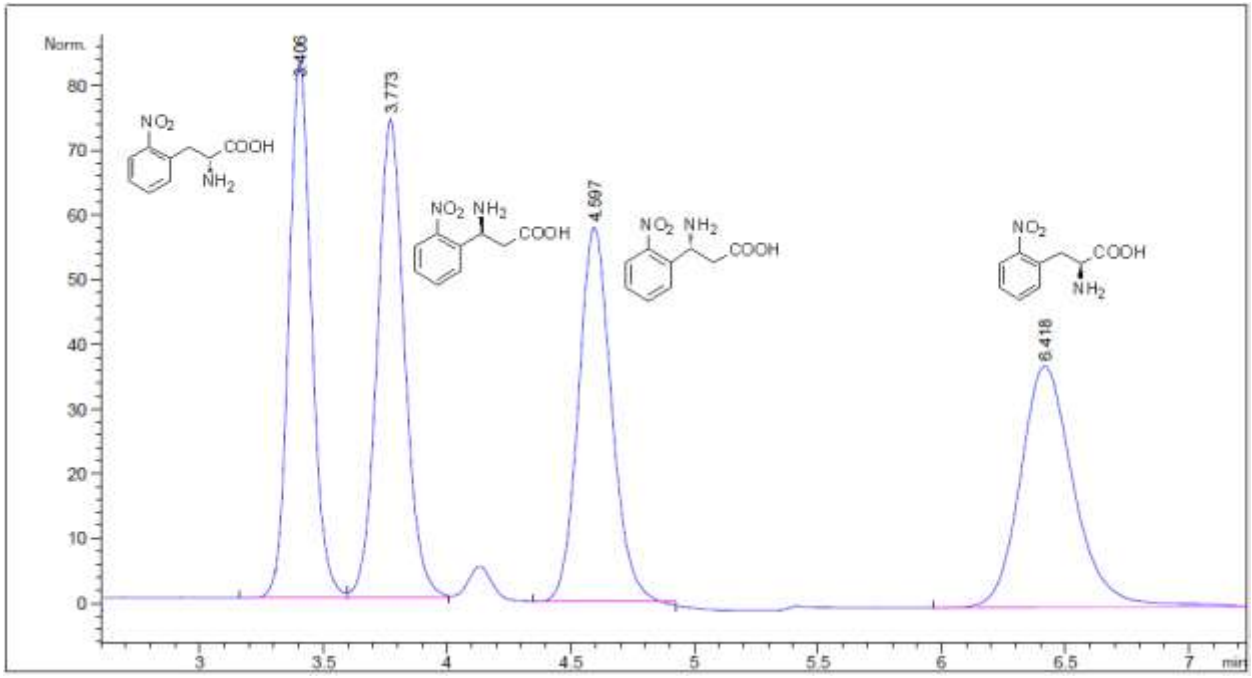
Products from (±)-4-chloro-α-phenylalanine (*rac*-**1i**) by *Pa*PAM after 20 h [Chiralpak ZWIX (+) column]



