Scientific synthetic report of the project
IMMOBILIZED WILD-TYPE AND MUTANT AMMONIA-LYASES AND AMINOMUTASES FOR THE PRODUCTION OF ALPHA- AND BETA-PHENYLALANINE ANALOGUES
2014

OBIECTIVE 1: Synthesis and characterization of substrates for the biotransformations

1. Chemical synthesis of unnatural racemic α- and β-arilalanines and their acrylate counterparts

In order to investigate the activity of phenylalanine ammonia-lyase (PAL) and phenylalanine aminomutase (PAM), a series of α- and β-aril- and heteroarilalanines were chemically synthesized starting from the corresponding aldehydes (Figure 1.).

1.1. Chemical synthesis of the racemic 2-amino-3-(hetero)aril propionic acids (Figure 1., Route 1.)

To a stirred solution of aldehydes (4 g) in methanol (40 mL) $NaBH_4$ was added in small portion at room temperature, until the entire amount of the aldehyde was transformed (controlled by TLC). Then the methanol was evaporated under reduced pressure, water was added to the products and the pH was adjusted to 2-3 with 1M HCl. The mixtures were extracted with dichloromethane; the organic phases were dried over Na_2SO_4 and concentrated under reduced pressure to obtain the corresponding alcohols **2a-c** which were used in the next step without further purification.

Into a solution of alcohols **2a-c** and benzotriazole (1.5 eq) in dry dichloromethane $SOCl_2$ (4 eq) was added in small portions. Before the addition is complete, benzotriazole hydrochloride started to precipitate. The mixtures were stirred further for 15 min. At the end the solid was filtered off and washed with dry CH_2Cl_2. The filtrates were washed with 10% HCl, water, 5% NaOH. The organic layers were separated and dried over anhydrous Na_2SO_4. The products were immediately used in the next step.

55% NaH oil suspension (0.65 g, 27 mmol) was added into dry DMF (40 mL) and it was vigorously stirred for 30 min, under argon at room temperature. Subsequently 2-acetylamino-malonic acid diethyl ester (2.77 g, 14 mmol) was added to the suspension, the mixture was stirred for 30 min, chloromethylene derivatives (2.68 g, 12.4 mmol) were added, then the mixtures were stirred for 3h at room temperature, for4 h at 60°C, and finally the products were poured into ice. The resulting precipitates were filtered off, washed with water and dried under reduced pressure.

The *N*-substituted-malonic acid diethyl esters (2 g) dissolved in methanol (7 mL) were added into 10% NaOH solution (10 mL), and then the mixtures were stirred for several hours at reflux temperature. The methanol was removed in vacuo , the reaction mixtures were extracted with

CH_2Cl_2 and the pH was adjusted to 1 with conc. HCl at -10°C. The resulting precipitates were filtered and dried in vacuo. The formed dicarboxylic acids were suspended in toluene and refluxed for 2 h. The solvent was removed in vacuo affording the pure products.

Into the *N*-protected amino acids (1 g) in 1,4-dioxane (15 mL) concentrated HCl (3 mL) was added and the mixtures were refluxed for 4 h, cooled to room temperature, obtaining the products which were filtered, dried and washed with Et_2O. Further purification should be realized by isoelectric precipitation of the amino acids hydrochlorides at pH 5-6.

2-amino-3-phenylpropionic acid: ^{1}H NMR (400 MHz, D_2O) δ 7.44 – 7.40 (m, 2H), 7.32 – 7.24 (m, 3H), 3.69 (t, J = 7.0 Hz, 1H), 3.25 (dd, J = 12.4, 7.1 Hz, 1H), 2.89 (dd, J = 12.3, 7.1 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 174.27, 135.46, 130.11, 128.78, 127.00, 56.13, 36.72. HRMS Calcd for $C_9H_{11}NO_2$ $(M+)^-$ 165.0794.Found 165.0802.

2-amino-3-(thiophen-2-yl)propionic acid**:** ^{1}H NMR (400 MHz, D_2O): *δ* 3.38-3.49 (m, 2H), 4.05-4.08 (m, 1H), 7.10 (s, 1H), 8.10 (s, 1H), 8.17 (s, 1H); ^{13}C NMR (101 MHz, D_2O): *δ* 35.7, 58.0, 125.4, 127.2, 128.0, 141.2, 181.6; HRMS Calcd for $C_7H_9NO_2S$ $(M+)^-$ 171.0354.Found 171.0349.

2-amino-3-(furan-2-yl)propionic acid**:** ^{1}H NMR (400 MHz, D_2O): *δ* 3.56-3.71 (m, 2H), 4.14-4.17 (m, 1H), 6.89 (s, 1H), 7.10 (s, 1H), 8.14 (s, 1H); ^{13}C NMR (101 MHz, D_2O): *δ* 34.1, 56.0, 107.9, 111.3, 142.8, 153.6, 181.8; HRMS Calcd for $C_7H_9NO_3$ $(M+)^-$ 155.0582. Found 155.0571.

2-amino-3-(2-chlorophenyl)propionic acid**:** ^{1}H NMR (400 MHz, D_2O) δ 7.53 – 7.46 (m, 1H), 7.39 – 7.30 (m, 3H), 4.34 (dd, *J* = 8.4, 6.4 Hz, 1H), 3.52 (dd, *J* = 14.4, 6.4 Hz, 1H), 3.27 (dd, *J* = 14.4, 8.4 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 171.55, 133.92, 131.97, 131.76, 129.87, 129.67, 127.60, 53.03, 33.82; HRMS Calcd for $C_9H_9ClNO_2$ $(M-H)^-$ 198.0316. Found 198.0331.

2-amino-3-(4-chlorophenyl)propionic acid: ^{1}H NMR (300 MHz, D_2O) δ 7.01 (d, *J* = 8.1 Hz, 2H), 6.88 (d, *J* = 8.0 Hz, 2H), 3.14 (t, *J* = 6.4 Hz, 1H), 2.62 (dd, *J* = 13.4, 5.6 Hz, 1H), 2.47 (dd, *J* = 13.4, 7.4 Hz, 1H); ^{13}C NMR (76 MHz, D_2O) δ 181.99, 136.66, 131.34, 130.63, 128.16, 57.09, 40.00; HRMS Calcd for $C_9H_9ClNO_2$ $(M-H)^-$ 198.0316. Found 198.0328.

2-amino-3-(2-nitrophenyl)propionic acid**:** ^{1}H NMR (400 MHz, D_2O) δ 8.11 (dd, *J* = 8.3, 1.4 Hz, 1H), 7.70 (td, *J* = 7.5, 1.4 Hz, 1H), 7.55 (ddd, *J* = 8.5, 7.6, 1.4 Hz, 1H), 7.49 (dd, *J* = 7.7, 1.4 Hz, 1H), 4.11 (t, *J* = 7.3 Hz, 1H), 3.53 (dd, *J* = 14.0, 6.9 Hz, 1H), 3.38 (dd, *J* = 14.0, 7.7 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 173.24, 148.71, 134.32, 133.17, 130.64, 129.05, 125.52, 55.26, 33.99; HRMS Calcd for $C_9H_9N_2O_4$ $(M-H)^-$ 209.0557 Found 209.0571.

2-amino-3-(3-nitrophenyl)propionic acid**:** ^{1}H NMR (400 MHz, D_2O) δ 8.19 (dd, *J* = 6.7, 1.3 Hz, 2H), 7.72 (dt, *J* = 7.7, 1.4 Hz, 1H), 7.64 – 7.59 (m, 1H), 4.39 (dd, *J* = 7.4, 6.0 Hz, 1H), 3.46 (dd, *J* = 14.7, 6.0 Hz, 1H), 3.35 (dd, *J* = 14.7, 7.5 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 171.18, 148.06, 136.19, 136.11, 130.24, 124.23, 122.99, 53.87, 35.18; HRMS Calcd for $C_9H_9N_2O_4$ $(M-H)^-$ 209.0557 Found 209.0567.

2-amino-3-(4-nitrophenyl)propionic acid: ^{1}H NMR (400 MHz, D_2O) δ 8.21 (d, *J* = 8.3 Hz, 2H), 7.52 (d, *J* = 8.3 Hz, 2H), 4.36 (t, *J* = 6.7 Hz, 1H), 3.44 (dd, *J* = 14.6, 6.0 Hz, 1H), 3.33 (dd,

J = 14.5, 7.5 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 171.22, 147.17, 142.23, 130.46, 124.12, 53.81, 35.44; HRMS Calcd for $C_9H_9N_2O_4$ $(M-H)^-$ 209.0557 Found 209.0571.

2-amino-3-(4-bromophenyl)propionic acid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.91 (d, J = 7.9 Hz, 2H), 8.64 (d, J = 7.9 Hz, 2H), 5.62 (dd, J = 7.5, 5.4 Hz, 1H), 4.71 (dd, J = 14.6, 5.5 Hz, 1H), 4.58 (dd, J = 14.6, 7.6 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 180.58, 135.25, 133.45, 132.88, 122.78, 56.49, 36.90. HRMS Calcd for $C_9H_{10}BrNO_2$ $(M-H)^-$ 242.9895 Found 242.9901.

