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Synthesis of Peptide Aldehydes via Enzymatic Acylation of Amino Aldehyde Derivatives

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Abstract—Two ways for semi-enzymatic preparation of the peptide aldehydes are proposed: (1) enzymatic acylation of amino alcohols with acyl peptide esters and subsequent chemical oxidation of the resulting peptide alcohols with DMSO/acetic anhydride mixture or (2) enzymatic acylation of the preliminarily obtained by a chemical route amino aldehyde semicarbazones. Subtilisin 72, serine proteinase with a broad specificity, distributed over macroporous silica, was used as a catalyst in both cases. Due to the practical absence of water in the reaction mixtures the yields of the products in both enzymatic reactions were nearly quantitative. The second way seems to be more attractive because all chemical stages were carried out with amino acid derivatives, far less valuable compounds than peptide ones. A series of peptide aldehydes of general formula *Z*-Ala-Ala-Xaa-al (where Xaa-al = leucinal, phenylalaninal, alaninal, valinal) was obtained. The inhibition parameters for these compounds, in the hydrolysis reactions of corresponding chromogenic substrates for subtilisin and α-chymotrypsin, were determined.

Introduction

Peptide aldehydes—peptide derivatives terminated with α-amino aldehydes—have been shown to be very strong competitive inhibitors of serine and thiol proteinases. These compounds were characterized as transition state analogues, based on their ability to form extremely stable tetrahedral adduct with the enzyme. The stability of such an adduct has been attributed to the formation of covalent hemiacetal bond between peptide aldehyde and serine or cysteine of the active center.[1–5] Peptide aldehydes are interesting, also, as intermediates in the synthesis of different peptide derivatives such as peptide isosteres, inhibitors for HIV proteinase, ligands for affinity chromatography of proteins etc.[6–9]

Chemical coupling of amino aldehyde derivatives (semicarbazone, acetals etc.) with peptides have been usually applied to prepare peptide aldehydes.[9–11] Occasionally peptide alcohols were oxidized to peptide aldehydes.[12,13] Recently we reported a convenient method for these derivatives preparation which consists in enzymatic acylation of amino alcohols with acylpeptide esters followed by chemical oxidation of the resulted peptide alcohols.[14] Subtilisin 72, a serine proteinase similar to Subtilisin Carlsberg, sorbed on macroporous silica, served as a catalyst for amino alcohols acylation. The yields of the enzymatic reactions were 70–99%, but in the stage of chemical oxidation a significant loss of the products took place. We decided to reverse the sequence of the reactions, so that the amino alcohol which is of less value than a peptide derivative was submitted to oxidation. Subsequent reaction—enzymatic acylation of amino aldehyde derivatives (e.g. of semicarbazone) with acyl peptide esters leads to the peptide aldehydes.

Here we present the peptide aldehyde synthesis following this "improved" scheme in comparison with the one discussed in our previous paper.[14]

Key words: Peptide aldehydes; enzymatic synthesis: peptide inhibitors; serine proteinases.

Abbreviations: Z-, benzyloxycarbonyl-; PHT-, phthalyl-; -ONSu, succinimidooxy-; -Sem, semicarbazone; -pNA, p-nitroanilide; DMSO, dimethylsulfoxide; Ac_2O, acetic anhydride; Py, pyridin; DMF, dimethylformamide; pheol, amino alcohol phenylalaninol; pheal, amino aldehyde phenylalaninal; Tris, Tris(hydroxymethyl)amino-methane. The notation of enzyme binding subsites corresponds to the Schechter and Berger nomenclature. Standard three abbreviations are used for amino acid residues, which belong to L-series.

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Results

Oxidation of amino alcohols

Although the oxidation of alcohols is well known reaction, the oxidation of peptide and amino alcohols needs some comments.

There are many reagents for oxidation of alcohol groups. The problem is to choose the reaction which is free from overoxidation danger, give optically pure product and proceed with a good yield. Among the oxidants which we tried, the most convenient turned out to be the mixture of DMSO and Ac_2O.[15] Using this method some peptide and acyl amino aldehydes were obtained (Table 1).

In the course of the oxidation the aliquots of the reaction mixture were examined by HPLC method. A broad pick of the aldehyde product was observed (Fig. 1). To all appearance such an unusual form of the pick may be explained by reversible hydration of the aldehyde function. Examination of ^{1}HNMR spectra of the products confirmed the presence of the aldehyde group in the peptide derivatives. Optical rotation of Z-Pheal, thus obtained, was fully identical to the literature data, so racemization does not occur during this oxidation.

The mixture of DMSO and Ac_2O gave satisfactory results, with some inconvenience:

1. both DMSO and Ac_2O should be freshly distilled;
2. the yield of the product does not exceed 70%. Moreover acylation of the alcohol group with the excess of Ac_2O could decrease the yield
3. the reaction mixture should be freeze-dried to remove the excess of DMSO which is the oxidant and the solvent simultaneously.

A number of reactions in which different derivatives of CrO_3 are used as the oxidant were described.[16–19] Among which the complex CrO_3 *Py seems to be the most attractive one. The non-polar solvents, like CH_2Cl_2 or $CHCl_3$, are usually applied. We found the optimal conditions for oxidation of Z-Leuol and Z-Valol with complex $CrO_3\times$Py in the presence of Ac_2O (Table 2).

Contrary to *N*-acylated amino alcohols, peptide alcohols are practically insoluble in CH_2Cl_2 and $CHCl_3$. In the polar solvents e.g. DMF, DMSO the result of the oxidation was still unsatisfactory. Thus the complex $CrO_3\times$Py is the most convenient oxidant for *N*-acylated amino alcohols, whenever the DMSO–Ac_2O mixture seems to be the more effective one for peptide alcohols. Note that the aldehyde product must be purified in both cases.