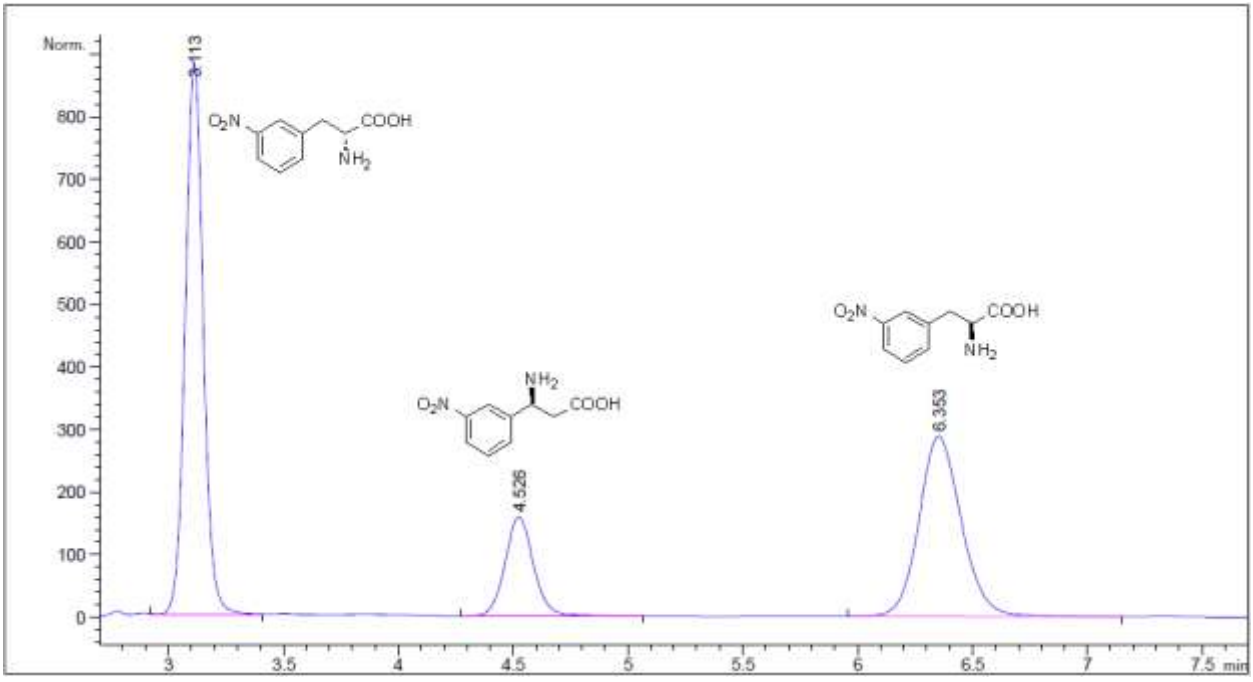
Enantioseparation of authentic (±)-4-chloro-α-phenylalanine (*rac*-**2i**) on Chiralpak ZWIX(+) column



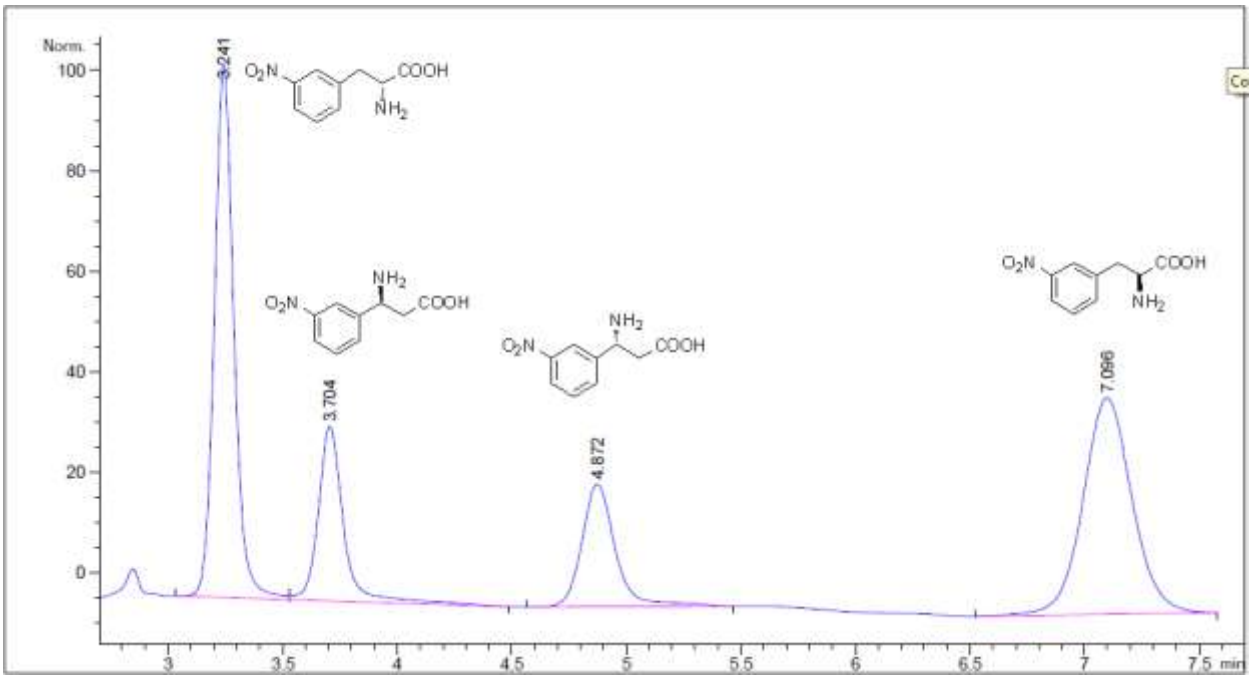
Products from (±)-4-nitro-β-phenylalanine (*rac*-**2j**) by *Pa*PAM after 20 h [Crownpak CR-I(+)column]