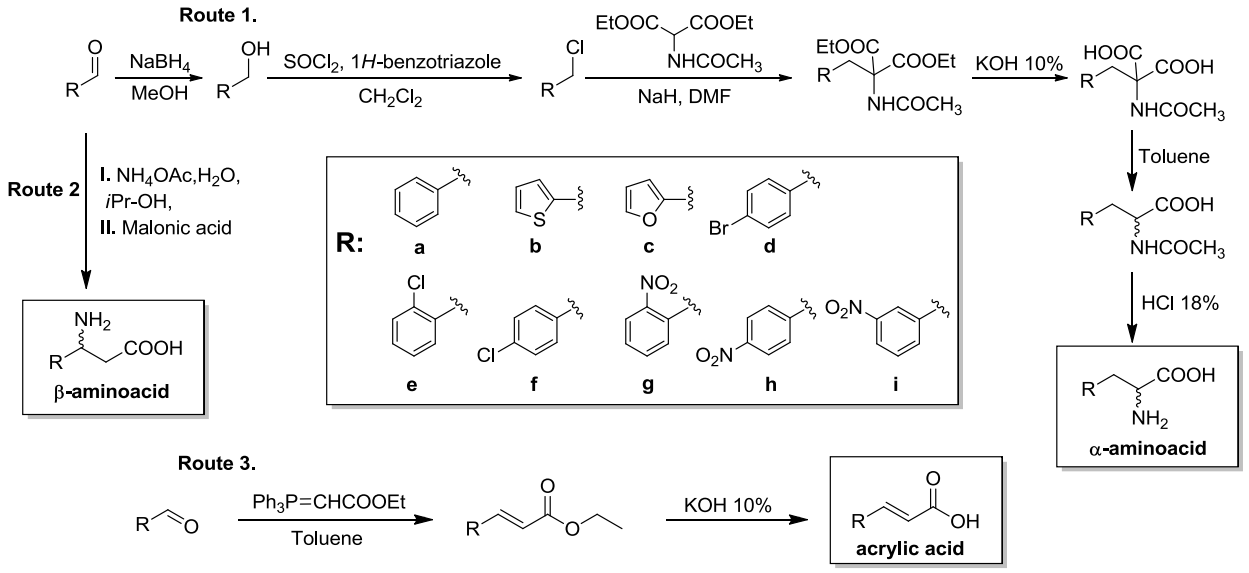


Figure 1. Synthesis of racemic substrates

1.2. Chemical synthesis of the racemic 3-amino-3-(hetero)aryl propanoic acids

The corresponding benzaldehyde derivatives (2 g), ammonium acetate (35.7 mmol) and a catalytic amount of water were stirred in isopropyl alcohol for 20 min at 50°C. Malonic acid (1.63 g, 15.7 mmol) was then added. During the addition the reaction mixture may solidify, therefore the usage of isopropyl alcohol in excess is recommended. After refluxing 8 h, the amino acids were separated by hot filtration, washed with isopropyl alcohol, and dried under reduced pressure.

3-amino-3-phenylpropanoic acid: ^{1}H NMR (400 MHz, D_2O) δ 7.47 – 7.42 (m, 2H), 7.39 (dd, J = 8.2, 6.6 Hz, 2H), 7.34 – 7.27 (m, 1H), 4.58 (t, J = 7.0 Hz, 1H), 2.95 (dd, J = 12.3, 7.0 Hz, 1H), 2.70 (dd, J = 12.4, 7.0 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 173.10, 141.51, 128.28, 127.67, 126.82, 53.03, 42.27. HRMS Calcd for $C_9H_{11}NO_2$ $(M+)^-$ 165.0794.Found 165.0802.

3-amino-3-(furan-2-yl) propanoicacid**:** ^{1}H NMR (400 MHz, DMSO) δ 7.71 (d, *J* = 1.7 Hz, 1H), 6.56 (d, *J* = 3.3 Hz, 1H), 6.47 (dd, *J* = 3.3, 1.8 Hz, 1H), 4.60 (dd, *J* = 9.5, 5.1 Hz, 1H), 3.10 (dd, *J* = 16.6, 5.2 Hz, 1H), 2.98 (dd, *J* = 16.6, 9.2 Hz, 1H); ^{13}C NMR (101 MHz, DMSO) δ 170.43, 149.75, 143.43, 110.91, 109.22, 44.38, 36.06; HRMS Calcd for $C_7H_8NO_3$ $(M-H)^-$ 154.0499. Found 154.0511.

***3-amino-3-(thiophen-2-yl) propanoicacid*:** ^{1}H NMR (400 MHz, D_2O) δ 7.50 (dd, *J* = 5.1 Hz, 1H), 7.24 (d, *J* = 3.6 Hz, 1H), 7.10 (dd, *J* = 5.1, 3.6 Hz, 1H), 4.96 (t, *J* =7.1 Hz, 1H), 2.95 (dd, *J* = 15.4, 6.4 Hz, 1H), 2.90 (dd, *J* = 15.4, 5.9 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 176.78, 138.15, 127.44, 127.08, 47.99, 40.77; HRMS Calcd for $C_7H_8NO_2S$ $(M-H)^-$ 170.0270. Found 170.0283.

3-amino-3-(2-chlorophenyl) propanoicacid: ^{1}H NMR (400 MHz, D_2O) δ 7.40 – 7.34 (m, 2H), 7.30 – 7.22 (m, 2H), 5.08 (dd, J = 8.0, 6.3 Hz, 1H), 3.06 (dd, J = 17.4, 7.9 Hz, 1H), 2.98 (dd, J = 17.4, 6.4 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 173.06, 132.89, 132.22, 131.01, 130.30, 128.02, 127.55, 47.90, 36.49; HRMS Calcd for $C_9H_9ClNO_2$ $(M-H)^-$ 198.0316. Found 198.0328.

3-amino-3-(3-chlorophenyl)propionic acid: ^{1}H NMR (600 MHz, D_2O) δ 7.48 (t, *J* = 1.9 Hz, 1H), 7.46 – 7.39 (m, 2H), 7.36 (dt, *J* = 7.5, 1.7 Hz, 1H), 4.75 (t, *J* = 7.2 Hz, 1H), 3.15 (dd, *J* = 17.3, 7.8 Hz, 1H), 3.06 (dd, *J* = 17.3, 6.6 Hz, 1H); ^{13}C NMR (151 MHz, D_2O) δ 173.11, 137.03, 134.41, 130.87, 129.66, 127.16, 125.42, 50.92, 37.43.

3-amino-3-(4-chlorophenyl) propanoicacid: ^{1}H NMR (400 MHz, D_2O) δ 7.50 – 7.40 (m, 4H), 4.82 – 4.73 (m, 1H and solvent peak), 3.16 (dd, *J* = 17.2, 7.7 Hz, 1H), 3.07 (dd, *J* = 17.2, 6.7 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 173.21, 134.91, 133.72, 129.35, 128.66, 50.84, 37.49; HRMS Calcd for $C_9H_9ClNO_2$ $(M-H)^-$ 198.0316. Found 198.0331.

3-amino-3-(2-nitrophenyl) propanoicacid: ^{1}H NMR (400 MHz, D_2O) δ 8.07 (d, *J* = 8.1 Hz, 1H), 7.82 (t, *J* = 7.5 Hz, 1H), 7.75 (d, *J* = 7.7 Hz, 1H), 7.64 (t, *J* = 7.7 Hz, 1H), 5.32 (dd, *J* = 7.9, 6.2 Hz, 1H), 3.27 (dd, *J* = 17.6, 7.9 Hz, 1H), 3.18 (dd, *J* = 17.6, 6.2 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 172.94, 148.28, 134.94, 130.90, 129.53, 128.21, 125.76, 46.47, 36.97; HRMS Calcd for $C_9H_9N_2O_4$ $(M-H)^-$ 209.0557 Found 209.0568.

3-amino-3-(3-nitrophenyl) propanoicacid: ^{1}H NMR (400 MHz, D_2O) δ 8.32 (d, *J* = 2.0 Hz, 1H), 8.25 (dd, *J* = 8.2, 2.2 Hz, 1H), 7.83 (d, *J* = 7.7 Hz, 1H), 7.67 (t, *J* = 8.0 Hz, 1H), 4.79 (s, 78H), 2.88 (dd, *J* = 15.9, 7.7 Hz, 1H), 2.80 (dd, *J* = 15.9, 6.8 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 177.21, 148.14, 139.52, 133.65, 130.38, 123.83, 122.00, 52.12, 41.54; HRMS Calcd for $C_9H_9N_2O_4$ $(M-H)^-$ 209.0557 Found 209.0570.

3-amino-3-(4-nitrophenyl) propanoic acid: ^{1}H NMR (600 MHz, D_2O) δ 8.17 (d, *J* = 8.7 Hz, 2H), 7.61 (d, *J* = 8.8 Hz, 2H), 4.86 (t, *J* = 7.1 Hz, 1H), 3.15 (dd, *J* = 17.5, 7.6 Hz, 1H), 3.06 (dd, *J* = 17.5, 6.6 Hz, 1H); ^{13}C NMR (151 MHz, D_2O) δ 172.82, 148.04, 142.20, 128.46, 124.40, 50.75, 37.43; HRMS Calcd for $C_9H_9N_2O_4$ $(M-H)^-$ 209.0557 Found 209.0568.

3-amino-3-(4-bromophenyl) propanoicacid: ^{1}H NMR (300 MHz, D_2O) δ 7.55 (d, J = 8.0 Hz, 2H), 7.28 (d, J = 8.1 Hz, 2H), 3.08 (dd, J = 17.2, 7.6 Hz, 1H), 2.98 (dd, J = 17.2, 6.7 Hz, 1H); ^{13}C NMR (75 MHz, D_2O) δ 178.23, 154.14, 138.72, 131.65, 129.41, 118.63, 121.00, 52.12, 41.54. HRMS Calcd for $C_9H_{10}BrNO_2$ $(M-H)^-$ 242.9895 Found 242.9907.

1.3. Chemical synthesis of (*E*)-acrylic acids.

To a stirred solution of aldehydes (10 mmol) in 20 mL dry toluene triphenyl-λ^5-phosphanilidene acetic acid ethyl ester (4.35 g, 12.5 mmol) was added. The reaction mixtures were refluxed for 4 h, and then the toluene was removed in vacuo. The precipitate was taken up

in a minimal amount of CH_2Cl_2 and the crude product was purified with column chromatography on silica gel using CH_2Cl_2 as eluent. In each case 2-5% (*Z*)-isomer was also formed.

The (*E*)-acrylic acid esters (0.5 g) were suspended in 10% KOH (10 mL) and the mixtures were refluxed for 4 h. After that the reaction mixtures were cooled to 0°C and the pH was adjusted to 1 using conc. HCl. The formed precipitate was filtered, washed with water and dried under reduced pressure.

(E)-3-phenylacrylic acid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.70 – 7.60 (m, 3H), 7.56 – 7.41 (m, 3H), 6.35 (d, J = 15.2 Hz, 1H); ^{13}C NMR (101 MHz, DMSO) δ 171.43, 146.21, 134.27, 129.77, 128.99, 128.86, 118.01. HRMS Calcd for $C_9H_8O_2$ $(M+)^-$ 148.0528. Found 148.0534.

