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Successful Application of the Lipase as a Catalyst in the Combinative Approach, Using an Efficient and Selective Iterative Procedure for the Chemo-Enzymatic Alpha-Amino Group Protection in the Natural Amino Acids, Representing the Ornithyl Model System as a Starting Research Pattern: Homogeneous and Heterogeneous Synthesis

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Abstract

The successful efficient combinative approach for iterative and selective chemo-enzymatic amino group protection was realized at mild conditions, using the lipase as a catalyst, without its regeneration and step isolation from the reaction mixture, which allows the elegant large-scale preparation of N-protected amino acids derivatives for the conventional peptide synthesis.

Keywords: Iterative procedure; Chemo-enzymatic synthesis; Lipase; **P**orcine **P**ancreatic **L**ipase (PPL); Solubility-controlled synthesis; Ornithine; Activated esters; Boc-protecting group; Fmoc-protecting group; Z-group; Bz(NO_2)-group

List of Abbreviations

BBO – **B**road**b**and **O**bserve

Boc - tret-**B**uty**l**oxy**c**arbonyl

Bz – **Bz**oyl

Bz(NO_2) – p-nitro-**Bz**oyl

COSY - **Co**rrelation **S**pectroscop**y**

DEPT-135 - **D**istortionless **E**nhanced **P**olarization **T**ransfer using a 135 degree decoupler pulse

DMF - **Dim**ethyl**f**ormamide

Et_2NH – diethylamine

Et_3N – triethylamine

Fmoc - 9-**F**luorenyl**m**ethyl**o**xy**c**arbonyl

HMBC - **H**eteronuclear **M**ultiple **B**ond **C**orrelation

HPLC – **H**igh **P**erformance **L**iquid **C**hromatography

HSQC - **H**eteronuclear **S**ingle **Q**uantum **C**orrelation

IR – **I**nfra**r**ed spectroscopy

NMR – **N**uclear **M**agnetic **R**esonance

Orn - **Orn**ithine

TBI – **T**riple **B**roadband **I**nverse

TLC – **T**hin **L**ayer **C**hromatography

UV – Ultra**v**iolet

Z - benzyloxycarbonyl

Introduction

The contemporary organic, particularly the peptide synthesis is inconceivable and impossible without protective groups, and require protection of the functionalities in the organic molecules. In the presence of at least two functional groups in the amino acids, their selective protection is necessary and is required, with goal – to avoid (protect) the process from the unwanted side reactions, as well as the carrying out of the synthesis in the desired direction. Utilization of the 9-fluorenylmethyloxycarbonyl moiety **(Fmoc)** as a protecting group for the amino function of the amino acids in the solid-phase peptide synthesis [1] of the benzyloxycarbonyl protection **(Z)** - also as N-protective group - in the realization of the peptide synthesis in solution (solution-phase peptide synthesis) [2-5] as well as the permanent p-nitro benzoyl protecting group **(Bz(NO_2)** - for increasing of the sensitivity in the UV-area during the peptide analysis [3], and also for synthesis of model compounds [6,7], as well as for protection of nucleosides and nucleotides, and finally - for increasing of the sensitivity and selectivity in the nucleoside, nucleotide and oligonucleotide synthesis and analysis [8], enforce the using of protected biomonomer derivatives, and particularly in this case - of the protected amino acids. **Fmoc**-group is labile in basic media, but it is stable in acidic conditions, respectively-it deprotects in the principle of the basic elimination (pyridine, or 10% diethylamine in DMF) [9]. From the other side, the classical **Z**-protective group, which is used since the early years ("daybreak") of the peptide synthesis, is exclusively stable in basic media and is very stable in acidic conditions – it deprotects with catalytic hydrogenation (H_2 and 5-10% Pd/C; 10-20% (88%HCOOH) in MeOH and Pd/C; as well as $HCOONH_4$ and Pd/C) [9]. p-Nitro benzoyl protective group

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is extremely stable in acidic and alkali media in the absence of strong nucleophiles. It deblocks with $NaNH_2$, H_2NNH_2 or sodium methylate (sodium methoxide) or potassium methoxide respectively [10]. Usually the acid (acyl or urethane, respectively carbamate) components are activated as chlorides, anhydrides, and also- as activated esters. Acid chlorides, which are used for introduction of the well-known acyl protective groups, are unstable, in the most cases - their preparation is difficult, as well as they have poor yields, during the procedure of their synthesis. Moreover, the selectivity of the reagents is negligible and respectively, the purity of the obtained products is too low. Acid chlorides (acyl chlorides) have a strong reaction ability (high reactivity), which leads to undesired side reactions, particularly in the presence of another nucleophilic centers (functional groups: OH, COOH and others), whereas the activated esters are stable, easy to prepare, the protective groups as activated esters are introduced at mild conditions, they have good selectivity, and finally leads to high yields and purity of the obtained products. However, only the utilization of activated esters in basic conditions and in the absence of enzyme is difficult, and sometimes unwanted - requires prolonged reaction time, whereas the reaction ability is too low, as well as the yields are poor, more crude reaction conditions are required (i.e., sometimes - increasing of the temperature and reflux), compared to the enzymatic and chemo-enzymatic methods. In the present manuscript, we describe an elegant, mild, suitable, convenient and selective method for alpha-amino group protection of the functional amino acids by the activated esters of the acyl (carbamate, respectively urethane) components (acyl (electrophile) synthons), playing a role as acyl synthetic equivalents of the most frequently used protecting groups in the peptide synthesis, and of a variety of alcohol nucleophile synthons, used as good nucleofuges, as well as by the iterative using of the lipase as a catalyst. We choose a combinative approach as a unique strategy for iterative synthesis, which concludes in the solvents system screening, selection of **P**orcine **P**ancreatic **L**ipase (PPL) (free or immobilized), and use of protected or unprotected amino component, depending on the electrophilic or nucleophilic enzyme specificity.

Experimental

General procedures, methods and materials

All reagents and solvents were purchased and used without further purification. Reverse phase HPLC analyses were performed on a Waters Liquid Chromatograph equipped with an absorbance detector model 441 set at 280 nm and a column Nucleosil 100-5C_{18} (12.5 cm × 4.6 mm) for analytical runs. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II+ 600MHz spectrometer using BBO or TBI probeheads. Chemical shifts are expressed in ppm and coupling constants in Hz. The precise assignments of the ^{1}H and ^{13}C NMR spectra were accomplished by measurement of 2D homonuclear correlation (COSY), DEPT-135 and 2D inverse detected heteronuclear (C–H) correlations (HSQC and HMBC). Chemical shifts are reported in δ (ppm). The analysis of first order multiplets in ^{1}H NMR spectra was speed up by the use of FAFOMA program [11].

Synthesis

Synthesis of activated esters of the protective groups as acyl components

Synthesis of N-hydroxysuccinimide.DCHA (HONSu.CHA): The synthesis of HONSu.DCHA was carried out according to the procedure of A. Paquet [12]. To 1.151 g (0.01 mol) of N-hydroxysuccinimide (HONSu) in acetone was added 1.986 ml (0.01 mol) of dicyclohexylamine. The mixture was stirred overnight and the precipitate was isolated by filtration, washed with cold acetone and thoroughly dried. Yield-2.845 g (96%). Mw. = 296.41 g/mol. Anal. calcd. for $C_{16}H_{28}N_20_3$: C 64.84, H 9.52, N 9.45; found: C 64.59, H 9.64, N 9.57.

Synthesis of 9-fluorenylmethyl succinimidyl carbonate (Fmoc-ONSu) (A): The synthesis of Fmoc-ONSu was accomplished according to the procedure for Fmoc protection, used by Paquet [12], as well as by Sigler et al. [13]. To a stirred solution of 1.407 g of Fmoc-chloride (5.44 mmol) in 10 ml chloroform was added 1.61 g (5.44 mmol, Mw.=296.41 g/mol.) of N-hydroxysuccinimide. DCHA (HONSu.DCHA) in portions. The stirring was continued overnight, and the precipitate was filtered off and washed with chloroform. The filtrate and washings were combined and washed with 1/3 of the volume each of 10% citric acid, 10% $NaHCO_3$, water and dried. The solvent was distilled off in vacuo and the residue was recrystallized from chloroform-ether to give 1.65 g (90%) of the title compound, Rf-0.910 (CH_2Cl_2:CH_3OH-9.5:0.5). M.p.151°C. Mw. = 337.33g/mol. Anal. calcd. for $C_{19}H_{15}NO_5$: C 67.65, H 4.48, N 4.15; found: C 67.57, H 4.41, N 4.27.

^{1}H NMR (600 MHz, DMSO-d6, 25°C): δ = 2.745(s, 4H, CH_2), 4.420(d, J=6.0 Hz, 1H, 8-CH), 4.824(t, J=6.0 Hz, 2H, 1-CH_2), 7.362(dt, J=1.0, 7.5 Hz, 2H, 2-CH and 7-CH), 7.446(t, J=7.4 Hz, 2H, 3-CH and 6-CH), 7.692(dd, J=0.6, 7.5 Hz, 2H, 1-CH and 8-CH), 7.922(d, J=7.5 Hz, 2H, 4-CH and 5-CH).

^{13}C NMR (150 MHz, DMSO-d6, 25°C): δ = 25.23(CH_2), 45.90(9-CH), 53.60(2-CH), 71.81(1-CH_2), 120.27(4-CH and 5-CH), 124.86(1-CH and 8-CH), 127.27(2-CH and 7-CH), 127.93(3-CH and 6-CH), 140.78(4a-C and 4b-C), 142.59(8a-C and 9a-C), 151.06(OCOO), 169.70(NCO). **TLC:** Rf-0.910 (CH_2Cl_2: CH_3OH-9.5:0.5).