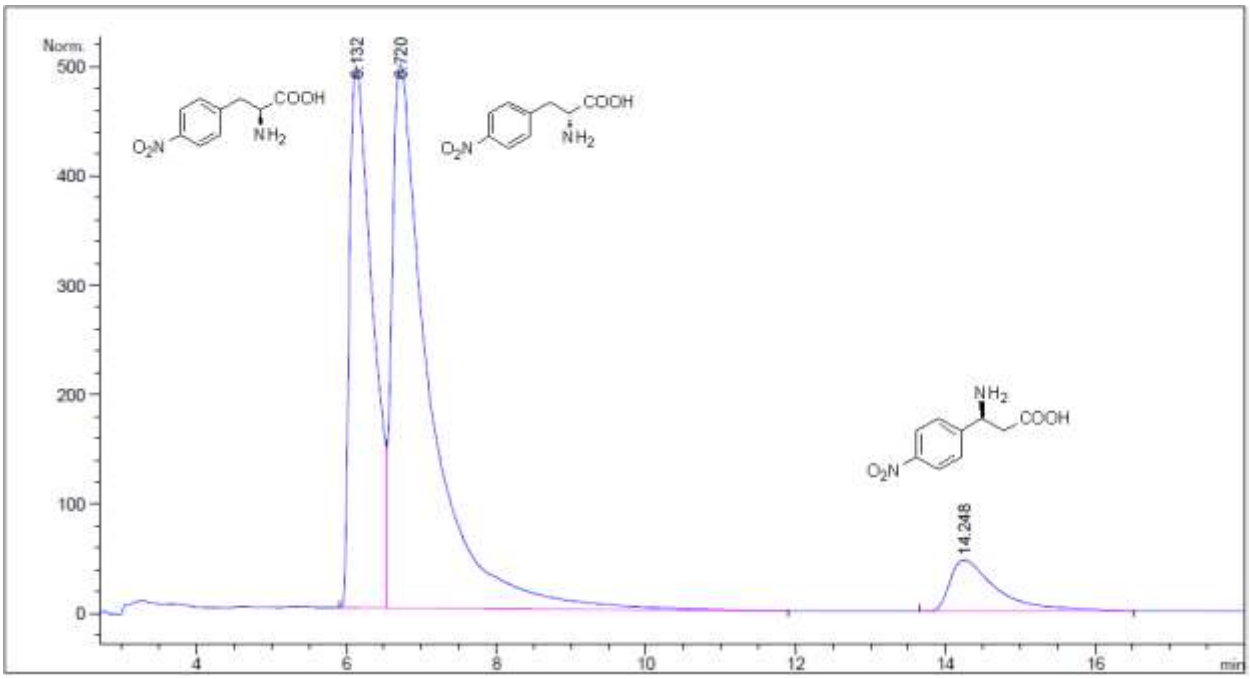
Enantioseparation of authentic (±)-4-nitro-α-phenylalanine (*rac*-**1j**)
and (±)-4-nitro-β-phenylalanine (*rac*-**2j**) onCrownpak CR-I(+) column