(E)-3-(furan-2-yl)acrylic acid**:** ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.96 (d, 1H), 7.22-7.23 (m, 1H), 7.34 (d, 1H), 7.81 (d, 1H), 8.25 (d, 1H); ^{13}C NMR (101 MHz, DMSO): δ 113.0, 113.6, 123.4, 128.1, 145.1, 152.1, 175.3. HRMS Calcd for $C_7H_6O_3$ $(M+)^-$ 138.0316. Found 138.0319.

(E)-3-(thiophen-2-yl)acrylic acid**:** ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.72 (d, *J* = 15.8 Hz, 1H), 7.29 (dt, *J* = 5.1, 1.0 Hz, 1H), 7.17 (dd, *J* = 3.8, 1.1 Hz, 1H), 6.97 (dd, *J* = 5.1, 3.6 Hz, 1H), 6.18 (d, *J* = 15.7 Hz, 2H); ^{13}C NMR (101 MHz, DMSO): δ 124.7, 128.3, 129.1, 130.5, 133.7, 141.0, 175.1. HRMS Calcd for $C_7H_6O_2S$ $(M+)^-$ 154.0088. Found 154.0080.

(E)-3-(2-chlorophenyl)acrylic acid**:** ^{1}H NMR (600 MHz, MeOD-d_4) δ 8.07 (d, *J* = 16.0 Hz, 1H), 7.78 (dd, *J* = 7.6, 1.9 Hz, 1H), 7.46 (dd, *J* = 7.7, 1.5 Hz, 1H), 7.36 (dtd, *J* = 17.8, 7.4, 1.6 Hz, 2H), 6.51 (d, *J* = 16.0 Hz, 1H); ^{13}C NMR (151 MHz, MeOD) δ 169.72, 141.47, 135.72, 133.79, 132.46, 131.13, 128.95, 128.56, 122.27; HRMS Calcd for $C_9H_6ClO_2$ $(M-H)^-$ 181.0051. Found 181.0065.

(E)-3-(4-chlorophenyl)acrylic acid**:** ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.48 (s, 1H), 7.72 (d, *J* = 8.5 Hz, 2H), 7.58 (d, *J* = 16.1 Hz, 1H), 7.46 (d, *J* = 8.5 Hz, 2H), 6.55 (d, *J* = 16.0 Hz, 1H); ^{13}C NMR (101 MHz, DMSO) δ 167.46, 142.55, 134.74, 133.23, 129.96, 128.95, 120.09; HRMS Calcd for $C_9H_6ClO_2$ $(M-H)^-$ 181.0051. Found 181.0065.

(E)-3-(2-nitrophenyl)acrylic acid**:** ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.06 (dd, *J* = 8.1, 1.3 Hz, 1H), 7.92 (dd, *J* = 7.9, 1.4 Hz, 1H), 7.85 (d, *J* = 15.8 Hz, 1H), 7.76 (td, *J* = 7.6, 1.3 Hz, 1H), 7.65 (ddd, *J* = 8.7, 7.5, 1.4 Hz, 1H), 6.53 (d, *J* = 15.8 Hz, 1H); ^{13}C NMR (101 MHz, DMSO) δ 167.33, 148.69, 139.29, 134.30, 131.24, 129.79, 129.68, 125.10, 124.25; HRMS Calcd for $C_9H_6NO_4$ $(M-H)^-$ 192.0291. Found 192.0301.

(E)-3-(3-nitrophenyl)acrylic acid**:** ^{1}H NMR (400 MHz, MeOD-d_4) δ 8.46 (t, *J* = 2.0 Hz, 1H), 8.25 (ddd, *J* = 8.3, 2.3, 1.0 Hz, 1H), 8.02 (dt, *J* = 7.8, 1.3 Hz, 1H), 7.75 (d, *J* = 16.1 Hz, 1H), 7.67 (t, *J* = 8.0 Hz, 1H), 6.66 (d, *J* = 16.0 Hz, 1H); ^{13}C NMR (101 MHz, MeOD) δ 168.07, 148.74, 142.04, 136.35, 133.36, 129.88, 124.05, 122.23, 121.27; HRMS Calcd for $C_9H_6NO_4$ $(M-H)^-$ 192.0291. Found 192.0303.

(E)-3-(4-nitrophenyl)acrylic acid**:** ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.14 (d, *J* = 8.5 Hz, 2H), 7.88 (d, *J* = 8.5 Hz, 2H), 7.59 (d, *J* = 16.1 Hz, 1H), 6.65 (d, *J* = 16.1 Hz, 1H); ^{13}C NMR (101 MHz, DMSO) δ 167.51, 148.39, 141.76, 141.19, 129.75, 124.40, 124.11; HRMS Calcd for $C_9H_6NO_4$ $(M-H)^-$ 192.0291. Found 192.0303.

(E)-3-(4-bromophenyl)acrylic acid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.49 (s, 1H), 7.68 – 7.51 (m, 5H), 6.57 (d, J = 16.0 Hz, 1H); ^{13}C NMR (101 MHz, DMSO) δ 167.87, 143.06, 133.99, 132.30, 130.61, 123.98, 120.59. HRMS Calcd for $C_9H_7BrO_2$ $(M-H)^-$ 225.9629. Found 225.0652.

5. Synthesis of non-aromatic compounds as novel potential substrates for the *Pc*PAL

The preliminary results based on computational studies showed that the PcPAL enzyme accepts a wide variety of novel substrates containing π-conjugated bonds. Among these propargyl glycine has special importance, due to its non-aromatic nature and wide application in the peptide chemistry. Therefore we elaborated the phenylalanine lyase mediated enantioselective synthesis of the propargyl-glycine.

Accordingly to the synthetic plan (Fig. 2) first we performed the chemical synthesis of acrylic acid derivative **2** and of the racemic aminoacid *rac*-**1**. Acrylic acid **2** was synthesized starting from propargyl alcohol in a one-pot procedure,containing the PDC oxidation and the subsequent Wittig reaction with triphenylphosphoilide, obtaining the corresponding ester derivative. The hydrolysis of the ester derivative was optimized, and due to the mild reaction conditions and the low stability of the acrylic acid **2**, we opted for the PLE (pig liver esterase) mediated enzymatic hydrolysis.

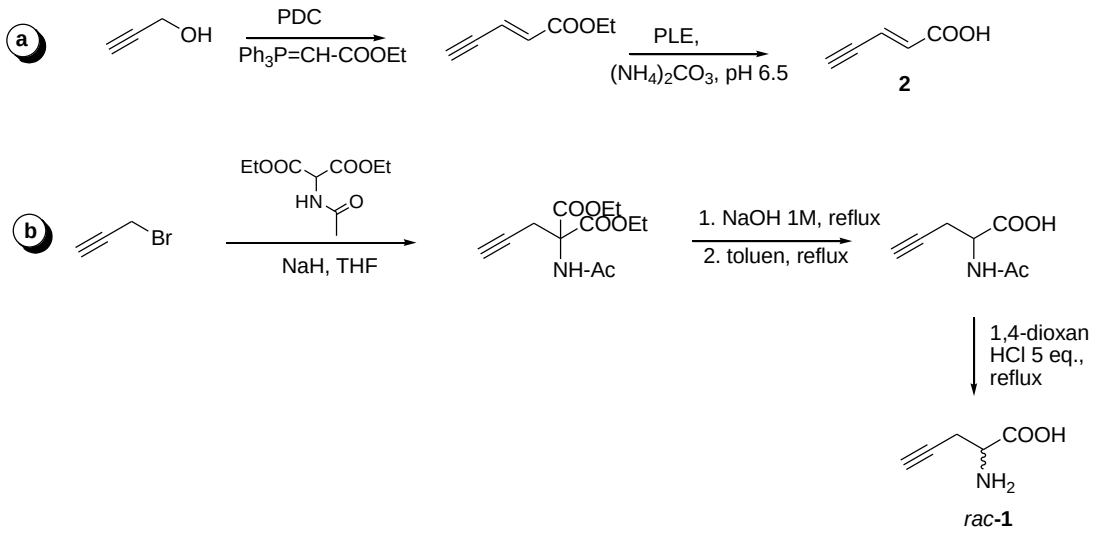


Figure.2. Synthetic plan of the novel non-aromatic substrates for the *Pc*PAL enzyme

The synthesis of racemic aminoacid 1 started from propargyl-bromide, which was coupled with the diethylacetamidomalonate activated by NaH. The obtained product was hydrolysed in alcaline conditions, followed by the decarboxylation of the diacidic compound, followed by the final acidic hydrolysis of the amide bond. The global yield was 35%.

The obtained compounds were characterized by ^{1}H, ^{13}C-RMN analysis and were further used as substrated in the *Pc*PAL mediated enzymatic transformations.

6. Synthesis of (*E*)-pent-2-en-4-inoic acid 2

9,5 g (56 mmol) Ph_3=CH-COOEt and 10,5 g (56 mmol) pyridinium-dichromate (PDC) were suspended in 100 ml dried dichloromethane. Into the suspension was added the solution of 1,5 g (53 mmol) propargyl-alcohol, disolved in 10 ml dichloromethane. The reaction was stirred at room temperature till completion (16 h), followed by the evaporation of the solvent. The product was purified by column chromatography using dichloromethane as eluent.

200 mg from the obtained ester derivative was suspended in 10 ml of $(NH_4)_2CO_3$ buffer (100 mM, pH 7.5), containing 20 mg of PLE (pig liver esterase). The reaction was stirred till completion at 200 rpm and 37 ^oC, maintaining the pH of the solution by adding 2 M NH_3 solution. The reaction was completed, when the decrease of pH stopped. At this point the pH was adjusted to 1, followed by extraction with ethyl acetate. The combined organic phases were dried over anhydrous sodium sulfate, the organic solvent was evapored under vacuum and the product **2** was purified by recrystallization with hexane.