Synthesis of benzyl 2, 4, 5-trichlorophenyl carbonate: Z-OTcp: This compound was prepared from 0.777 ml (0.928 g, 5.44 mmol, Mw. = 170.59 g/mol, d_4^{20} = 1.195 g/cm^3) of benzyl chloroformate and 2.061 g (5.44 mmol, Mw = 378.77 g/mol) of trichlorophenol. DCHA salt (prepared by the same manner as N-hydroxysuccinimide. DCHA (HONSu.DCHA)) as described for the synthesis **A** of ester: 9-fluorenylmethyl succinimidyl carbonate (Fmoc-ONSu). Yield 1.55g (86%) of the title compound. Rf–0.953 (CH_2Cl_2:CH_3OH-9:1). Rf–0.786 (CH_2Cl_2:CH_3OH-9.5:0.5). Mw. = 331.58 g/mol. Anal. calcd. For $C_{14}H_9O_3Cl_3$: C 50.71, H 2.74, Cl 32.08; found: C 50.67, H 2.72, Cl 32.01.

^{1}H NMR (600 MHz, DMSO-d6, 25°C): δ = 5.115(s, 2H, OCH_2, Ar (Ph)), 7.031-7.426(m, 5H, 2-CH, 3-CH, 4-CH, 5-CH and 6-CH, Ar (Ph)), 7.492(s, 1H, 6-CH, Tcp), 7.715(s, 1H, 3-CH, Tcp).

^{13}C NMR (150 MHz, DMSO-d6, 25°C): δ = 70.38(OCH_2, Ar (Ph)), 122.98(2-C-Cl, Tcp), 123.57(6-CH, Tcp), 126.14(4-CH), 126.89(2-CH and 6-CH), 127.45(3-CH and 5-CH), 129.37(4-C-Cl, Tcp), 132.05(3-CH), 133.24(5-C-Cl, Tcp), 135.03(1-C, Ar (Ph)), 147.13(1-C, Tcp), 152.55(OCOO). **TLC:** Rf–0.953 (CH_2Cl_2:CH_3OH-9:1), Rf–0.786 (CH_2Cl_2:CH_3OH-9.5:0.5).

Synthesis of benzyl ethylene glycol carbonate: Z-O-CH_2CH_2-OH: This compound was synthesized from 0.777 ml (0.928 g, 5.44mmol, Mw. = 170.59 g/mol, d_4^{20} = 1.195 g/cm^3) of benzyl chloroformate and 1.456 g (5.984 mmol, Mw. = 243.39 g/mol) – 1.1 equiv. excess of ethylene glycol. DCHA salt - to avoid the dieters preparation, as described for the synthesis of ester: 9-fluorenylmethyl ethylene glycol carbonate: Fmoc-OCH_2CH_2-OH. Yield 0.79 g (74%) of the title compound. Rf–0.876 (CH_2Cl_2:CH_3OH-9:1). Rf–0.708 (CH_2Cl_2:CH_3OH-9.5:0.5). Mw. = 196.2 g/mol. Anal. calcd. For $C_{10}H_{12}O_4$: C 61.22, H 6.17; found: C 61.18, H 6.12.

^{1}H NMR (600 MHz, DMSO-d6, 25°C): δ = 3.93-3.98(m, 2H, CH_2-OH), 4.42-4.45(t, J=9.2 Hz, 2H, OCH_2), 5.189(s, 2H, OCH_2, Ar (Ph)), 5.623(bs, 1H, OH), 7.143-7.624(m, 5H, 2-CH, 3-CH, 4-CH, 5-CH and 6-CH, Ar (Ph)).

^{13}C NMR (150 MHz, DMSO-d6, 25°C): δ = 59.78(CH_2-OH), 67.81(OCH_2), 69.56(OCH_2, Ar (Ph)), 127.49(4-CH), 127.86(2-CH and 6-CH), 128.46(3-CH and 5-CH), 136.43(1-C, Ar(Ph)),154.37(OCOO). **TLC:** Rf–0.876 (CH_2Cl_2:CH_3OH-9:1) Rf–0.708 (CH_2Cl_2:CH_3OH-9.5:0.5).

Synthesis of p-nitrobenzoyl cyanomethyl ester: Bz(NO_2)-OCH_2CN: The procedure on cyanomethylation of Bz(NO_2)-OH includes reaction of the acyl component with $ClCH_2CN$ at room temperature and different solutions, which vary from polar aprotic to hydrophobic. To a solution of 0.127 g (0.759 mmol) of Bz(NO_2)-OH in DMF/CH_2Cl_2 were added 2 equiv. - 0.211 ml respectively (1.518mmol) triethylamine, in ice cold bath. After 10 minutes 2 equiv. – 0.096 ml (1.518 mmol) of chloroacetonitrile were added to the reaction mixture. The temperature of the reaction was left to reach at room t°C and the reaction mixture was left overnight. It was then evaporated under reduced pressure and dissolved in ethylacetate. The organic phase was washed three times with 0.5N HCl, brine, saturated solution (10%) of $NaHCO_3$ and again with brine. Finally the organic phase was dried over sodium sulfate and evaporated to dryness in vacuo. Yield-0.136 g (87%). Rf-0.854 (CH_2Cl_2:CH_3OH-9:1). Mw. = 206.16 g/mol. Anal. calcd. for $C_9H_6N_2O_4$: C 52.44, H 2.93, N 13.59; found: C 52.39, H 2.84, N 13.67.

^{1}H NMR (600 MHz, DMSO-d6, 25°C): δ = 5.214(s, 2H, OCH_2), 8.109(d, J=8.8 Hz, 2H, 3-CH and 5-CH), 8.351(d, J=8.8 Hz, 2H, 2-CH and 6-CH).

^{13}C NMR (150 MHz, DMSO-d6, 25°C): δ = 49.65(OCH_2), 115.72(CN), 123.57(3-CH and 5-CH), 128.96(2-CH and 6-CH), 138.94(1-C), 149.21(4-C), 173.39(COOC).

A general procedure for the synthesis of N-protected ornithine derivatives

To a solution of 5 or 8-10 mol (or 0.5 or 0.8-1 mol)[ש] from the one component (either: an amino- or an acyl compound, depending on the nucleophilic specificity) and the enzyme (free or immobilized lipase•), in a proper solvent (used as reaction media) (carbonate buffer/organic solvent, pH 9.0-9.3) ((Figures 1 and 2) from the discussion part), a solution of 1 mol (or 0.1 mol) from the another (amino- or acyl) component was added[ϑ]. The reaction was carried out at room temperature for about 1-12 hours depending on the nature of the substrate and the enzyme, as well as depending on the nucleophilic specificity and the reaction conditions*. After the reaction was finished (TLC, HPLC) the solution was filtered from the formed precipitate[#] of the product, which was then washed and the filtrate was entered directly into the next cycle of the process, with addition by one mol from every reagent (Figures 3 and 4). When immobilized lipase was used[€] (in the case when $CHCl_3$ or CH_2Cl_2 were used as solvents (reaction media) and R_2 = H) (Figures 5 and 6 from the discussion (below the basic line)), the formed precipitate of the product was filtered together with the immobilized lipase, after which the precipitate (the immobilized lipase together with the product) was washed with $CHCl_3$ or with CH_2Cl_2 and after that-it was washed with polar solvent or with water. The product was found in the water phase (the polar solvent), which after acidification of the reaction mixture and after precipitation-it was then filtered and rinsed, whereas the immobilized enzyme was entered to a new cycle, together with the component from the filtrate (the acyl component), and an addition of equimolar quantities from each of the components for the every new cycle was realized. When immobilized lipase was used together with the polar solvent / Na_2CO_3, which formed the media (in the case, where R_2 = Me (CH_3)) (Figure 5) from the discussion, the procedure was the same, but the washings were vice versa[€]. The product precipitated together with the lipase, after which they were filtered, washed with a polar solvent (the same, consisted the reaction media) and the precipitate was filtered with $CHCl_3$ or CH_2Cl_2. At this time the immobilized lipase remains undissolved and after that it was entered to the new cycle together with the excess from the amino component (which composes the "pool"), but the product dissolves in the organic solvent, and after evaporation in vacuo it was obtained in pure form. In the case of low enzyme nucleophilic specificity (or high enzyme electrophilic specificity), where a carboxy unprotected amino acid was used as an amino component in a hydrophobic organic solvent ($CHCl_3$ or CH_2Cl_2, R_2 = H) and immobilized lipase was used as a catalyst, at the first stage the product precipitates together with the immobilized lipase and with the amino component excess (Figure 7) from the discussion. They were filtered and rinsed using $CHCl_3$ or CH_2Cl_2. At the second stage, by the second filtration with the polar media (H_2O - acetone), the product with the amino component excess dissolved, whereas the lipase was insoluble and was entered to the next cycle. The product was purified from the excess of the amino component by acidification and third filtration with a polar solvent combination (H_2O- acetone), which allows for the product precipitation and its separation by the filtering, but the excess of the amino component was dissolved in the polar solvents and was directly introduced to the next cycle (Figure 7) from the discussion. When the reaction proceeds in polar solvents (water- organic mixed reaction media) using the same conditions and requirements as in the previous example (Figure 8) (discussion), at the end of the reaction and filtering of the reaction mixture-the lipase was separated (insoluble), whereas the product and the amino component excess passed over the filter in the polar organic media. After acidification and second filtration (also the washing with water) the product was precipitated and was separated from the amino component excess (4 equiv., composing the "pool"), which was introduced to the next cycle together with the immobilized enzyme (regenerated in some cases if it is required) and with additionally addition of 1 equiv. from the acyl and the amino component. In the cases, when the immobilized lipase displays the high nucleophile (or the low electrophilic specificity by the acyl component) specificity using polar solvents media (R_2 = H) (Figure 9) (discussion), the enzyme and the excess from the acyl component precipitated after the first filtration and washing with water, but the product was dissolved in the filtrate (polar solvent + water phase). Sometimes the additionally washing of the polar phase with a hydrophobic organic solvent was necessary – to purify the product and to avoid the impurity from the acyl component. After acidification of the water phase, the product was precipitated and filtered, whereas the immobilized lipase with the acyl component excess were introduced to the next cycle after the first filtration, together with the addition of 1 equiv. of the amino and acyl components. Finally, after the last cycle (10^{th}-15^{th})[†] the deactivated enzyme and the unused "pool" were discarded, except with the cases, when they could be regenerated.