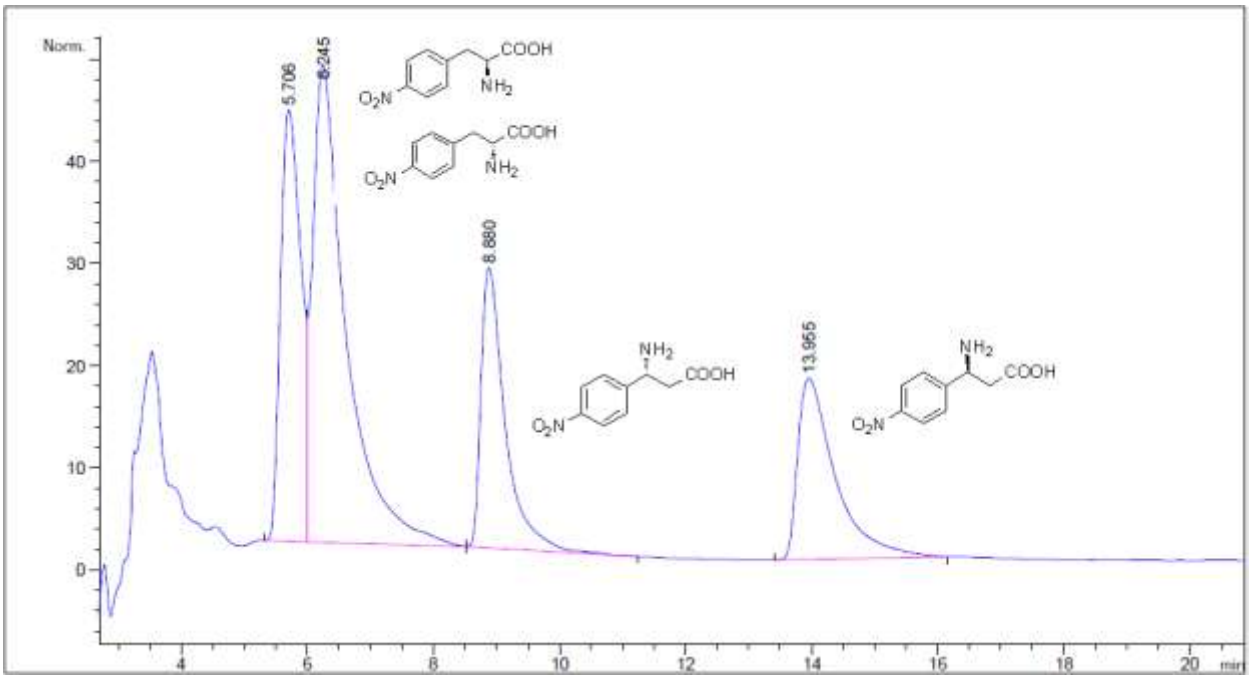
Products from (±)-3-nitro-α-phenylalanine (*rac*-**1k**) by *Pa*PAM after 20 h
[Crownpak CR-I(+) column]



Enantioseparation of authentic (±)-3-nitro-α-phenylalanine (*rac*-**1k**)
and (±)-3-nitro-β-phenylalanine (*rac*-**2k**) on Crownpak CR-I(+) column



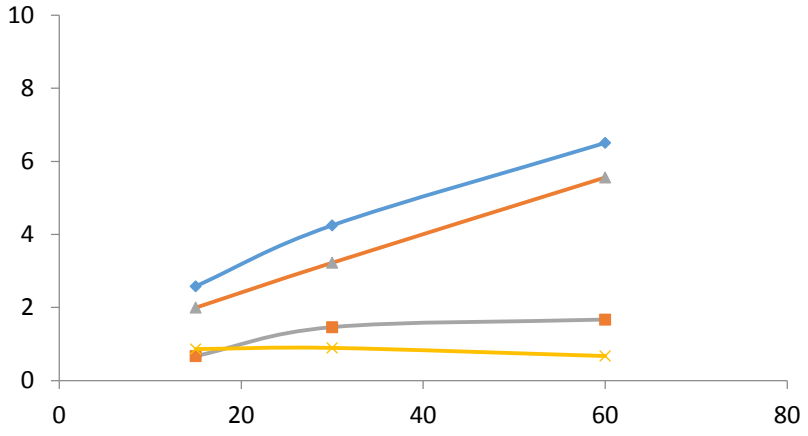
Products from (±)-4-nitro-α-phenylalanine (*rac*-**1l**) catalyzed by *Pa*PAM, after 20 h
[Chiralpack ZWIX(+) column]