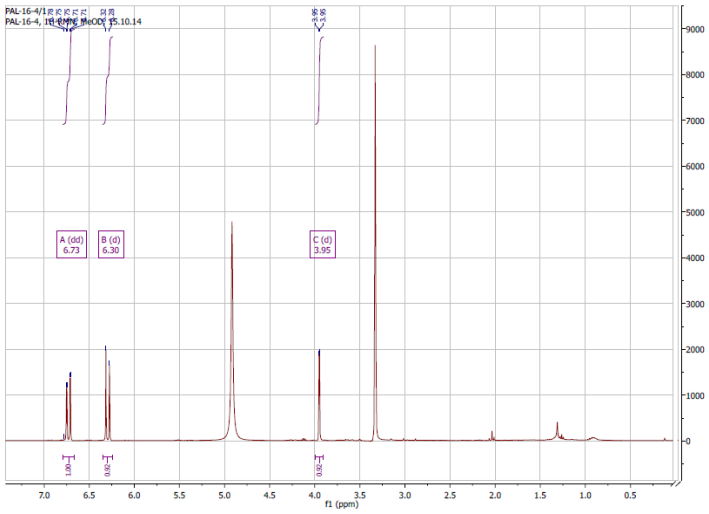


Figure 3. ^{1}H-NMR spectra of product **2**.

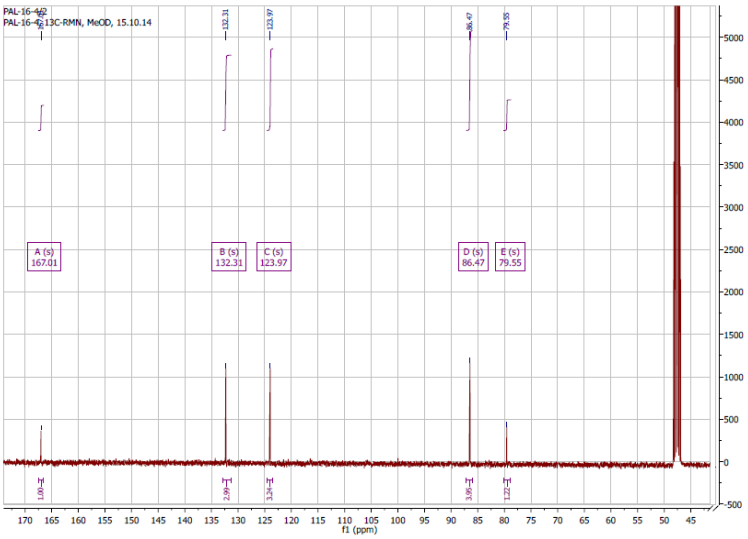


Figure 4. ^{13}C-NMR spectra of acrylic derivative **2**.

7. Synthesis of racemic propargyl glycine *rac*-1

Under inert atmosphere, 19,5 mmol diethylacetamido-malonate was added into the stirred suspension obtained from 0,85 g (20 mmol) 55% NaH and 20 ml dried DMF. The mixture was stirred for 30 min at room temperature, followed by the dropwise addition of 19 mmol propargyl bromide. The reaction mixture was stirred at room temperature for 3 h, and 4 h at 60 ^{o}C. The cooled solution was turned to a water-ice mixture (100 g). The obtained precipitate was filtered, dried under vacuum and the diethylacetamido-malonate derivative was recrystallized from hexane. 0,5 g from the pure product was disolved in 5 ml MeOH nad 3 ml of 10% NaOH was added. The reaction mixture was stirred overnight at reflux temperature, followed by the evaporation of the organic solvent. The obtained oil was extracted with ethyl-acetate/water. The pH of the water phase was adjusted to 1 and the precipated produced was extracted with ethyl-acetate. The combined organic phase was dried over anhydrous sodium sulfate, followed by the evaporation of the solvent. The product obtained was redisolved in toluene and stirred at reflux temperature for 4 h, obtaining the pure *N*-acetyl propargyl glycine.

1 ml of conc. HCl was added into the solution of 100 mg *N*-acetyl propargyl glycine in 15 ml 1,4-dioxane. The reaction mixture was refluxed for 4 h, followed by the evaporation of the organic solvent. The viscous lichid obtained was washed with cold acetone, obtaining a white precipiate which was filtered and dried under vacuum and identified as the clorhydrate salt of the racemic propargylglycine *rac*-**1**.

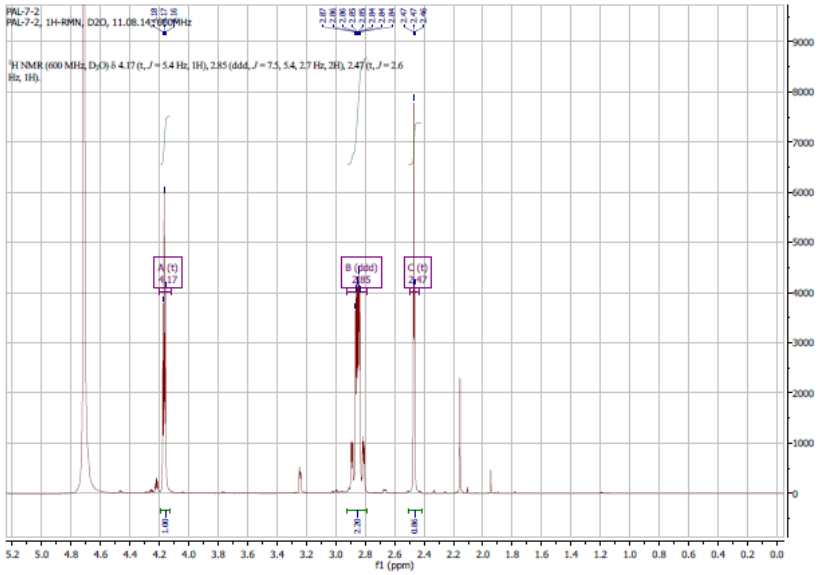


Figure 5. ^{1}H-NMR spectra of the racemic propargyl-glycine *rac*-**1**.

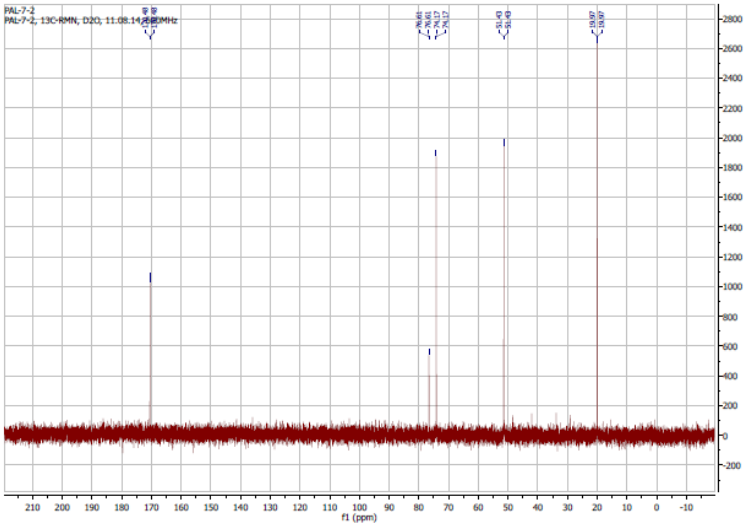


Figure 6. ^{13}C-NMR spectra of the racemic propargyl-glycine *rac*-**1**.

Enzymatic transformations

The synthesized novel substrates **2**, *rac*-**1** were tested in the ammonia addition (Figure 7a) and elimination (Figure 7b) reaction catalyzed by *Pc*PAL.

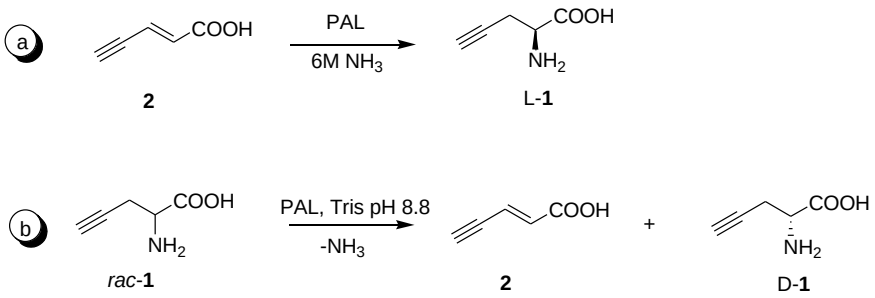


Figure 7. a) addition of ammonia using acrylic acid **2** as substrate and **b**) elimination of ammonia from *rac*-**1** catalyzed by *Pc*PAL

By measuring the UV-VIS spectra of compounds **1,2**, the wavelength of 242 nm was selected to monitor the production or consumption of acrylate **2** in the ammonia elimination, respectively ammonia addition reactions mediated by PAL. At this wavelength the acrylic derivate **2** shows absorption maximum, while the racemic aminoacid **1** has a minimal absorbtion, neglectible in comparison with the absorbtion of **2**.

To determine the enantioselectivity of the enzymatic transformation and the enantiomeric excesses of the D- and L- aminoacids obtained from the elimination, respectively addition reactions, we developed a chiral cromatographic separation method for the enantiomers

of the racemic aminoacids *rac*-**1** (Fig. 8). The developed optimal HPLC separation conditions: chiral column Chiralpak Zwix+, eluent: 50 mM DEA and 6% CH_3COOH in MeOH/ MeCN/H_2O, 49/49/2 (v/v), detection by ELSD:

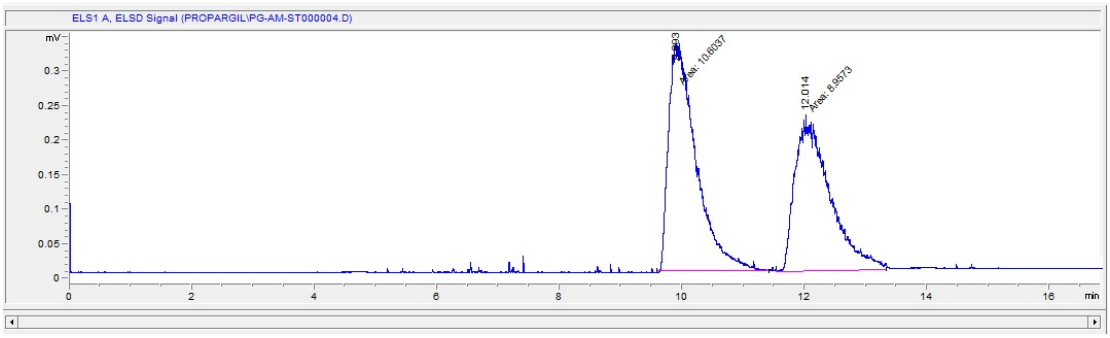


Figure 8. The chromatogram of the racemic propargylglycine *rac*-**1**.