[ש] *Despite the described difference and incompatibility in the ratio of the molar order of magnitude between the procedures for the activated ester preparation and the large-scale iterative procedures for the amino acid derivatives protection, large amounts of activated esters were used in the procedures for alpha-amino group protection of L-ornithine derivatives.*

• *(When immobilized lipase was used, an enzyme suspension has existed.*

[ə] *(When $CHCl_3$ or CH_2Cl_2 were used as reaction media and R_2 = H, under the existence of high nucleophilic specificity, a suspension from the appearing precipitate of the starting amino component was initially formed, which disappeared with the time course).*

***** *In the case under the existence of hydrolytic product traces (when a methyl ester of the amino acid was used as an amino component) the unwanted sub-product was needed to be removed by additional work-up of the reaction mixture.*

[#] *(When R_2 = H, the solution was acidified before filtration).*

[€] *When a two-phase system with immobilized or with free lipase was used (in the case when R_2 = H, CH_3), in most cases the filtration was not indispensable, but the direct washing of the water phase with an organic solvent, and its entering to a new cycle was only necessary. The filtration was needed only in the case, when insoluble immobilized lipase was used, to avoid the insoluble lipase from the solution, before its introducing to the next cycle.*

[ŧ] *The reaction on every cycle was monitored and studied by the kinetic measurement of its reaction rate. After every following cycle, the enzyme activity was decreased.*

Fmoc-Orn(Boc)-OH

Mw. = 454.52g/mol. Anal. calcd. For $C_{25}H_{30}N_2O_6$: C 66.06, H 6.65, N 6.16; found: C 66.04, H 6.61, N 6.04.

^{1}H NMR (600 MHz, DMSO-d6, 25°C): δ = 1.369(s, 9H, CH_3), 1.40-1.47(m, 2H, 4-CH_2), 1.52-1.58(m, 1H) and 1.66-1.72(m, 1H, 3-CH_2), 2.906(q, J=6.5 Hz, 2H, 5-CH_2), 3.907(ddd, J=4.6, 8.0, 13.0 Hz, 1H, 2-CH), 4.21-4.23(m, 1H, 9-CH), 4.25-4.30(m, 2H, OCH_2), 6.816(t, J=5.5 Hz, 1H, 5-CH_2NH), 7.329(t, J=7.4 Hz, 2H, 2-CH and 7-CH), 7.416(t, J=7.4 Hz, 2H, 3-CH and 6-CH), 7.643(d, J=8.0 Hz, 1H, 2-CHNH), 7.724(d, J=7.5 Hz, 2H, 1-CH and 8-CH), 7.894(d, J=7.6 Hz, 2H, 4-CH and 5-CH), 12.578(bs, 1H, COOH).

^{13}C NMR (150 MHz, DMSO-d6, 25°C): δ = 26.20(4-CH_2), 28.08(3-CH_2), 28.21(CH_3), 39.97(5-CH_2), 46.57(9-CH), 53.60(2-CH), 65.54(OCH_2), 77.34(C), 120.06 and 120.08(4-CH and 5-CH), 125.23 and 125.25(1-CH and 8-CH), 127.02(2-CH and 7-CH), 127.58(3-CH and 6-CH), 140.64 and 140.66(4a-C and 4b-C), 143.74 and 143.79(8a-C and 9a-C), 155.53 and 156.07(NHCOO), 173.82(COOH). **TLC:** Rf-0.352 (CH_2Cl_2 : CH_3OH-9:1), Rf-0.320 (CH_2Cl_2 : CH_3OH-9.5:0.5). M.p. 113-115°C.

Fmoc-Orn (Boc)-OCH$_3$

Mw. = 468.55 g/mol. Anal. calcd. For $C_{26}H_{32}N_2O_6$: C 66.65, H 6.88, N 5.98; found: C 66.54, H 6.82, N 5.94.

^{1}H NMR (600 MHz, DMSO-d6, 25°C): δ = 1.369(s, 9H, CH_3), 1.39-1.47(m, 2H, 4-CH_2), 1.54-1.60(m, 1H) and 1.65-1.71(m, 2H, 3-CH_2), 2.88-2.93(m, 2H, 5-CH_2), 3.616(s, 3H, OCH_3), 4.009(ddd, J=4.9 Hz, J=7.9 Hz, J=9.4 Hz, 1H, 2-CH), 4.224(t, J=7.1Hz, 1H, 9-CH), 4.295(d, J=7.1Hz, 2H, OCH_2), 6.810(t, J=5.7 Hz, 1H, 5-CH_2NH), 7.333(dt, J=7.4 Hz, J=0.9 Hz, 2H, 2-CH and 7-CH), 7.418(t, J=7.4 Hz, 2H, 3-CH and 6-CH), 7.714(dd, J=7.4 Hz, J=2.8 Hz, 2H, 1-CH and 8-CH), 7.790(d, J=7.8 Hz, 1H, 2-CHNH), 7.895(d, J=7.6 Hz, 2H, 4-CH and 5-CH).

^{13}C NMR (150 MHz, DMSO-d6, 25°C): δ = 25.98(4-CH_2), 27.91(3-CH_2), 28.19(CH_3), 39.97(5-CH_2), 46.56(9-CH), 51.78(OCH_3), 53.56(2-CH), 65.55(OCH_2), 77.37(C), 120.08(4-CH and 5-CH), 125.18(1-CH and 8-CH), 127.01(2-CH and 7-CH), 127.58(3-CH and 6-CH), 140.67(4a-C and 4b-C), 143.69 and 143.74(8a-C and 9a-C), 155.53 and 156.03(NHCOO), 172.82($COOCH_3$). Rf–0.878 (CH_2Cl_2:CH_3OH-9:1). **TLC:** Rf–0.652 (CH_2Cl_2:CH_3OH-9.5:0.5). M.p. 119-120°C.

Bz(NO$_2$)-Orn(Boc)-OH

Mw. = 381.39 g/mol. Anal. calcd. For $C_{17}H_{23}N_3O_7$: C 53.54, H 6.08, N 11.02; found: C 53.45, H 6.12, N 11.01.

^{1}H NMR (600 MHz, DMSO-d6, 25°C): δ = 1.362(s, 9H, CH_3), 1.43-1.53(m, 2H, 4-CH_2), 1.69-1.86(m, 2H, 3-CH_2), 2.92-2.96(m, 2H, 5-CH_2), 4.34-4.38(m, 1H, 2-CH), 6.830(t, J=5.8 Hz, 1H, 5-CH_2NH), 8.105(d, J=8.8 Hz, 2H, 3-CH and 5-CH), 8.334(d, J=8.8 Hz, 2H, 2-CH and 6-CH), 8.972(d, J=7.3 Hz, 1H, 2-CH_2NH), 12.71(bs, 1H, COOH).

^{13}C NMR (150 MHz, DMSO-d6, 25°C): δ = 26.37(4-CH_2), 27.83(3-CH_2), 28.20(CH_3), 39.35(5-CH_2), 52.68(2-CH), 77.37(C), 123.48(3-CH and 5-CH), 128.92(2-CH and 6-CH), 139.54(1-C), 149.05(4-C), 155.54(NHCOO), 164.90(NHCO), 173.31(COOH). **TLC:** Rf–0.320 (CH_2Cl_2:CH_3OH-9:1). M.p. 97.5°C.

Bz(NO$_2$)-Orn(Boc)-OCH$_3$

Mw. = 395.41g/mol. Anal. calcd. For $C_{18}H_{25}N_3O_7$: C 54.68, H 6.37, N 10.63; found: C 54.63, H 6.31, N 10.41.

^{1}H NMR (600 MHz, DMSO-d6, 25°C): δ = 1.362(s, 9H, CH_3), 1.43-1.53(m, 2H, 4-CH_2), 1.72-1.78(m, 2H, 3-CH_2), 2.92-2.96(m, 2H, 5-CH_2), 3.651(s, 3H, CH_3), 4.42-4.86(m, 1H, 2-CH), 6.829(t, J=5.6 Hz, 1H, 5-CH_2NH), 8.106(d, J=8.8 Hz, 2H, 3-CH and 5-CH), 8.337(d, J=8.8 Hz, 2H, 2-CH and 6-CH), 9.105(d, J=7.3 Hz, 1H, 2-CH_2NH).

^{13}C NMR (150 MHz, DMSO-d6, 25°C): δ = 26.14(4-CH_2), 27.67(3-CH_2), 28.19(CH_3), 39.23(5-CH_2), 51.92(OCH_3), 52.68(2-CH), 77.39(C), 123.51(2-CH and 6-CH), 128.96(3-CH and 5-CH), 139.24(1-C), 149.13(4-C), 155.54(NHCOO), 165.00(NHCO), 172.31($COOCH_3$). **TLC:** Rf–0.523 (CH_2Cl_2:CH_3OH-9:1). M.p. 88-89°C.

Z-Orn(Boc)-OH

Mw. = 366.41 g/mol. Anal. calcd. For $C_{18}H_{26}N_2O_6$: C 59.00, H 7.15, N 7.65; found: C 58.94, H 7.12, N 7.42.