Enantioseparation of authentic (±)-4-nitro-α-phenylalanine (*rac*-**1l**)
and (±)-4-nitro-β-phenylalanine (*rac*-**2l**) on Chiralpak ZWIX(+) column

5. Enzymatic reaction starting from (±)-β-phenylalanine 2a and (*S*)-β-phenylalanine (*S*)-2a

The time course profiles of the product formation in *Pa*PAM catalysed reactions from (*S*)-β-phenylalanine and (±)-β-phenylalanine were determined HPLC on Gemini NX-C-18 column



Time course profiles: (A) conversion of (*S*)-β-phenylalanine [5 mM, (*S*)-**2a**] into (*S*)-α-phenylalanine [(*S*)-**1a**, ◆] and cinnamic acid (**2a**, ▲); (B) conversion of (±)-β-phenylalanine (10 mM, **2a**) into (*S*)-α-phenylalanine [(*S*)-**1a**, ■] and cinnamic acid (**2a**, ×)

a. Immobilization of *Pc*PAL on MNPs

Into a TRIS buffer solution (3 mL, 0.1 M, pH 8.8) epoxy-MNPs [MagneCat-250GP14 (magnetic nanoparticles functionalized with epoxy groups, diameter 250nm)n 108 mg] and were dispersed by ultrasonication (35 kHz, 20 min). The MNP suspension was added to a solution of *Pc*PAL (3 mg mL^{-1}, în TRIS buffer: 4 mL, 0.1 M, pH 8.8), and the reaction mixture was shaken for 24 h (25 °C, 450 rpm). The enzyme-coated nanoparticles were colected with a magnet and the solution was removed. The MNP preparation was washed with TRIS buffer 3 × 4 mL, 0.1 M, pH 8.8) and ethanol (4 mL). The obtained biocatalyst was dried at room temperature for 2 h. After immobilization a negligible quantity of protein was detected, determind by the Bradford method.

b. Covalent immobilization of *Pc*PAL on $SwCNT_{COOH}$ via GDE based linker

$SwCNT_{COOH}$ (carboxy single walled carbon nanotubes) was incubated with carbonyldiimidazole (CDI) with shaking (at 1350 rpm at room temperature overnight), with occasional sonication to avoid bundled SwCNT formation. After CDI activation, the sample was filtered on membrane filter and then washed with CH_2Cl_2.

To the CDI-activated $SwCNT_{COOH}$, propane-1,3-diamine was added in distilled water and the reaction mixture was shaken (at 1350 rpm at room temperature overnight), with occasional sonication to avoid bundled SwCNT formation. After the propane-1,3-diamine coupling, the sample was filtered on membrane filter and then washed with distilled water.

A solution of glycerol diglycidyl ether (GDE)) was added to the propane-1,3-diamine-coupled $SwCNT_{COOH}$, and the reaction mixture was shaken (at 1350 rpm at room temperature overnight), with occasional sonication to avoid bundled SwCNT formation. After incubation the sample was filtered on membrane filter and then washed with CH_2Cl_2.

To the resulted bisepoxide-activated $SwCNT_{COOH}$, PAL was added and the mixture was shaken at room temperature at 1350 rpm, overnight. After the PAM-immobilization, the resulted biocatalyst ($SwCNT_{COOH}$-PAL^{II}) was filtered off on a membrane filter and washed with distilled water. The amount of PAL immobilized on the bisepoxide-activated $SwCNT_{COOH}$ was determined from the total mass of the enzyme in the solution before the immobilization and in the unified filtrates after the immobilization (measured spectrophotometrically using the Bradford method).

Biotransformation of the substrates with immobilized PAL and PAM for the synthesis of both enantiomers of α- and β arilalanines

Phenylalanine ammonia-lyases (PAL; EC 4.3.1.24) are homotetrameric enzymes performing the non-oxidative deamination of L-phenylalanine into (*E*)-cinnamic acid, while phenylalanine 2,3-aminomutases (EC 5.4.3.x) are catalyzing the izomerization of L-phenylalanine ori L- ori D-β- phenylalanine depending on the origin of the enzyme (**Fig 3**). PAL has been used as biocatalyst for the synthesis of L-α- amino acids from acrilates and in processes

of enzimatic kinetic resolution forming D-α-amino acids from racemic mixtures, and the PAL-Pam tandem was used for the synthesis of L- sau D-β-arilalanines (PAM).