The ammonia elimination reaction (Figure **7b)** was optimized at analytical scale, with different substrate (10-100 mM) and enzyme (1-10 mg/ml) concentrations. Both native and immobilized enzyme was tested. The native enzyme was immobilized through reticulation or covalent binding on magnetic nanoparticles. The reactions were monitored by the developed UV and HPLC methods. The determined optimal conditions were used for the preparative scale enzymatic synthesis: 100 mg of the racemic aminoacid *rac*-**1** was disolved in 20 ml Tris buffer (40 mM Tris, 140 mM NaCl, pH 8.8), followed by the addition of 3 ml solution of *Pc*PAL enzyme (c=4 mg/ml). The mixture was incubated for 80 h at 30 ^{0}C. In case of using the immobilized enzyme, the enzyme was removed by using a magnet. Using the reticulated or covalently bound enzyme to non-magnetic nanotubes, the removal of enzyme was achieved through filtration, while the native enzyme was removed by adsorption of active carbon. The pH of the filtrate was adjusted to 1 and the acrylic acid **2** was extracted with ethylacetate, while the D-aminoacid 1 was purified from the water phase by cation-exchange chromatography using Amberlyst 15 as anionic resin. The enantiomeric excess determined with the chiral HPLC method is > 98% (Fig. 9).

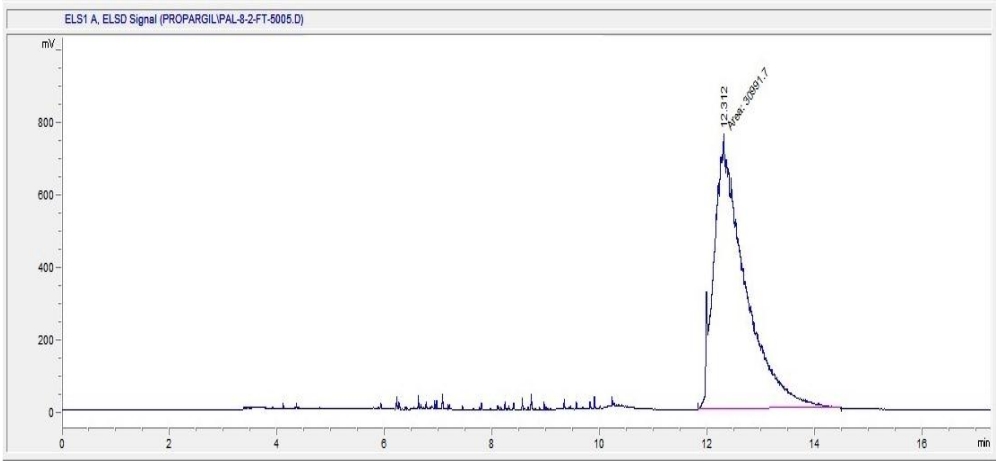


Figure 9. The chromatogram of D-propargylglycine obtained from the ammonia elimination reaction catalyzed by *Pc*PAL

The ammonia addition reaction (Figure **7a)** was optimized at analytical scale, with different substrate (10-100 mM) and enzyme (1-10 mg/ml) concentrations. Both native and immobilized enzyme was tested. The determined optimal conditions were: substrate concentration of 5 mM and enzyme concentration of 1 mg/ml. Thus the preparative scale reactions were performed with 50 mg of acrylic acid **2** in a solution of 6M NH_3, obtaining a conversion of 90% after 36 hours at 30 ^{0}C. The produced L-aminoacid was purified with the Amberlyst-15 based cation-exchange chromatography, obtaining a a yield of 68%. The determined enantiomeric excess was > 99 % (Fig. 10).

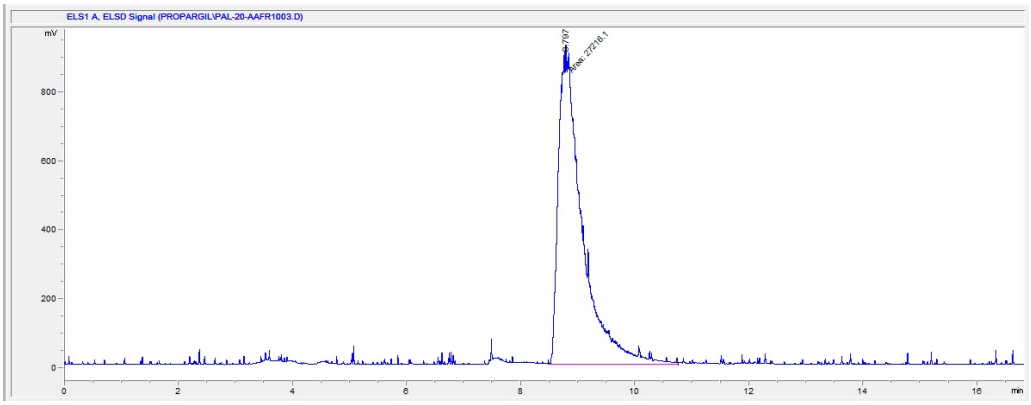


Figure 10. The chromatogram of L-propargylglycine produced in the ammonia addition reaction catalyzed by the *Pc*PAL

Therefore we succesfully performed the chemoezymatic synthesis of both L- and D-propargylglycine using the native and the immobilized *Pc*PAL. The results obtained are of significant importance, since according to our knowledge the reported substrate scope of the PcPAL enzyme consists of aromatic, heteroaromatic derivatives, the propargylglycine and its acrilic acid derivative being the first non-aromatic compound accepted as substrate by the enzyme..

3. Immobilization of the *Pc*PAL enzyme

a. to functionalized nanoparticles

Magnetic nanoparticles MagneCat-GP14, functionalized with epoxi groups (108 mg) were dispersed in TRIS buffer (3 ml, 0.1M, pH 8.8) with ultrasound sonication (35kHz, 20 min) to get stable suspension. The PcPAL solution (4 ml, 3 mg ml-1, in TRIS buffer 0.1M, pH 8.8) was added to the suspension and the mixture was shaken (450 rpm) for 24 h. After the sample was collected with neodymium magnet, the supernatant decanted and the residue washed three times with TRIS buffer and once with ethanol. The immobilized enzyme was dried at room temperature for 2 h.

b. Covalent binding of PAL to $SwCNT_{COOH}$ via GDE-based linker

$SwCNT_{COOH}$ (single walled carbon nanotube) was incubated with carbonyldiimidazole with shaking (at 1350 rpm at room temperature overnight), with occasional sonication to avoid bundled SwCNT formation (Fig. 11b, step *i*). 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 (step *ii*). 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 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 (step *iii*). 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 (step *iv*). After the PAL-immobilization, the resulted biocatalyst was filtered off on a membrane filter and washed with distilled water. The amount of PAL immobilized on the bisepoxide-activated $SwCNT_{COOH}$ was calculated using the Bradford method.

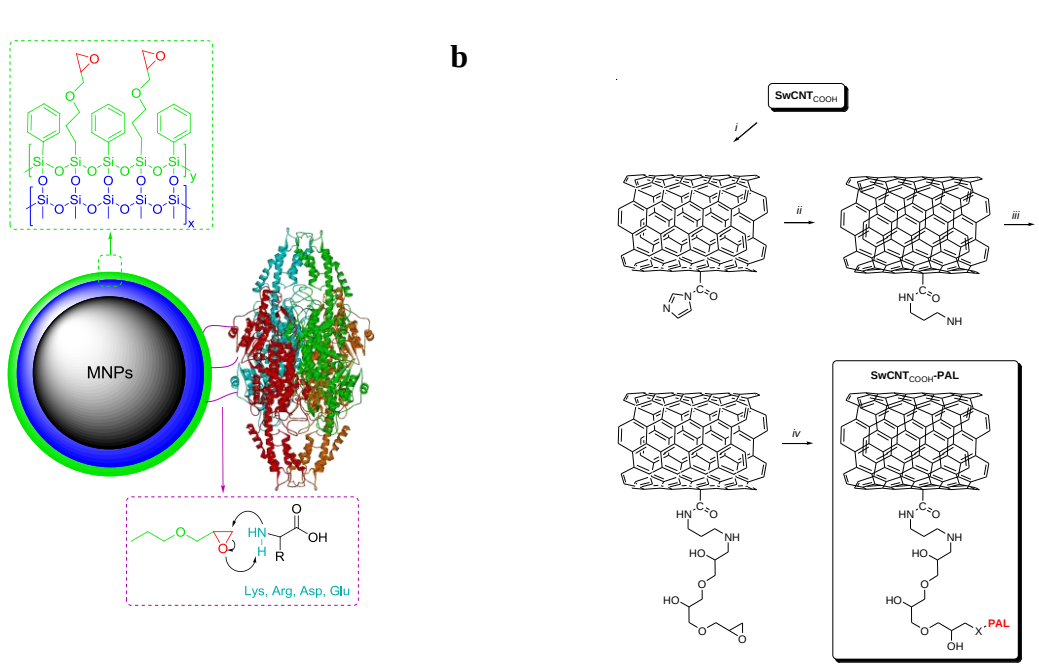


Figure 11. a. *Pc*PAL immobilization on magnetic nanoparticles epoxy-functionalized. **b**. PAL immobilization on $SwCNT_{COOH}$ *via* linker. Reactants and solvents: *i*) CDI in CH_2Cl_2; *ii*) $H_2N(CH_2)_3NH_2$ in water; *iii*) glycerol diglycidyl ether in CH_2Cl_2; *iv*) PAL in Tris buffer (0.1M, pH 8.8).