^{1}H NMR (600 MHz, DMSO-d6, 25°C): δ = 1.361(s, 9H, CH_3), 1.45-1.54(m, 2H, 4-CH_2), 1.67-1.81(m, 2H, 3-CH_2), 2.90-2.94(m, 2H, 5-CH_2), 4.11-4.19(m, 2H, OCH_2), 4.33-4.37(m, 1H, 2-CH), 6.832(t, J=5.8 Hz, 1H, 5-CH_2NH), 7.036(t, J=7.5Hz, 1H, 4-CH), 7.691(d, J=8.1 Hz, 2H, 3-CH and 5-CH), 8.229(d, J=7.8 Hz, 2H, 2-CH and 6-CH), 8.971(d, J=7.3 Hz, 1H, 2-CH2NH), 12.73(bs, 1H, COOH).

^{13}C NMR (150 MHz, DMSO-d6, 25°C): δ = 26.33(4-CH_2), 27.81(3-CH_2), 28.21(CH_3), 39.34(5-CH_2), 52.67(2-CH), 77.38(C), 123.47(3-CH and 5-CH), 128.91(2-CH and 6-CH), 139.53(1-C), 149.05(4-CH), 155.55(NHCOO), 164.91(NHCO), 173.32(COOH). **TLC:** Rf–0.310 (CH_2Cl_2:CH_3OH-9:1). M.p. 82.5°C.

Z-Orn(Boc)-OCH$_3$

Mw. = 380.44 g/mol. Anal. calcd. For $C_{19}H_{28}N_2O_6$: C 59.99, H 7.42, N 7.36; found: C 60.03, H 7.39, N 7.37.

^{1}H NMR (600 MHz, DMSO-d6, 25°C): δ = 1.361(s, 9H, CH_3), 1.40-1.49(m, 2H, 4-CH_2), 1.72-1.76(m, 2H, 3-CH_2), 2.91-2.95(m, 2H, 5-CH_2), 3.652(s, 3H, CH_3), 4.396(d, J=7.4Hz, 2H, OCH_2), 4.41-4.87(m, 1H, 2-CH), 6.828(t, J=5.6 Hz, 1H, 5-CH_2NH), 7.132(t, J=7.9Hz, 1H, 4-CH), 7.809(d, J=7.8 Hz, 2H, 3-CH and 5-CH), 8.233(d, J=8.1 Hz, 2H, 2-CH and 6-CH), 9.109(d, J=7.3 Hz, 1H, 2-CH_2NH).

^{13}C NMR (150 MHz, DMSO-d6, 25°C): δ = 26.13(4-CH_2), 27.66(3-CH_2), 28.18(CH_3), 39.21(5-CH_2), 51.93(OCH_3), 52.67(2-CH), 77.38(C), 123.53(2-CH and 6-CH), 128.95(3-CH and 5-CH), 139.23(1-C),

149.13(4-CH), 155.56(NHCOO), 165.01(NHCO), 172.32($COOCH_3$). **TLC:** Rf–0.543 (CH_2Cl_2:CH_3OH-9:1). M.p. 78-79°C.

Discussion

Enzymatic peptide synthesis has many advantages over the classical methods for peptide synthesis, which concludes in enhanced selectivity and specificity towards the substrates and reagents, stereo-selectivity and stereo-specificity towards the prepared products, low energy consumption, mild reaction conditions, elegant synthetic approaches etc. Usually, the most of enzymes work in water media (buffer), which allows for their activity and stability. However, the water neighborhood and proximity impedes the normal dissolving of some reagents and substrates, which are water insoluble. This demands and enforces the using of organic solvents in combination with water (water miscible or water immiscible), which however is not sufficient in some cases- during the synthesis of very hydrophobic products from hydrophobic substrates. Lipases are ones of the few enzymes that can also work only in organic solvent, retaining its own native structure and catalytic activity, which is suitably for synthesis of such hydrophobic substrates. The solvent selection is one of the most important factors for the successful realizing of the enzyme synthesis. Besides the water, the organic solvent allows for the successful dissolving of substrates, which are water insoluble, as well as for product precipitation during the thermodynamically-controlled synthesis. Most of lipases, used in the organic synthesis require a fixed, certain and precise amount of water for the normal catalysis, due to the enzyme activity and stability. During the carrying out of enzyme peptide synthesis, a controlled product precipitation is described, depending on its solubility, which allows keep its concentration bellow the equilibrium values [14]. This allows for the shifting of the reaction equilibrium towards the formation of the product. Usually, the precipitated product is collected by filtration of the reaction medium, which consists besides the product and the enzyme, also the unreacted excess from one of the components, which usually discards [15-19]. This methodology suffers from many disadvantages, due in many cases to the expensive reagents (costliness of the acyl or amino components), composing the related"pool" which means, that enzyme synthesis cannot be carried out in the presence of the observed low nucleophilic or electrophilic specificity, that would require the presence of a large excess from one, or from the another of the components (nucleophilic (amino) or electrophilic (acyl) compounds), which after remains unused**.** In some cases, this problem can be resolved by the reagents regeneration after additionally working up of the reaction mixture. However, the enzyme recycling and the recovery of its activity, is one of the general and the most important basic problems, which still remains unresolved. To achieve a low-cost industrial preparation of chiral active pharmaceutical products, the enzyme should be used in many times without its complicated regeneration and sharply lose of its native structure and activity. The alternative using of immobilized enzyme in water-organic media is complicated, due to the difficult and prolonged separation of the immobilized enzyme from the another components in the reaction mixture [20], as well as because of the slow reagents diffusion, and finally - due to the low and poor enzyme activity - many times longer reaction time is required [20,21]. Moreover, some methodologies for enzyme immobilization require expensive matrices utilization and a specific methodology for immobilization: **1)** adsorption to the outside of an inert material - on glass, alginate beads or matrix; **2)** entrapment in insoluble beads or microspheres, such as calcium alginate beads; **3)** covalently cross-linkage of the enzyme to a matrix through a chemical interaction by a spacer molecule like poly(ethylene glycol), that helps reduce the steric hindrance by the substrate. Utilization of the alternative procedure for enzyme synthesis allows the partially, and in more cases the fully solving of this problem, which consists in the iterative addition of equivalent amounts from the acyl- and amine- components to the solution ("electrophile pool" or "nucleophile pool"), consisting an enzyme and excess of the electrophilic or nucleophilic components depending on the method (Figure 1)**.** Therefore the problem is resolved by the repeatedly enzyme using, and by the utilization of an iterative procedure (Figure 1). As a catalyst in the synthesis procedure, the selection directed us towards the application of **P**orcine **P**ancreatic **L**ipase (PPL) (Figure 2). On the other hand the lipase is a triacylglycerol hydrolase **(EC 3.1.1.3),** which catalysts hydrolysis reactions of lipids (triglycerides, TAG) in nature. The enzyme reactions in the most cases are equilibrated, which is specific and relevant for all hydrolases. Besides hydrolysis reaction in the water presence, also another nucleophilysis (alcoholysis, aminolysis) reaction is possible, if a stronger nucleophile, instead of water exists in the reaction mixture. Moreover, it is clearly known, from the law of mass action that in the presence of a multifold

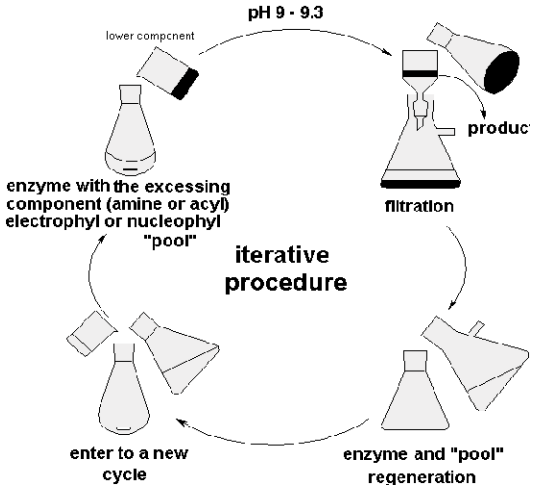

Figure 1: A general scheme for the iterative procedure for chemo-enzymatic semi-synthesis of N-amino acid derivatives (N-functional group protection of the amino acids).

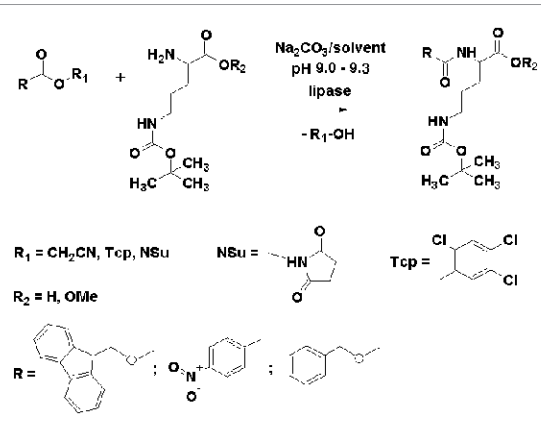

Figure 2: Scheme of the synthesis of N-protected amino acids derivatives. It is used in the case a δ-Boc-protected L-Ornithine: H-Orn(Boc)-OR_2 as an amino acyl component. In the case of Fmoc-group and Z-group – p-nitrophenyl activated esters: Fmoc-ONP (Fmoc-ONp) and Z-ONP (Z-ONp), as well as pentachlorophenyl esters: Fmoc-OPcp, Z-OPcp were also employed, whereas everyone of the protected groups were also introduced by their 1-(benzotriazole-1-yl) (hydroxybenzotriazole-1-yl; 1-hydroxy benzotriazole) activated ester: Fmoc-OBt, Z-OBt, Bz(NO_2)-OBt*.