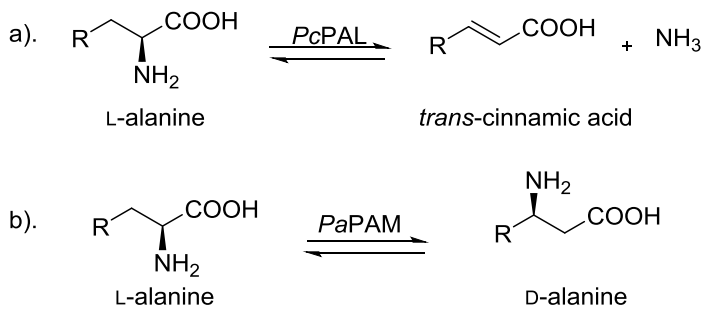


Fig. 3. Reactions catalyzd by PAL (**a**)) and PAM (**b**).).

*Pc*PAL și *Pa*PAM are the most active, stable and selective in their purified form and immobilized in the two mentioned forms were used in continuous-flow reactors, in tubular reactors, for obtaining both anatiomers of some unnatural analogues of α- și β arilalanine. Thus we proposed two methods. (*R*)-β-phenylalanine was obtained starting from the racemic β phenylalanine, using *Pa*PAM and *Pc*PAL in tandem.

*Pa*PAM transforms (*S*)-β-phenylalanine in (*S*)-α-phenylalanine, which is converted in cinnamic acid in the presence of *Pc*PAL (Fig. 4b). Obtaining the (*S*)-β-arylalanine implyes two steps. În the first step *Pa*PAM catalyse the transformation of)*S*)-α-arylalanine, obtained în the ammonia addition reaction to the coresponding cinnamic acid, to (*S*)-β arilalanine, but the reaction stops at the equilibrum concentration of these. Thus the immobilized *Pa*PAM was removed from the reaction mixture and *Pc*PAL was added for the transformation of the unreacted (*S*)-α-arylalanine în acrilic acid (**Fig. 4a.**). The enantiopure amino acids were purified on ion exchangers.

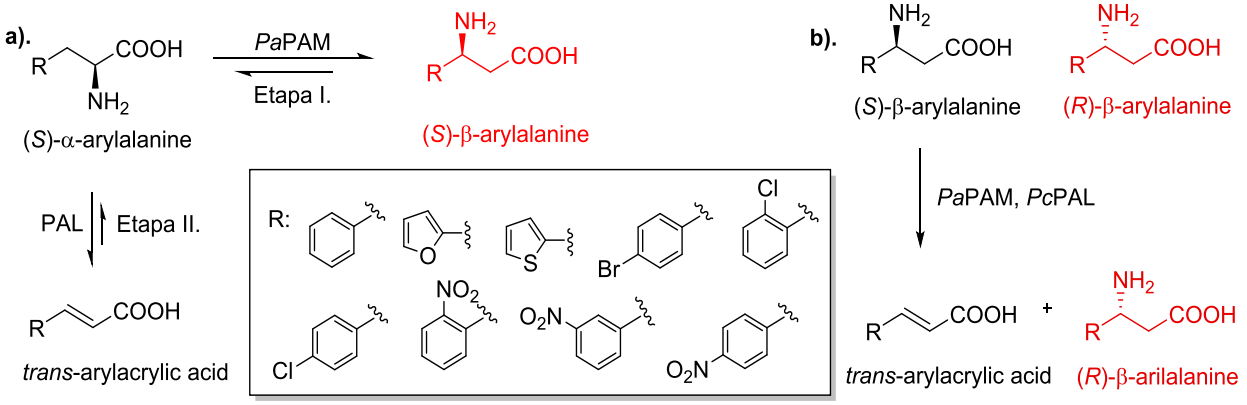


Fig. 4. Synthesis of (*S*)-β-arylalanine (**a**) and synthesis of (*R*)-β-arylalanine (**b**).