4. The influence of several additives on the *Pc*PAL activity

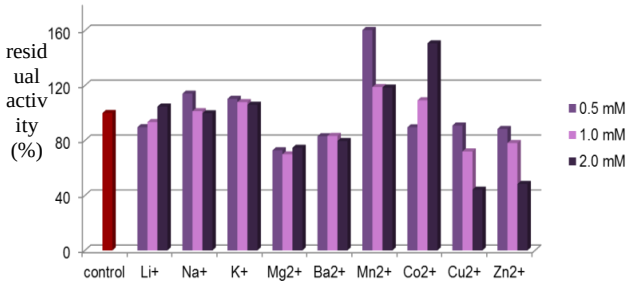
Several organic solvents and a series of mono- and divalent metallic ions were studied through *Pc*PAL catalyzed reactions in order to obtain *trans*-cinnamic acid from L-phenylalanine. In order to determine if the presence of the additives mentioned above lead to increase the performance of phenylalanine alanine ammonia lyase from parsley, multiple experiments were designed.

4.1. The effect of metal ions on the *Pc*PAL activity

The effect of the following metal ions: Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, was investigated by pre-incubating L-phenylalanine (10 mM) at 30 ºC for 5 min with 0.5 mM, 1.0 mM and respectively 2.0 mM metal ions, in 0.1 mM Tris-HCl pH 8.8. The reactions were started after 5 min by adding purified *Pc*PAL. Formation of cinnamic acid was monitored spectrophotometrically at 290 nm for 5 min longer.

Table 4.1. The effect of metal ions on lyase activity

	residual activity (%) (10 mM L-Phe)		
metallic ion	**0.5 mM**	**1.0 mM**	**2.0 mM**
Li^{+}	91.7	91.5	91.2
Na^{+}	114.1	101.3	99.8
K^{+}	110.3	107.9	106.0
Mg^{2+}	72.8	69.9	74.7
Ba^{2+}	83.1	83.3	79.6
Mn^{2+}	160.4	118.9	118.5
Co^{2+}	89.6	109.2	150.7
Cu^{2+}	90.9	72.0	44.2
Zn^{2+}	88.4	78.1	48.3



metallic ions

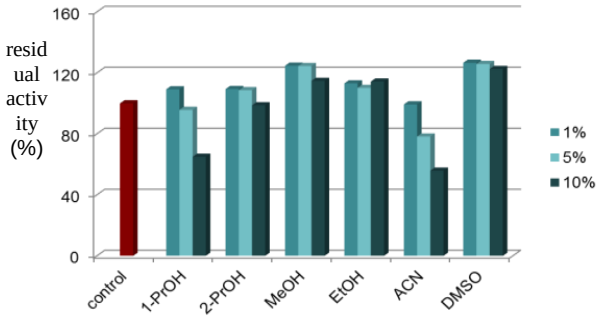
The results obtained in the experiments carried out in presence of the metallic ions prove that Mn^{2+} and Co^{2+} enhanced considerably the activity of the enzyme, while others like Na^{+} and K^{+} caused a negligible increase of the enzyme activity. Other metal ions like Mg^{2+}, Cu^{2+}, Zn^{2+} proved to inhibit at small concentration the lyase activity.

4.2. The effect of organic solvents on the *Pc*PAL activity

The organic solvents: propanol (1-PrOH), isopropanol (2-PrOH), methanol (MeOH), ethanol (EtOH), acetonitrile (ACN) and dimethyl sulfoxide (DMSO) were tested at different concentrations (1%, 5% and 10%) in order to determine the enzyme stability in their presence.

Table 4.2. The effect of organic solvents on lyase activity

	residual activity (%) (10 mM L-Phe)		
organic solvent	**1%**	**5%**	**10%**
1-PrOH	109.2	95.7	64.9
2-PrOH	109.4	108.6	98.7
MeOH	124.7	124.5	114.7
EtOH	113.1	110.1	114.3
ACN	99.3	78.2	55.8
DMSO	126.6	125.8	122.5

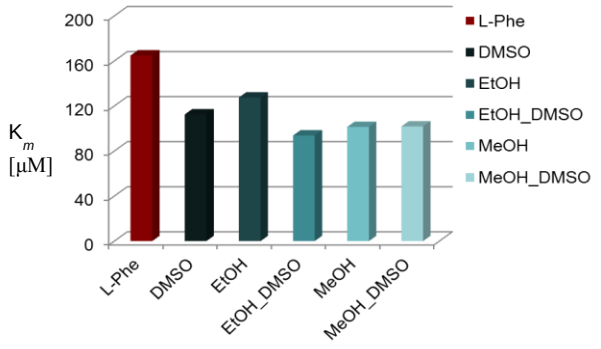


The investigation performed in the presence of polar solvents revealed that *Pc*PAL showed increasing stability and activity in presence of ethanol, methanol and dimethyl sulfoxide, while 10% of other solvents as propanol and acetonitrile reduced with almost 50% the enzyme activity. After incubating the substrate with and without the organic solvents at 30 ºC for 5 min, the enzymatic reaction was initiated by *Pc*PAL addition. The acrylate formation was continously monitored using the spectrophotometer at 290 nm for 5 min.

The table bellow include the kinetic parameters, K_m and v_{max}, obtained for *Pc*PAL in presence of the substrate, and the substrate in combination with certaine solvents mentioned above.

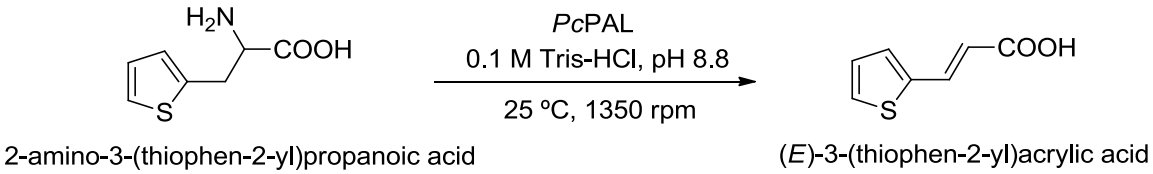
Table 4.3. Determination of kinetic parameters

organic solvent (1%)	**K_m** [μM]	**v_{max}** [μM/s]
L-Phe	164.83	2.50E-01
DMSO	112.50	2.50E-01
EtOH	127.53	2.50E-01
EtOH_DMSO	93.77	2.50E-01
MeOH	101.38	2.50E-01
MeOH_DMSO	101.82	2.50E-01



4.3. Enzymatic screening

In order to investigate the effect of methanol, ethanol, Mn^{2+} and the combination of the metallic ion with the two solvents on the enzyme activity, the *rac*-2-amino-3-(tiophen-2-yl)propanoic acid was selected as model substrate.



2-amino-3-(thiophen-2-yl)propanoic acid (*E*)-3-(thiophen-2-yl)acrylic acid

Scheme 4.1. The enzymatic elimination of ammonia from *rac*-2-amino-3-(tiophen-2-yl)propanoic

Table 4.4. Enzymatic screening of *rac*-2-amino-3-(tiophen-2-yl)propanoic acid in presence of *Pc*PAL and several additives

	substrate	Mn^{2+}	MeOH	EtOH
reactions	**5 mM**	**0.5 mM**	**10%**	**10%**
1	✓	–	–	–
2	✓	✓	–	–
3	✓	–	✓	–
4	✓	–	–	✓
5	✓	✓	✓	–
6	✓	✓	–	✓

The enantiomeric separation of the racemic 2-amino-3-(thiophen-2-yl)propanoic acid and the determination of the conversion value at different periods of time was performed on a Chiralpak ZWIX(+) HPLC column (4.0 × 250 mm) using acetonitrile: methanol (50 mM DEA, 6% CH_3COOH): H_2O (49:49:2, v/v/v) at 1.0 mL/min flow rate as mobile phase.

Table 4.5. The conversions obtained after 24 hours in the ammonia elimination reactions catalyzed by *Pc*PAL

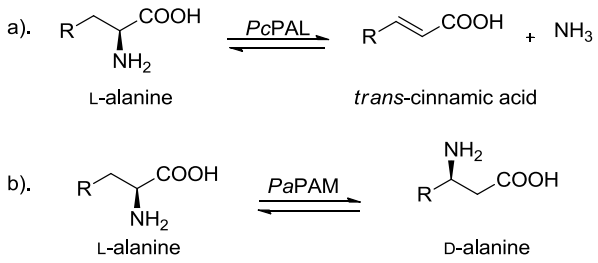
	conversion (%)					
time (hours)	1	2	3	4	5	6
2	28	29	27	28	27	26
3	32	30	31	30	32	31
7	33	39	40	38	37	38
24	35	43	**50**	47	47	46

Following the enzymatic screening and after analyzing the HPLC chromatograms, it was found that the elimination reaction of ammonia from the racemic mixture occurred optimal by using 10% MeOH (c = 50%, after 24 hours)

OBJECTIVE 2: BIOTRANSFORMATION OF SUBSTRATES USING NATIVE AND MUTANT PAL AND PAM.

The biotransformation of substrates using native and mutant PAL and PAM

Phenylalanine ammonia-lyases (PAL;EC 4.1.3.24) catalyse the non-oxidative deamination of L-phenylalanine in (*E*)-cinnamic acid, while phenylalanine 2,3-aminomutases (EC 5.4.3.x) catalyse the isomerization of L-phenylalanine to form L- or D-β-phenylalanine, depending on the origin of the enzyme (Scheme 1.). Even though PALs are frequently found in plants, where they have an essential role in forming phenylpropanoids, only a few bacterial PALs have been identified so far. PAL and PAM are used as biocatalysts for the synthesis of L-α-amino acids from acrylates (PAL), in kinetic resolution processes for obtaining D-α-amino acids starting from their racemates (PAL), or for the synthesis of L- or D-β-arylalanines (PAM).



Scheme.1. PAL (**a).**) and PAM (**b). mediated reactions**).

*Pc*PAL and *Pa*PAM purified enzymes were used for obtaining both enantiomers of several unnatural analogues of α- and β-arylalanine.