excess from the nucleophile, besides the water, and which is different from the water, the reaction proceeds in the nucleophilysis (towards alcoholysis, aminolysis) direction. If $\mathbf{k_w}$ and $\mathbf{k_n}$ are the values of the rate constants of the hydrolysis and the aminolysis parallel reactions, the preparative yield can be calculated and evaluated, using the equation [22 and 23] **(1)**):

$$YP = \frac{K_n(N)}{K_n(N) + K_w(W)} \times 100\% \quad \textbf{(1)}$$

$$YP = \frac{K_n(N)}{(N)(K_n + K_w(W)/(N))} \times 100\% \quad \textbf{(2)}$$

$$\frac{K_n + K_w(W)}{(N)\,Yp} = K_n \times 100$$

$$\frac{K_w(W)}{(N)\,Yp} = K_n \times 100 - K_n$$

If in 1000 ml H_2O we have 55. 56 mol, then we calculate with the following substitution:

$$\frac{K_w(55,56)}{(N)\,Yp} = K_n \times 100 - K_n$$

$$\frac{K_w(55,56)}{(N)\,Yp} = K_n \times 100 - Yp \times K_n$$

$$(N) = \frac{K_w \times (55,56) \times Yp}{K_n 100 - Y \times K_n}$$

$$(N) = \frac{K_w \times (55,56) \times Yp}{K_n 100 - Y \times K_n}$$

$$(N) = (55,56) \times Yp$$

$$\frac{K_n 100 - Yp \times K_n}{K_w}$$

$$(N) = Y_p \times 55,56$$

$$\frac{100 - Yp \times K_n}{K_w}$$

Therefore, the necessary yield **Yp,** may be obtained, using the nucleophile concentration **(2)**):

$$(N) = \frac{Yp \times 55,562}{(100 - Yp)(K_n / K_w)}$$

i.e., depends from the constants ratio of the aminolysis towards the hydrolysis reactions $\mathbf{k_n}$ / $\mathbf{k_w}$. Depending on the enzyme nucleophilic specificity, this equation varies in some extent, and has different values [22, 23]. From the equation 2) follows, that under low nucleophilic specificity (poor and unsatisfactory values of the ratio: $\mathbf{k_n}$ / $\mathbf{k_w}$), high preparative yields may be obtained, using a high nucleophile concentration. By analogy, under high nucleophilic enzyme specificity, high yields of the final desired product may be obtained, using high electrophile and low nucleophile concentrations. When the reaction is carried out in medium, consisting the so called "electrophile pool" or "nucleophile pool", with the single filtration of the final product or with the single filtration of one of the reagents (media components), respectively with the addition of equivalent amounts from the corresponding components on the second, as well as on the next cycle, a repeated synthesis may be achieved many times, by the iterative procedure and with time and again enzyme (lipase) regeneration and utilization. One of the components (electrophile or nucleophile), depending on the nucleophilic or electrophilic specificity needs to be about 5 or 8-10 equivalents in excess, according to the other reagent (Figures 3-6). In spite of the different mechanism of a lipase-catalyzed reaction of peptide synthesis (Log ***P***, which characterizes the hydrophobic properties of organic solvents used in the selection of the solvents system (mixed aqueous-organic, or anhydrous organic media), as well as the dielectric constants of the respective co-solvent system can not explain the effect of the organic solvents well) according to the protease-catalyzed synthesis, they have some mutual and general characters. The peptide synthesis, catalyzed by the lipases is realized on the similar principle and by the same manner as the kinetically-controlled protease catalyzed amide synthesis, due to their esterase activity. The advantages of lipases over the proteases in the protection of the alpha-amino group in the amino acids, as well as in the peptide synthesis conclude: **1)** in the increased stability in hydrated and anhydrous organic solvents (hydrophilic or hydrophobic). This stability supports using of lipase in the synthesis of products in anhydrous environments, where the reaction would give products in high yields by the kinetically or thermodynamically controlled synthesis; **2)** the prepared amides (N-protected amino acids and peptides) formed in solution cannot be hydrolyzed by the lipase, which does not have protease activity. It is well known, from the classical enzymatic peptide synthesis, that the water per cent has a great effect on the yield of the product in the protease-catalyzed peptide bond formation. Higher water content favors the higher enzyme activity, but leads to an increase in the hydrolysis of the amide bond and particularly in the hydrolysis of the peptide bond. Lipase has no such disadvantage, since it has only esterase, but not amidase activity. Cleavage of peptide bonds as an undesirable side reaction would not take significant place in the process of amide and peptide bond synthesis, using the lipase as a catalyst; **3)** despite of the proteases, which in the most cases (with few exceptions) can accept only L-amino acids, lipases exhibit affinity and selectivity towards a variety of natural and unnatural L- and D-amino acids, which allows the preparation of peptides, containing unusual amino acids. Moreover, because of the wide range of lipase selectivity towards the reagents and substrates (electrophilic and nucleophilic specificity according to the acyl and nucleophilic components), it allows the

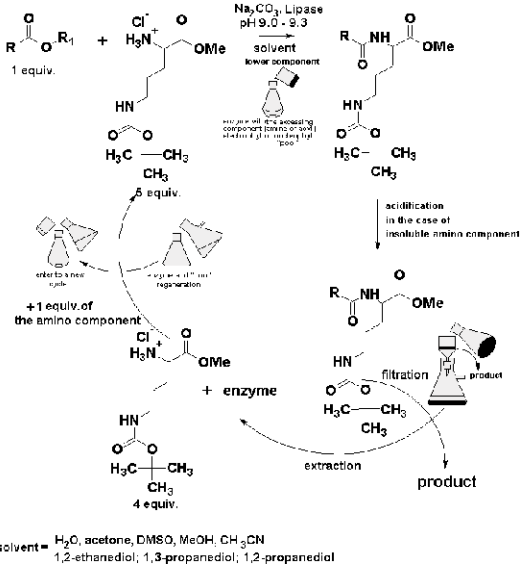

Figure 3: An iterative procedure for protection, using the excess of the protected amino acid (L-Ornithine) as an amino component ("nucleophile pool") and free lipase as a catalyst. After completing the synthesis and after acidification of the reaction mixture, filtering and washing of the precipitated product with water, the combined water phases were extracted with CH_2Cl_2 or EtOAc to increase the yield and avoid the waste and loss of the product.

industrial large-scale preparation of a variety of pharmaceutical compounds as substances, used in the pharmaceutical industry. The screening of solvents system plays an important role in the lipase-catalyzed synthesis of amides and peptides, and particularly in this case – in the process of preparation of N-protected amino acids derivatives. The solvent media has an influence not only on the yield of the product, but also on the enzyme stability and the substrate solubility, as well as the product behaviour, by its precipitation from the reaction mixture. It is well known, that lipase prefers hydrophobic solvents (anhydrous or with a few (small) buffer content): pentane, hexane, cyclohexane, heptane, octane, nonane, decane, benzene, toluene, dichloromethane, chloroform, tetrachloromethane, 1,1,1-trichloroethane, ethyl acetate, diethyl ether, di-n-butyl ether, diisobutyl ether, diisopropyl ether etc. When the reaction is carried out in a biphasic water (buffer)-organic system using the free Porcine Pancreatic Lipase (PPL), or when the synthesis reaction proceeds only in hydrophobic organic solvent, using immobilized lipase, the success of the reaction and the yield depends on the i.e., partition coefficient [24,25]:

$$P = \left[\frac{C_0 - C}{C}\right] \times \left[\frac{V_0}{V - V_0}\right]$$

where **P** is a partition coefficient, $\mathbf{C_0}$ is an initial concentration of compound, **C** is the equilibrium concentration of compound, $\mathbf{V_0}$ is the total volume of system (organic phase + water (buffer phase) – in the case of using free Porcine Pancreatic Lipase (PPL) and two- phase system, or organic phase + support – when immobilized Porcine Pancreatic Lipase (PPL) was used in a system, composed from one phase), and **V** is a volume of the organic phase. Also, the **partition coefficient** may be described as a ratio of concentrations of unionized compound between the two solutions. To measure the **partition coefficient** of ionizable solutes, the pH of the aqueous phase is adjusted such that the predominant form of the compound is unionized. The logarithm of the ratio of the concentrations of the unionized soluble compound in the solvents is called **log *P***: The log P values is also known as a measure of lipophilicity.

$$\log P_{oct/wat} = \log\left(\frac{[solute]_{octanol}}{[solute]^{un-ionized}_{water}}\right)$$

The unionized solute may be as the dissolved solid compound, as well as it may be another solvent, composing the solvents system.

However, these conditions (solvents systems) are convenient in the preparation of esters of hydrophobic products from hydrophobic reagents and substrates, whereas in the synthesis of amino acid derivatives, as well as in the peptide synthesis (aminolysis reactions) - more suitable are turn out - more polar organic solvents, as the one of the substrates usually is hydrophilic, but the another substrate and in many cases the products are hydrophobic. The application of more polar solvents allows the product precipitation and its bringing out from the equilibrium system, during the thermodynamically-controlled synthesis. Nevertheless, that the reaction rate is negligibly slower compared to the using of more hydrophobic solvents in the hydrophobic esters preparation, the hydrophilic solvents have some advantages in the peptide synthesis. Moreover in the water[¥] - alcohol (MeOH, ethylene glycol, 1,2-propylene glycol etc.) combined solvents system (media), the aminolysis reaction is accelerated by the alcohol and water assisted catalysis, due to the homogeneous or mixed (water : alcohol) cluster creation and proton shuttling mechanism, accelerating of the tetrahedral intermediate formation [26]. Due to the presence of a stronger nucleophile (amino group) than the alcohol or water components in the reaction media, the hydrolysis reaction does not proceed, and the obtained product (amide) is more thermodynamically stable. Also, because of the nature of the synthesized product (amide), it is not susceptible to the nucleophilic attack and hydrolysis reaction by lipase, which is one of the most well-known disadvantages in the carrying out of synthesis, using proteases.