The conversions were determinde by high pressure liquid chromatography (HPLC) using an achiral column Gemini NX-C-18 column (150 × 4.6 mm × 5 µm). Mobile phase: A: NH_4OH buffer (0.1 M, pH 9.0) / B: MeOH, flow rate: 1 mL/min. The separation conditions and the extinction coefficients are presented in table 4. The enantiopurity of the compounds was

determined in chiral conditions, using Crownpak CR-I(+) (150 × 3.0 mm × 5 µm) as chiral column and $HClO_4$ (pH 1.5):acetonitrile 70:30 as mobile phase, flow rate 0.4mLmin^{-1}.

Table 4. HPLC conditions and response factors

Aryl moiety in 1,2,3	Eluent*	λ	Response factor**		
	[% B]	[nm]	**2** *vs***1**	**3** *vs***1**	**3***vs***2**
Phenyl	10 to 39 in 12 min	220	1.988	0.161	0.084
Thiophen-2-yl	10 to 39 in 12 min	250	1.146	0.413	0.387
4-Bromophenyl	20 to 54 in 12min	220	1.857	0.372	0.222
2-Fluorophenyl	10 to 39 in 12 min	220	1.249	0.139	0.119
3-Fluorophenyl	10 to 39 in 12min	220	3.039	0.156	0.05
4-Fluorophenyl	10 to 39 in 12 min	220	1.388	0.123	0.095
2-Chlorophenyl	10 to 39 in 12 min	220	–	–	0.386
3-Chlorophenyl	15 to 50 in 15 min	220	0.817	0.265	–
4-Chlorophenyl	15 to 50 in 15 min	220	0.948	0.679	–
2-Nitrophenyl	10 to 50 in 15 min	260	–	–	0.019
3-Nitrophenyl	10 to 50 in 15 min	260	2.238	0.144	–
4-Nitrophenyl	10 to 50 in 15 min	220	1.138	1.228	–

*Eluent **A**: NH_4OH buffer (0.1 M, pH 9.0); **B**: MeOH

** **1**– *rac*-α-arylalanine; **2**– *rac*-β-arylalanine; **3** – arylacrylate

Synthesis of *(R)-β arylalanines*

The racemic *β* amino acid (2 mg) was dissolved in $(NH_4)_2CO_3$ (0.1M, pH 8.8, 1 mL), and into the mixture immobilized *Pa*PAM (0.6 mg) and *Pc*PAL (0.4 mg) were added. The reaction mixture was incubated at 30°C under continuous shaking. In different time intervals samples were taken (70 µL), and 70 µL of methanol was added for the precipitation of the protein. The solution was filtered, was diluted with mobile phase (160 µL) and was injected to HPLC.

Tabel 5. *Rac*-β-arylalanines transformed by *Pa*PAM and *Pc*PAL

Substrate	time (h)	Conv. (%)
(±)-*β*-phenylalanine	4	15.56
	19	22.41
(±)-*β*-tiophen-2-yl alanine	4	4.78
	19	5.69
(±)-2-fluoro-*β*-phenylalanine	4	18.22
	19	23.55
(±)-3-fluoro-*β*-phenylalanine	4	13.68
	19	14.35
(±)-4-fluoro-*β*-phenylalanine	4	7.28
	19	10.34
(±)-2-chloro-*β*-phenylalanine	4	18.81
	19	24.65

Results are indicating that the nature and the position of the substituents can strongly modify the *Pa*PAMs activity, and the efficacy of the reactions in tandem. Thus *β*-phenylalanines *orto*-

substituted and heteroaryl alanines are good substrates for *Pa*PAM, while in case *β*-phenylalanines *meta and para*-substituted low conversion were obtained.

Synthesis of (S)- β-arylalanines

(*S*)-β-arilalanine is synthesized starting from (*S*)-α-arilalanine, which is prepared with the addition of ammonia (6M $(NH_4)_2CO_3$) to the corresponding acrylic acid, mediated by *Pc*PAL.

The corresponding cinnamic acid (2 mg) is dissolved in a solution of $(NH_4)_2CO_3$ (6M, pH 10, 1 mL), *Pc*PAL (0.4 mg) was added and the reaction mixture was incubated at 30°C under continuous stirring. After the reaction time the enzyme is inactivated by heating the solution to 95°C for 5 minutes. The enzyme is removed by filtering, and the obtained solution is freezed at 80°C (30 min) and it is liophilized overnight.