First, the influence of the substrate concentration upon the reaction velocity and the inhibitory effect of the acrylate on the *Pa*PAM mediated biotransformations was investigated. It was found that in the investigated concentration range, the concentration of the substrate has no significant influence on the conversion of the reactions. Similarly, the inhibitory effect of the increasing acrylate concentration upon *Pa*PAM was negligible (**Figure 1.**)

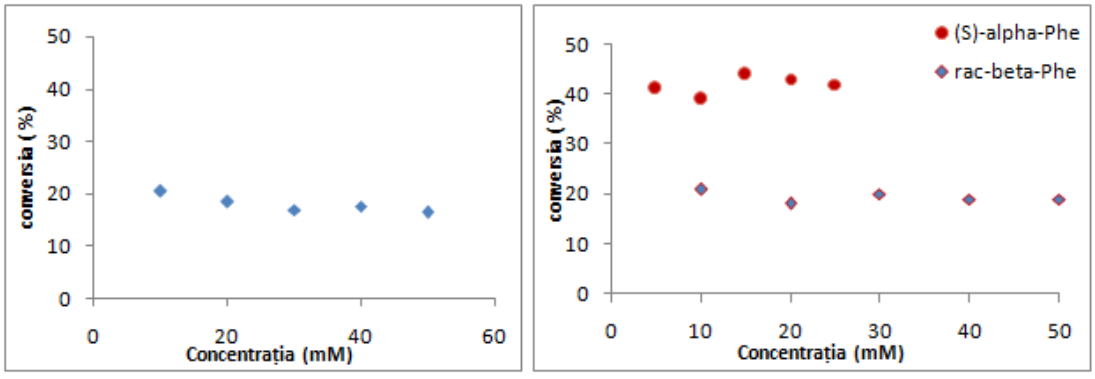


Figure.1. a) . The effect of cinnamic acid concentration on conversion **b)** The effect of substrate concentration on conversion

In order to obtain both enantiomers (*R*) and (*S*) of β-arylalanine, we proposed two methods. Thus, (*R*)-β-phenylalanine was obtained starting from the racemic β-phenylalanine, using *Pa*PAM and *Pc*PAL in tandem. *Pa*PAM enzyme transforms (*S*)-β-phenylalanine in (*S*)-α-phenylalanine, which in the presence of *Pc*PAL is converted to cinnamic acid (**Figure 2.b.**).

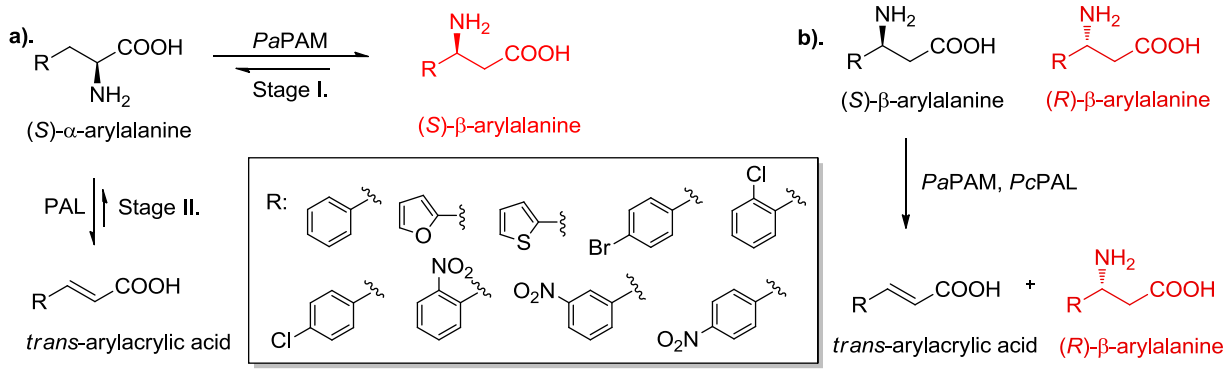


Figure 2. The synthesis of (*S*)-β-arylalanine **(a).**) and the synthesis of (*R*)-β-arylalanine **(b).**)

Enantiopure (*R*)-β-phenylalanine was isolated by using cationic ion exchange chromatography. The monitoring of the reactions was realized using high performance liquid chromatography (HPLC), with a CHIRALPACK ZWIX(+) column, and MeOH(50mM FA+ 100mM DEA):ACN:H_2O 49:49:2 (v:v:v) as eluent, respectively (**Figure 3.**).

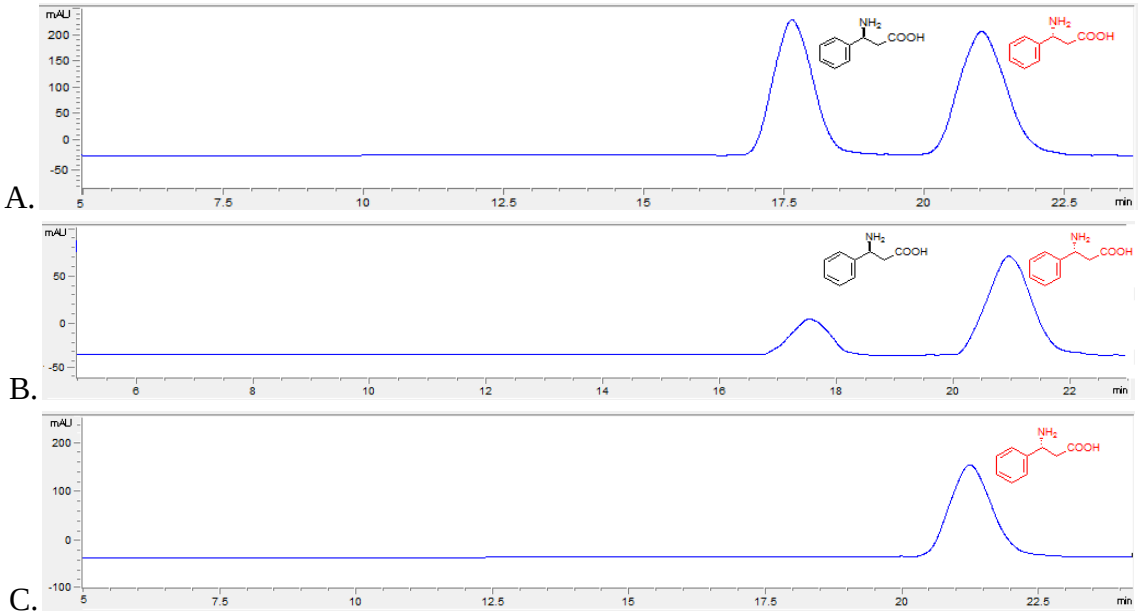


Figure 3. *Rac*-β-phenylalanine transformed by *Pa*PAM. A.after 2h, B.after 24h, C.after 48h

In order to extend the method to unnatural alanines, we considered it necessary to first analyze the reactions catalysed by *Pa*PAM (**Figure 4.**). We observed that the nature and position of the substituents significantly modifies the activity of the enzyme. By comparison to the unsubstituted *rac*-β-phenylalanine, the *orto*- substituted phenylalanines and the heteroaryl-alanines, respectively, are good substrates for *Pa*PAM, unlike *para*- substituted phenylalanines where the enzyme presents no activity.

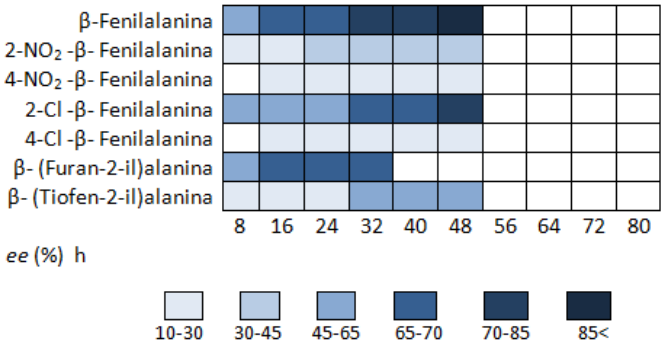


Figure 4. *Rac*-β-arylalanines transformed by *Pa*PAM

By applying the method described above to different unnatural analogues of *rac*-β-arylalanine, it was observed that the transforming rate of (*S*)-β-phenylalanine into acrylic acid depends on the behaviour of *Pa*PAM discussed above (**Table 1.**).

Table 1. Unnatural analogues transformed using *Pa*PAM-*Pc*PAL in tandem

Substrates	**T(h)**	***ee*$_\beta$ (%)**
β- (Tiofen-2-il)alanina	35	86
β- (Furan-2-il)alanina	48	66
2-Cl-β-Fenilalanina	16	99
4-Cl-β-Fenilalanina	48	-
2-NO_2-β-Fenilalanina	98	77
3-NO_2-β-Fenilalanina	98	51
4-NO_2-β-Fenilalanina	48	-
4-Br-β-Fenilalanina	98	10

In order to obtain (*S*)-β-arylalanine, we started from (*S*)-α-arylalanine, which was prepared by adding ammonia (6M NH_3 aqueous solution) to the corresponding acrylic acid, catalysed by *Pc*PAL.

This method has two steps. In the first step, *Pa*PAM catalyses the transformation of (*S*)-α-arylalanine to (*S*)-β-arylalanine, but the reaction stops at their equilibrium concentrations. Because of this,the *Pa*PAM enzyme was inactivated and removed from the reaction medium and *Pc*PAL was addea in order to transform the unreacted (*S*)-α-arylalanine into the acrylic acid (**Figure 2.**). Ion exchange chromatography was used for isolating (*S*)-β–phenylalanine in its optical pure form.

Regarding the reactions catalysed by *Pa*PAM (**Figure 5.**), only thiofen- and *para*-Cl-phenylalanine, respectively, were transformed with acceptable enantiomeric excesses. *Meta*- and *para*-substituted alanines gave moderate results, while *orto*-substituted compounds presented no reactivity. In the case of furyl-2-alanine *Pa*PAM presents significant lyase activity, therefore the transformation of α-alanine takes place with a higher rate toward the formation of furyl-2-acrylic acid.

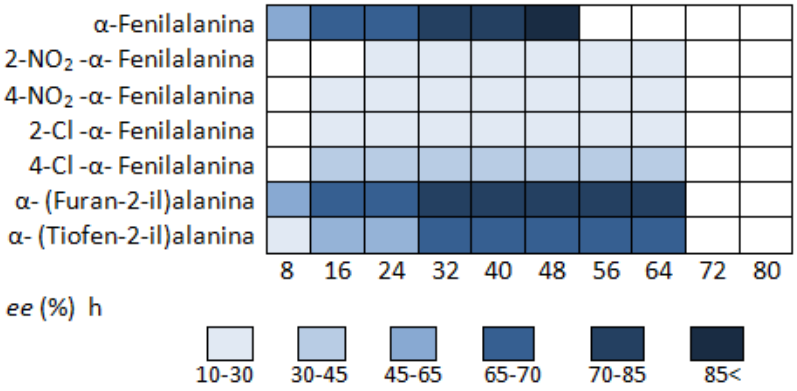


Figure 5. *Rac*-α-arylalanines transformed using *Pa*PAM

The monitoring of the reactions was realized using high performance liquid chromatography (HPLC), with a CHIRALPACK ZWIX(+) column, and MeOH(50mM FA+ 100mM DEA):ACN:H_2O 49:49:2 (v:v:v) as eluent (**Figure 6.**).