[¥] *The water content plays an important role in the successful realizing of an amide (alpha-amino group protection) and peptide synthesis. Li-Qiang Zhanga et al. [27] described that when they used DMF and DMSO as solvents for the lipase-catalyzed peptide synthesis, the optimum water content had to be about 50%. When the water content was greater than 60%, the yield of their tripeptide product decreased rapidly because of the hydrolysis of the ester substrate. Moreover, when the water content was lower than 50%, the percent of the polar organic solvent in the system was insufferable for the lipase, leading to the denaturation of the enzyme and lowering of the yield of the peptide product.* Of course, the utilization of hydrophobic solvents in one phase (anhydrous) and in two-phase systems is also possible in the case, when the lipase has high nucleophilic specificity (or high electrophilic and high nucleophilic specificities together), and if the molar ratio between the acyl and amino components is 5 equiv.: 1 equiv., and when the amino component is not protected at the carboxyl group, which will allow the product precipitation from the reaction mixture, and will realize the iterative procedure, whereas the acyl component (which forms the "electrophile pool") remains in solution. Only in the case of using of immobilized **P**orcine **P**ancreatic **L**ipase **(PPL)**, it precipitates together with the product (Figure 5 and 6), and the additionally working up of the reaction mixture is necessary, by washing with organic solvent (Figure 5), or by acidification at the last step (before the third filtration) (Figure 6). During the second filtration with acetone/water the product dissolves and passes over with the filtrate, whereas the immobilized lipase remains undissolved and discards, after which it enters to the

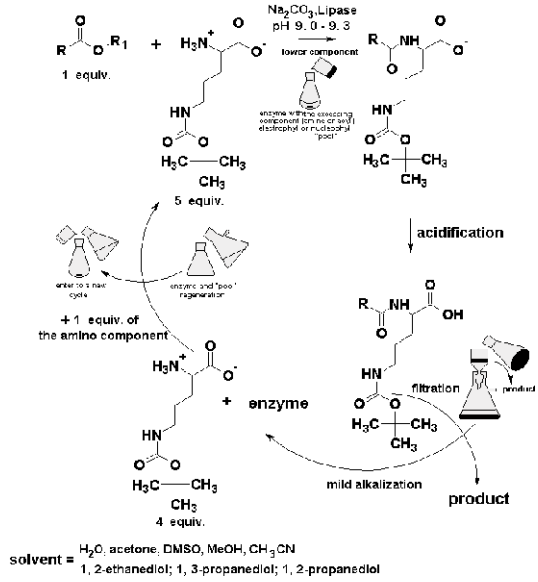

Figure 4: An iterative procedure for protection, using the excess of the carboxy-free amino acid (L-Ornithine) as an amino component ("nucleophile pool") and free lipase as a catalyst. After completing of the synthesis and after acidification of the reaction mixture, filtering and washing of the precipitated product with water, the combined water phases were extracted with CH_2Cl_2 or EtOAc to increase the yield and avoid the product loss.

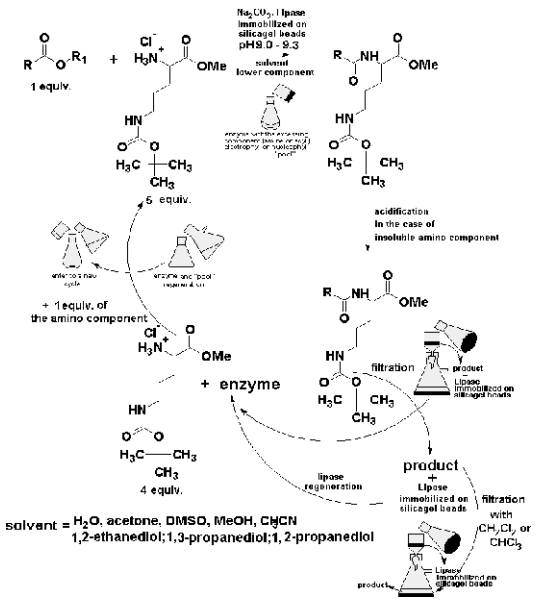

Figure 5: An iterative procedure for protection, using the excess of the protected amino acid (L-Ornithine) as an amino component ("nucleophile pool"), as well as immobilized lipase as a catalyst.

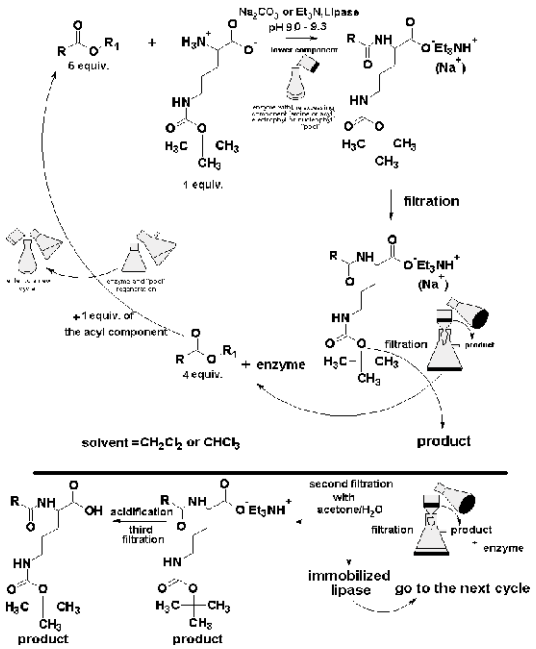

Figure 6: An iterative procedure for protection, using an excess of the acyl component ("electrophile pool") and utilizing a carboxy-free amino acid (L-Ornithine) as an amino component, as well as free or immobilized lipase as a catalyst.

next cycle. After acidification, the product precipitates and discards by the third filtration. Despite the good lipase selectivity towards the L- and D-amino acids methyl esters and amides, it is not well studied the lipase specificity according to the carboxy free amino- components. This will be the object for the next studies by our laboratory. **P**orcine **P**ancreatic **L**ipase (PPL), as many other lipases and proteases is a Ser-hydrolase, which catalyzes hydrolysis of esters through the initially formation of the acyl-enzyme complex, and then by an acyl-enzyme intermediate creation through the tetrahedral transition state, by the nucleophilic attack of β-OH of L-Serine in the enzyme active center, in which an ester bond exists between the L-Ser-hydroxyl of the enzyme and the acyl synthons. This complex is susceptible to the nucleophile attack of water (hydrolysis) or other stronger nucleophiles (nucleophilysis). The most successful solvent combination depends on the procedure for protection (synthesis), which in turn is determined and restricted by the nucleophilic specificity and respectively by the "pool" species and identity, nature, specificity and character of the components and therefore depending on the component excess. The most general, frequently and commonly utilizing solvents combination, which were used is a mixture of polar solvents with the water solution of sodium carbonate (pH 9-9.3): DMSO/Na_2CO_3, acetone/Na_2CO_3, CH_3CN/Na_2CO_3, DMF/Na_2CO_3, MeOH/Na_2CO_3, ethylene glycol/Na_2CO_3, propylene glycol/Na_2CO_3, isopropylene glycol/Na_2CO_3 – one phase system (in the case, when the enzyme is dissolved) [28]☼, as well as a combination of non-polar solvents with water solution of sodium carbonate (pH 9-9.3): $CHCl_3$/Na_2CO_3 or CH_2Cl_2/Na_2CO_3 – two phase system, or one phase: $CHCl_3$ or CH_2Cl_2 and triethylamine as a base (in the presence of immobilized lipase) [28]. As a result of the using of different nucleophilic and electrophilic components - the approach is chosen, which therefore depends on the different nucleophilic and electrophilic specificity, hence the conclusion is that an excess (different molar ratios) from the amino component - nucleophile ("nucleophile pool") or acyl component - electrophile ("electrophile pool") is allowed to be used and had to be added to the reaction mixture (Figure 2). After completing of the synthesis, depending on the used enzyme (free or immobilized lipase), different components were filtered - in the first case of using of free lipase, under the low nucleophilic specificity and in the presence of a carboxymethyl ester of the amino component (nucleophile) or in the presence of a free amino acid (R_2 = Me, H), the product is filtered, whereas the enzyme and one of the reagents, which is in excess (it characterizes and constructs the "pool" type and content, in these cases the amino component excess ("nucleophile pool")), remain in solution (Figures 3 and 4). Sometimes at the starting of reaction the acyl component was insoluble, depending on the acyl component and solvent, but with the progress in the reaction time - it mildly and slowly dissolves. When immobilized lipase is used, it is filtered together with the obtained product (Figure 5), after which they are separated by the washing with $CHCl_3$ or CH_2Cl_2 and by dividing of the product from the immobilized lipase, which is introduced to the next cycle, sometimes after an additionally regeneration. In the other case, under the presence of high nucleophilic specificity, and under the using of free or immobilized on silica beads lipase – the product and the enzyme (in the case of immobilized lipase) are precipitated and filtered, whereas one of the reagents, which is in excess and which composes and characterizes the "pool" remains in solution (Figure 6). After that a second filtration follows, to discharge (discard) and separate the product from the enzyme, and precipitate the product from the solution, and after washing and regeneration of the immobilized lipase, it is entered again into the "pool", and is again introduces to the next cycle together with the other components. Depending on that, we choose the different solvent media - this determines the selection of the combinative approach - as combinations of different solvents and an excess from a given component, as well as a different type of lipase (free [dissolved in solution] or immobilized). On the other hand - the lipase consistence (condition and ability) characterizes the type of the synthesis. When free lipase, dissolved in solution was used, homogeneous synthesis was carried out, due to the presence of all of the components in the homogeneous phase of the mixture. In the case of the immobilized

lipase utilization, the synthesis was already heterogeneous, due to the fact, that the enzyme and the other components are found in different phases.