Table 6. Sybthesis of (*S*)-α-alanines by the ammonia addition reaction catalyzed by *Pc*PAL

Substrate	time (h)	ee_p (%)	Conv. (%)
Cinnamic acid	48	99	91
tiophen-2-yl acrylic acid	48	99	97
2-fluoro acrylic acid	48	99	98
3-fluoro acrylic acid	48	99	95
4-fluoro acrylic acid	48	99	92
3-chloro acrylic acid	48	99	95
4-chloro acrylic acid	48	99	91
3-nitro acrylic acid	48	93	96
4-nitro acrylic acid	48	85	99

In the second step the previously obtained (*S*)-α-arilalanine was dissolved in a solution of $(NH_4)_2CO_3$ (0.1M, pH 8.8, 1 mL) *Pa*PAM (0.6 mg) was added and the reaction was incubated at 30°Cunder continuous stirring. After the reaction the *Pa*PAM is removed by filtration and *Pc*PAL (0.4 mg) is added in the reaction mixture. The reaction is incubated at 30°C and it is monitored by cromatography.

It must be mentioned that in this case *Pa*PAM is not transforming the α-alanines substituted in orto with Cl and NO_2, but the alanines meta and para substituted are accepted as substrate.

Table 7. Synthesis of (*S*)-*β*-alanines from (*S*)-α-alanines catalyzed by *Pa*PAM and *Pc*PAL

Substrate	time (h)	$ee_β$ (%)	Conv.[1] (%)	Conv.[2] (%)
(*S*)-α-phenylalanine	17	99	47.74	2.52
	48	99	57.95	5.48
(*S*)-tiphen-2-yl α-alanine	17	99	24.52	13.5
	48	99	26.42	14.51
(*S*)-2-fluoro- α-alanine	17	99	2.77	32.30
	48	99	2.92	43.65
(*S*)-3-fluoro- α-alanine	17	99	6.68	65.54
	48	99	7.06	69.94
(*S*)-4-fluoro- α-alanine	17	99	28.68	11.94
	48	99	34.46	14.50
(*S*)-3-chloro- α-alanine	17	99	9.71	6.47
	48	99	9.81	6.56
(*S*)-4-chloro- α-alanine	17	99	13.16	10.87
	48	99	13.32	10.87

(*S*)-3-nitro- α-alanine	17	-	-	-
(*S*)-4-nitro- α-alanine	17	-	-	-

[1]Conversion of the transformation (*S*)-α in (*S*)-*β*;
[2]Conversion of the transformation (*S*)-*α* in acrilic acid

Using the MagneChip reaction system these procedures were optimized in continuous flow for the synthesis of both enantiomers of α- and β arylalalnines. The concentration of the substrate was adjusted so the maximum conversion to be achieved. The study was not yet published, the results are in an article under evaluation, therefore the results can not be presented here.

Articles published in 2016

1. Ferenc Ender, Diána Weiser, Botond Nagy, Csaba László Bencze, Csaba Paizs, Péter Pálovics and László Poppe: **Microfluidic multiple cell chip reactor filled with enzyme-coated magnetic nanoparticles – An efficient and flexible novel tool for enzyme catalyzed biotransformations**; *Journal of Flow Chemistry* **2016,** *6(1)*, 43-52

2. A. Varga, G. Bánóczi, B. Nagy, L. C. Bencze, M. I. Toșa, Á. Gellért, F. D. Irimie, J. Rétey, L. Poppe, C. Paizs: **Influence of the aromatic moiety in α- and β-arylalanines on their biotransformation with phenylalanine 2,3-aminomutase from *Pantoea agglomerans;*** *RSC Advances*, **2016**, **00**, 1-9

3. Andrea Varga, Alina Filip, László-Csaba Bencze, Péter Sátorhelyi, Evelin Bell, Beáta G. Vértessy, László Poppe, Csaba Paizs: **Expression and purification of recombinant phenylalanine 2,3-aminomutase from *Pantoea agglomerans;*** *Studia Universitas Babes-Bolyai, Ser.* Chemia **2016**, *LXI (2)*, 7-19