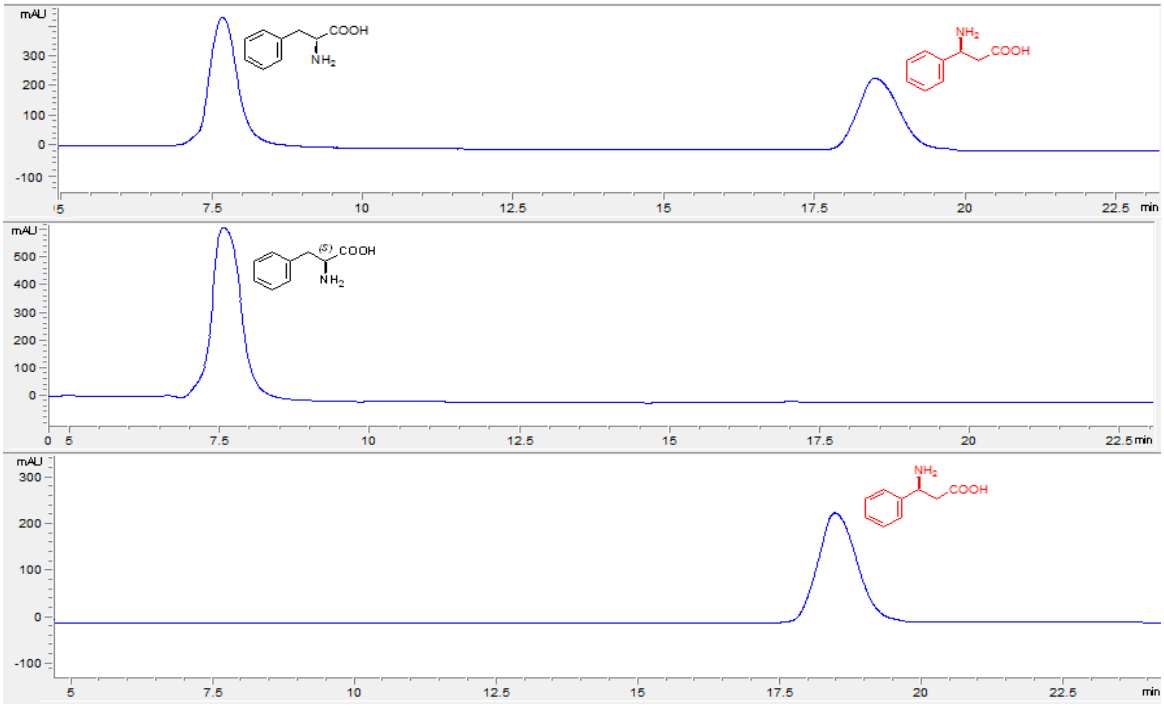
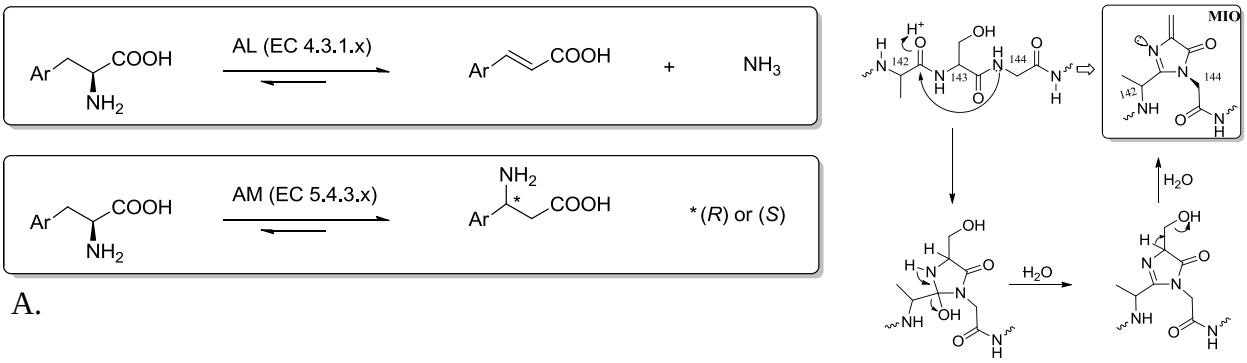


Figure 6. (*S*)-α-phenylalanine transformed using *Pa*PAM. A.after 2h, B.after 24h, C.after 48h

4. THE MODELING OF MIO-DEPENDENT AMMONIA-LYASES AND 2,3-AMINOMUTASES BY HOMOLOGY. MECHANISTIC COMPARISONS

Besides ammonia-lyases PAL and HAL, 2,3-aminomutases (PAM and TAM) which catalyse the selective formation of β-amino acids (**Figure 4.1A**) also contain as a prosthetic group 3.5-dihydro-5-methyliden-4*H*-imidazole-4-one (MIO), a strong electrofil, formed by the intramolecular ciclization of a tripeptide Ala-Ser-Gly (**Scheme 4.1B**).
The Protein Database (PDB) contains the structures determined through X-ray diffraction of 10 different enzymes in which this prosthetic group was identified: HAL from *Pseudomonas putida*, PAL from *Rhodosporidium toruloides*, *Petroselinum crispum*, *Anabaena variabilis* and *Nostoc punctiform*, TAL from *Rhodobacter sphaeroides*, TAM from *Streptomyces globisporus* and PAM from *Taxus canadensis*, *Taxus chinensis* and *Pantoea agglomerans*.



A.

B

Scheme 4.1. A. The scheme of the reactions catalyzed by lyases (AL) and 2,3-mutases (AM); **B.** The formation of the MIO prosthetic group, common to enzymes

The sequences of the chosen enzymes were analyzed and presented in **Table 4.1**. We compared not only the enzymes which catalyse the same type of reactions, but also enzymes which catalyse different reactions.

The analysis led to a series of interesting observations. For example, by overlaying the active site of *Pl*HAL and that of *Av*PAL (**Figure 4.1A**) it can be observed that the Gln452 and Glu412 residues are localized at the same position, close to Tyr314. Gln411 residue from *Pl*HAL is in the same position as Asn451 from *Av*PAL.

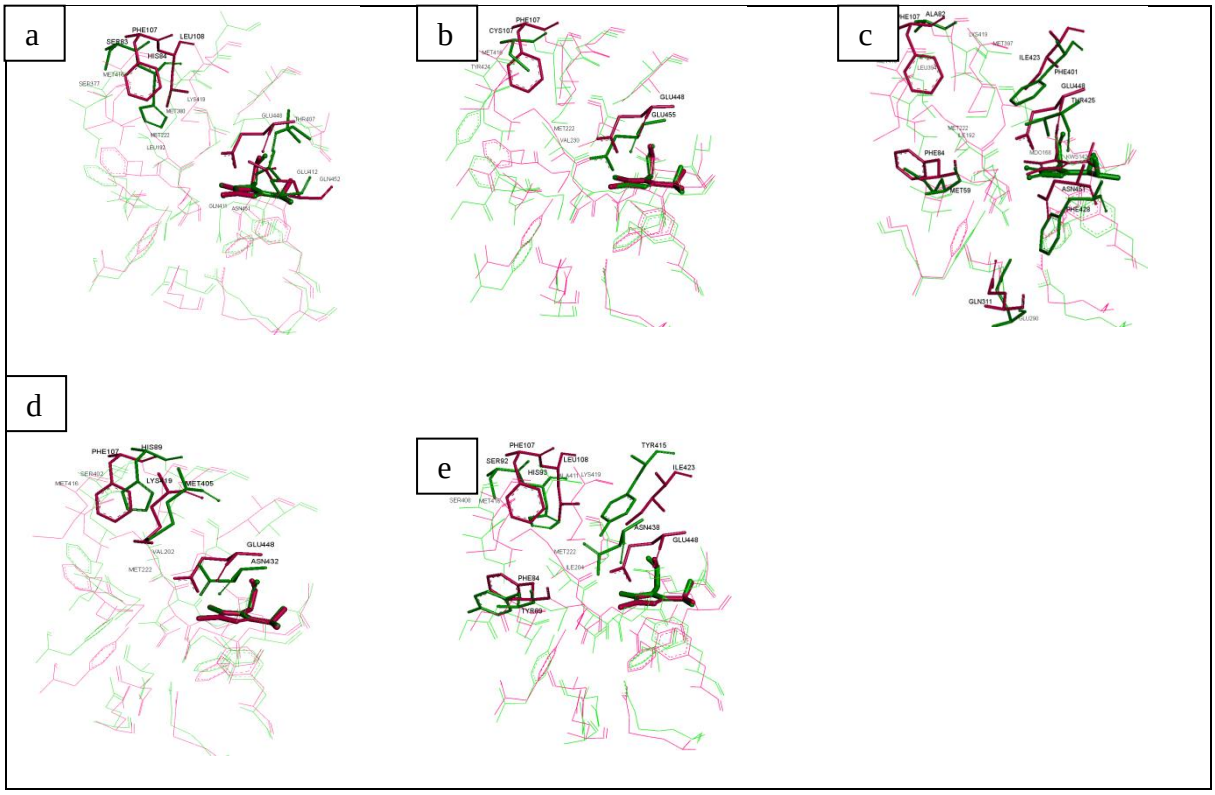


Figure 4.1. The catalytic site of aromatic ammonia-lyases and aminomutases (green) by comparison to the active site of *Av*PAL (3CZO, pink). **a**. The homology model of *Pl*HAL **b**. Crystalline structure of *Tc*PAM **c.** Homology model of *Sm*PAM **d.** Crystalline structure of *Rs*TAL **e**. Crystalline structure of *Sg*TAM.

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