☼ *So et al. describe 3-methyl 3-pentanol, containing 5% (v/v) buffer, as the most convenient solvents system for peptide synthesis (in their case they synthesized peptides, containing D-amino acids)[29].*

Scheme of the synthesis of N-protected amino acids derivatives (Figure 2): *It is used in the case δ-Boc-protected L-Ornithine: H-Orn(Boc)-OR_2 as an amino acyl component. In the case of Fmoc-group and Z-group – p-nitrophenyl activated esters: Fmoc-ONP (Fmoc-ONp) and Z-ONP (Z-ONp), as well as pentachlorophenyl esters: Fmoc-OPcp, Z-OPcp were also employed, whereas everyone of the protected groups were also introduced by their 1-(benzotriazole-1-yl) (hydroxybenzotriazole-1-yl; 1-hydroxy benzotriazole) activated ester: Fmoc-OBt, Z-OBt, Bz(NO_2)-OBt*.*

**Full protocols related to the synthesis and analysis of all noted activated esters will be described elsewhere in the forthcoming article⌂ which is in progress [30].*

⌂ *In the noted article [30] the electrophilic specificity of Porcine Pancreatic Lipase (PPL), according to a variety of esters of the described well-known protective groups is studying by the kinetic measurements of the lipase catalyzed reaction of alpha-amino group protection.*

To prove our proposed theory, on the first stage-the studies were carried out using H-Orn(Boc)-OMe(H) as a substrate for the model reaction of the iterative procedure. Because of the presence of the side chain δ-NH_2 group functionality, we previously decided to protect this side chain function with Boc-protection and to preserve its

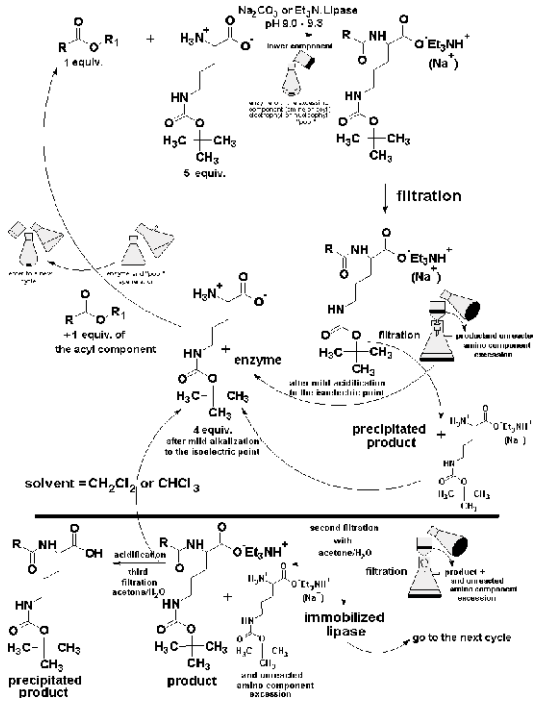

Figure 7: An iterative procedure for protection, using an excess of the amino component (a free amino acid) ("nucleophile pool") in the case of low enzyme nucleophilic (or high electrophilic) specificity, as well as free (above the basic line) or immobilized (below the basic line) lipase as a catalyst.

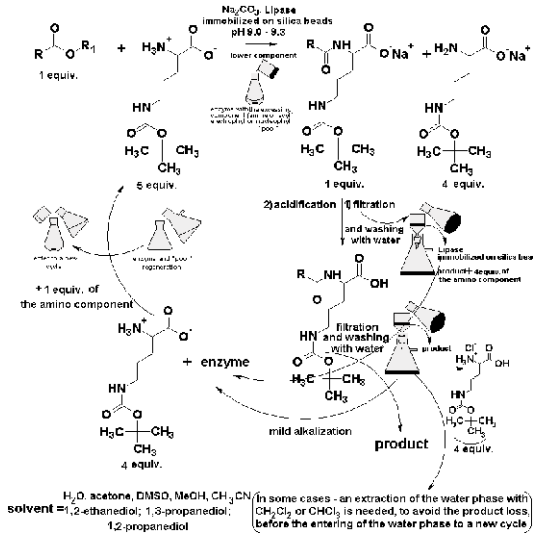

Figure 8: Effective iterative procedure for protection of alpha-amino acid using an excess of the carboxy-free amino acid (L-Ornithine), composing the "nucleophile pool" in the case of low enzyme nucleophilic specificity and immobilized on silica beads lipase as a catalyst.

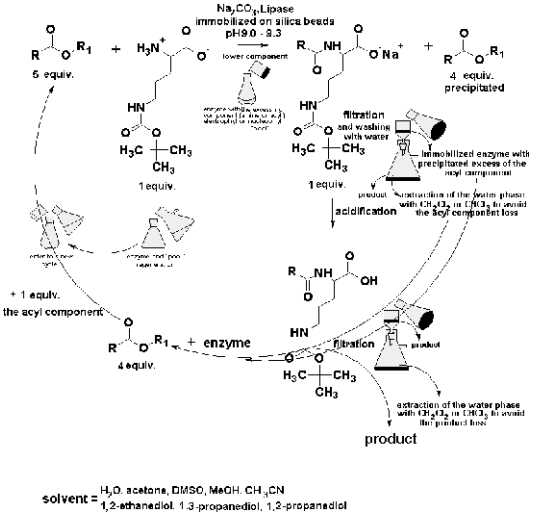

Figure 9: Effective iterative procedure for protection of alpha-amino acid (L-Ornithine) using an excess of the acyl component, composing the "electrophile pool" in the case of low enzyme electrophilic (high nucleophilic) specificity and immobilized on silica beads lipase as a catalyst. As in Figure 8 polar solvents were used as a reaction media.

interaction as nucleophile – to avoid the undesired side reaction. To prove our predicted observations - activated esters of Fmoc-, Z- and Bz(NO_2)-protective groups were used to protect the α-amino group of the above mentioned amino acid. Because of the high specificity of the **P**orcine **P**ancreatic **L**ipase (PPL) towards the above noted synthetic equivalents (activated ester of Fmoc-, Z- and p-nitrobenzoyl protective groups)₸ (Figure 2), and concretely towards the alcohol synthon and in some extent towards the acyl synthon of these protective groups, we choose the molar ratio between the acyl- and amino- components to be 1 equiv.: 5 equiv. (Figures 3-5). Different conditions were selected, depending on the combinative approach (a different solvent system, free or immobilized lipase, a carboxy- free, or a carboxy- protected

amino- component.) When a carboxy- protected L-Orn was used as an amino- component, the reaction rate was slowly higher in some extent, probably due to the better enzyme affinity towards the protected L-Orn as its methyl ester [30].

[ᵀ] *A variety of acyclic aliphatic esters of Fmoc-, Z- and Bz(NO_2)-protective groups: n-butyl, n-pentyl, n-hexyl, n-heptyl, n-octyl, n-nonyl, n-decyl, n-undecyl, n-dodecyl, as well as their isomers with a branched carbon chain were synthesized, and their activity in the lipase catalyzed aminolysis reaction (protection of the alpha-amino group) were studied [30]. Matos et. al. revealed [31], that esters of longer, straight chain alcohols, were better substrates than those of shorter chain alcohols, probably due to the unity properties of this long-chain alcohols as better leaving groups (nucleophuges). The results will be published elsewhere [30]. Also it is forwarded, that the synthesis of cyclic aliphatic, aromatic as well as aliphatic-aromatic esters (alcohol synthons) of the above noted protective groups (acyl synthons) as synthetic equivalents and the study of their reactive ability will be realized.*

(Figure 3) *After completing the synthesis and after acidification of the reaction mixture, filtering and washing of the precipitated product with water, the combined water phases were extracted with CH_2Cl_2 or EtOAc to increase the yield and avoid the waste and loss of the product.*

Fmoc-Orn(Boc)-OMe(H), Z-Orn(Boc)-OMe(H) and Bz(NO_2)-Orn(Boc)-OMe(H) were synthesized and used as intermediates, which were found a general application for the two pathways for the synthesis of substrates for the model ribosome reactions [7,32], we prove the crucial role of vicinal *sin*-oriented 2'-OH group in the intramolecular aminolysis reaction of adenosine derivative: 2'/3'-O-[Bz(NO_2)-Orn(Boc)]-5'-O-Piv-Ado [6,7,32], and the general and important role of A76 2'-OH in the ribose ring of peptidyl-tRNA for acceleration of peptidyl transferase reaction, during the protein biosynthesis on ribosome [33,34]. On the other hand the amino acid was used as its methyl ester, as well as it was used with its carboxyl free functional group, which determines the combinative approach, related to the selection of solvents media and lipase determination (free or immobilized). Despite the worldwide using of amino acids amides as amino components (acyl acceptors, nucleophiles) in order to avoid the undesired hydrolytic reaction, we decided to use as amino components the unprotected and protected as methyl ester amino acids. In the iterative procedure for protection, using a methyl ester, in a few of cases - traces of the unwanted hydrolytic product were observed (hydrolysis of the methyl ester of the amino acid as an undesired side reaction). *So et al.* used also methyl esters of amino acids as good acyl acceptors [29]. If the reaction proceeded within the range of 1-2 hours depending on the electrophilic or nucleophilic enzyme specificity and the nature of the components, only traces of hydrolytic product were observed (0.1 - 0.3%). About 0.5% was observed up to 3 hour. The hydrolysis of the amino acid methyl ester is much more embarrassed, than the aminolysis reaction of activated esters of a variety of protective groups, probably due to the higher enzyme selectivity towards the bulky acyl synthon, and towards the hydrophobic alcohol synthon, playing the role as a good nucleophuge (leaving group) in the acyl ester synthetic equivalent of the acyl components[ɬ]. Thus, the significant percent of hydrolytic product, due to the hydrolysis reaction is possible only in the cases, when the methyl esters of substrates are incubated in the reaction mixture with lipase for a time, more than 48 hours and under different conditions [32]. [ɬ] *In rare cases (using a methyl ester of the amino acid as an amino (nucleophile) component, and a molar ratio 1 equiv.:5 equiv. between the acyl and amino components), when traces of a dimmer formation were observed as an undesired side product as a result of aminolysis reaction on the carboxy methylated group of amino acid, its removing from the reaction mixture was necessary, by the additionally working up procedure.*

(Figure 4) *After completing of the synthesis and after acidification of the reaction mixture, filtering and washing of the precipitated product with water, the combined water phases were extracted with CH_2Cl_2 or EtOAc to increase the yield and avoid the product loss.*

In the case, when a negligible amount of the hydrolytic product exists, two ways for the solving of the problem are possible, by the product purification (additionally working up of the reaction mixture). By the first manner – if the hydrolytic products (H-Orn(Boc)-OH and <HN-Orn(Boc)-OH, where <HN- is the alpha-amino group protection) are soluble in the filtrate as their sodium salts, they may be automatically introduced to the next cycle, and finally – after the last cycle to be discarded together with the reaction mixture (enzyme, which has lost its activity and the respective "pool")[θ], except the relevant product, which precipitates and filtrates. In the second pathway depending on its solubility, <HN-Orn(Boc)-OH has to be avoided from the reaction mixture by the additionally working up of the mixture at every cycle. If the hydrolytic product (formed as undesired, during the side hydrolytic reaction) is insoluble - it precipitates together with the product and the compounds are purified by the additionally selective extraction. If <HN-Orn(Boc)-OH is soluble in the reaction mixture as sodium salt, it may be entered to the next cycle and at the last cycle to be discarded, or it may be additionally worked up by acidification. In these conditions the carboxyl group of the hydrolytic product is protonated and <HN-Orn(Boc)-OH precipitates together with the main product, where by the additionally extraction with hydrophobic organic solvent and washing of the organic phase, the desired product may be purified.

[θ] *Of course, the "pool" and the lipase can be regenerated, if it is possible.*

The studies of the combinative approach on the iterative procedure of the alpha-amino group protection, using free (unprotected) amino acids side chain functionalities are in progress in our laboratory and the completed results will be published elsewhere (Figure 5). These results will have a solely aim and purpose – to prove the selectivity of the reaction related to the alpha-amino group protection of amino acids with many functionalities, having the same strong or stronger nucleophilic properties, together with the alpha-amino group. The possibilities of protection of a side chain functional group in the amino acids as an undesired side reaction depends on the electrophilic (acyl) and nucleophilic (amino) enzyme specificity, which determines the proportional ratio between the acyl- and amino- components. For example: under an existence of high electrophilic and high nucleophilic specificity (1equiv. acyl component : 1,2 equiv. amino component), under low electrophilic and low nucleophilic specificity (1equiv. acyl component : 1,2 equiv. amino component), as well as under high electrophilic and under low nucleophilic specificity (1 equiv. acyl component : 5 equiv. amino component), in the presence of OH-group in the side chain of L-Ser and L-Thr, and in the presence of SH-group in the side chain of L-Cys, these OH-groups and SH-group in the above noted amino acids cannot be acylated. Only under low electrophilic and high nucleophilic specificity (5 equiv. of acyl component : 1 equiv. of amino component), side chain acylation is possible as an undesired reaction. Of course, also when high electrophilic and high nucleophilic specificity are observed, as well as using molar ratio of the acyl according to the amine component: 5 equiv.:1 equiv. (applying the iterative procedure), side chain functionality acylation is possible as an unwanted side reaction, but not possible when the molar ratio between

the acyl and the amine component is 1 equiv.:5 equiv. By the same manner the guanidine side chain functionality of L-Arginine, ε-amino group of L-Lysine, p-hydroxybenzyl (phenol) side chain functionality of L-Tyrosine, side chain indole ring of L-Tryptophane, and side chain imidazole ring of L-Histidine would have been having the same behaviour, using these reaction conditions. Therefore, the lipase selectivity towards the alpha-amino group of the amino acids in the presence of side chain functionalities will be the object for discussion in the next article. The study of the nucleophilic specificity of lipase according to the different L- and D-amino acids is in progress in our laboratory and the results will be published elsewhere. Moreover, in the mentioned article, the results with the kinetic experiments of lipase catalyzed reactions of the alpha-amino group protection in the amino acids with previously protected side chain functional groups with hydrophobic and bulky functionalities will be published, allowing to the proposed changing of the enzyme selectivity towards these amino acids[ϖ]. It is worth to be noted, that when the ratio between the acyl and amino components is 1.2 equiv. : 1 equiv., or 1equiv. : 1.2 equiv. (under low electrophilic and low nucleophilic specificity of the lipase, as well as under high electrophilic and high nucleophilic specificity) – the iterative procedure is not possible. When low electrophilic and low nucleophilic specificity of the lipase towards the acyl and amino components is presented, the reaction proceeds very slowly with poor yields, and sometimes is not possible. Under high electrophilic and high nucleophilic specificity of the components, the iterative procedure may be realized employing a molar ratio related to 1 equiv. : 5 equiv., or 5 equiv. : 1 equiv., according to the combinative approach, and followed the procedure, which is selected (solvent media, free or immobilized lipase etc.).

[ϖ] *Full details concerning the synthesis and kinetic experiments of a lipase catalyzed alpha-amino group protection are included in the forthcoming manuscript, which is in preparation [30].*

So et al. [29] noted that **P**orcine **P**ancreatic **L**ipase (PPL) has a good affinity and selectivity towards H-***D***-Ala-NH_2, H-***D***-Ser-OMe, H-***D***-Thr-OMe, H-***D***-His-OMe and H-***β***-Ala-OMe as acyl acceptors [29]. In their studies the noted D-amino acid derivatives were characterized as good nucleophiles. Moreover, the enzyme should have high nucleophilic specificity towards the more hydrophobic L-amino acids (with hydrophobic side chains), whereas the enzyme specificity according to the D-amino acids will be particularly studied. It is worth noting, that **P**orcine **P**ancreatic **L**ipase (PPL) exhibits higher electrophilic specificity towards the bulky and hydrophobic protective groups (of acyl type components), having the more hydrophobic ester group (nucleophuge, leaving group). This determines the observed different ratio in the reactions of protection of the alpha-amino group of L-ornithine with different protective groups, having different alcohol synthons, with the preferences towards more hydrophobic alcohol moieties. Detailing experiments, including kinetic measurements of the lipase catalyzed model reaction of protection of L-ornithine with different activated and unactivated esters of the above mentioned protective groups are in progress and will be published elsewhere (Figure 7).

Moreover, sometimes in the presence of a large excess of the acyl component towards the nucleophile component (5 equiv. : 1 equiv. or higher molar ratio) probably OH- and SH-groups, as well as the other above noted side chain functionalities can be acylated by lipase. This problem may be partially resolved, by the carrying out of the lipase catalyzed reaction of protection at pH<7, as at these conditions the side chain δ-guanidino- (Arg) and δ-amino- (Orn), ε-amino- (Lys), and at pH<6.5 imidazolyl- (His) and indolyl- (Trp) functionalities will be protonated, and only OH- (Ser, Thr, Tyr) and SH- (Cys) groups will be revealed and susceptibilited to the attack by the acyl component, to some extent. Despite this, if the protection reaction is carried out at pH<6, using Ser, Thr, Tyr and Cys, only OH- (Ser, Thr, Tyr) and SH- (Cys) groups will be acylated, but if then pH of the reaction mixture increases, O-N acyl migration may be described, as in these conditions the alpha-amino group will be deprotonated and will participate in intramolecular aminolysis reaction as a stronger nucleophile, and the obtained amides will be more termodinamically stable, than their precursor esters. These model reactions will give us more details about the mechanism of catalysis by the **P**orcine **P**ancreatic **L**ipase (PPL). However, at these conditions, the lipase catalyzed synthesis reaction will be substantially slowered, due to the different pH-optimum of catalysis of the **P**orcine **P**ancreatic **L**ipase (PPL) (pH 8.1-9.3). But this will allows for the synthesis of O-acylated drug precursors (for example some erithromycin antibiotics and other compounds that have many functionalities), which will or won't realize O-N acyl migration by the varying of pH-conditions, depending on the necessity. By this way the synthesis of drugs and prodrugs will be possible, allowing for the originally developed methods for compounds synthesis in the pharmaceutical industry. Everything of this will be the object for the next studies.

Conclusion

The successful efficient and selective chemo-enzymatic amino group protection was carried out at mild conditions, using lipase as a catalyst and allowing to the easy and pure preparation of N-protected amino acids derivatives for conventional peptide synthesis and appropriate various using. During the solubility-controlled enzyme peptide synthesis, the product precipitates from the solution to keep its concentration below the equilibrium. The methodology allows the iteration and efficacy, using the lipase without its regeneration and step isolation from the reaction mixture. The method will be developed for the multi-scale carrying out of the homogeneous and heterogeneous synthesis, applying the combinative procedure for the successful amino group protection, as well as for the creation of amide (peptide) bond, during the process of peptide synthesis.

A combinative approach for effective and selective iterative procedure for chemo-enzymatic alpha-amino group protection in the natural amino acids was successfully applied. To prove the approach utility and the procedure efficacy, an Ornithyl model system was initially represented. As a catalyst, the lipase was employed as an enzyme, in media consisted from different solvents and under the existence of a "nucleophile pool" or an "electrophile pool", depending on the nucleophilic enzyme specificity, and according to the reagents. When dissolved lipase was used, the so called homogeneous synthesis was applied, whereas the heterogeneous synthesis was realized by the using of lipase, which was immobilized on silica beads. The method allows as the multifold enzyme utility, without additionally regeneration, as well as exclusively mild conditions of the carrying out of the reaction (successful reaction procedure), high yields (almost 100%) and purity of the obtained products, and also – high reaction selectivity and specificity. By this way, the routine preparation of N-protected amino acids derivatives is advantaged, which are necessary for the peptide synthesis.

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