It is known that acyl amino aldehyde is the rather unstable compound which easily suffers racemization. The racemization is especially intensive when acyl amino or peptide aldehydes are purified on silica, whereas aldehyde derivatives, e.g. semicarbazone, are stereo stable.[17,20,21] Therefore, it would be reasonable to convert the peptide aldehydes after oxidation in corresponding semicarbazones before their purification. After removing the Z-protecting group semicarbazones of amino aldehydes, were used as nucleophiles in enzymatic acylation.

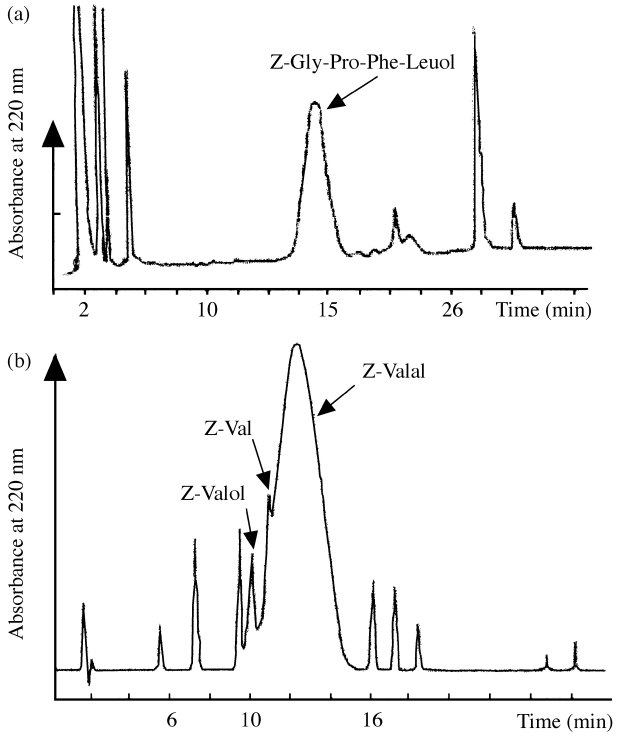


Figure 1. Chromatograms of the reaction mixtures. (a) Oxidation of Z-Gly-Pro-Phe-Leuol with DMSO-Ac_2O mixture. Condition applied: Z-Gly-Pro-Phe-Leuol 18.7 μmol, DMSO 330 μl, Ac_2O 35 μl, 24 h, 16°C. (b) Oxidation of Z-Valol with CrO_3 *Py complex. For details see Experimental.

Table 1. Oxidation of amino and peptide alcohols (RCH_2OH) to the corresponding aldehydes with the mixture of DMSO and Ac_2O

Starting alcohol	Reagents molar ratio RCH_2OH: Ac_2O	Time (H)	Temperature (C°)	Yield assessed HPLC (%)
Z-Pheol	1:10	3	37	81
CHO-Pheol	1:10	2	37	49
PHT-Pheol	1:10	20	37	51
Z-Ala-Ala-Pheol	1:20	2.5	37	67
Z-Ala-Ala-Valol	1:10	5	16	62
Z-Ala-Ala-Leu-Pheol	1:20	24	16	45
Z-Gly-Pro-Phe-Leuol	1:20	24	16	62

Table 2. Oxidation of *N*-acylated amino alcohols (RCH_2OH) to the corresponding amino aldehydes with the complex $CrO_3\times$Py and Ac_2O at 16°C

Starting amino alcohol	Reagents molar ratio RCH_2OH:$CrO_3\times$ Py:Ac_2O	Time (min)	Yield assessed HPLC (%)
Z-Leuol	1:1:4	18	81
Z-Valol	1:1:3	20	88

Enzymatic reactions

Since subtilisin 72 possesses a rather broad specificity, its S_1'-subsite is capable of binding different amino components, e.g. amino alcohols. On the other hand β-amino alcohol has no carbonyl group, therefore, its interaction with the enzyme S_1'-subsite is handicapped. To compensate for this shortcoming, the 10-fold excess of the amino component needed to ensure a good yield of the peptide alcohol. Although enzymatic acylation of amino alcohols has been described earler,[14] some details of this reaction should be discussed briefly.

When amino alcohol is acylated with acyl tripeptide esters (e.g. Z-Gly-Pro-Phe-OCH_3 or Z-Ala-Ala-Leu-OCH_3), the latter become exhausted rather early in the course of the reaction (Fig. 2). Although the 5% DMF in acetonitrile was used as a solvent and no water was introduced in it, the hydrolysis of the starting peptide esters occurs, which led to the formation of Z-Gly-Pro-Phe-COOH or Z-Ala-Ala-Leu-COOH. Perhaps this is to be attributed to the water traces present in the solvent and/or bound by this hydrophilic silica support. The peptide, thus formed, is still capable to act as an acylating agent under equilibrium conditions. This was demonstrated by the direct experiment (Fig. 3). Thus, the yield of the tripeptidyl amino alcohol is the result of the following reactions:

$$\text{Z-Ala-Ala-Leu-OCH}_3 + \text{H-Pheol} \rightarrow \text{Z-Ala-Ala-Leu-Pheol} + \text{CH}_3\text{OH} \quad (1)$$

$$\text{Z-Ala-Ala-Leu-COOH} + \text{H-Pheol} \rightarrow \text{Z-Ala-Ala-Leu-Pheol} + \text{H}_2\text{O} \quad (2)$$

The amino alcohol acylation with N-protected dipeptide esters proceeds in a similar way (Fig. 4), but in this case the reaction

$$\text{Z-Ala-Ala-COOH} + \text{H-Pheol} \rightarrow \text{Z-Ala-Ala-Pheol} + \text{H}_2\text{O} \quad (3)$$

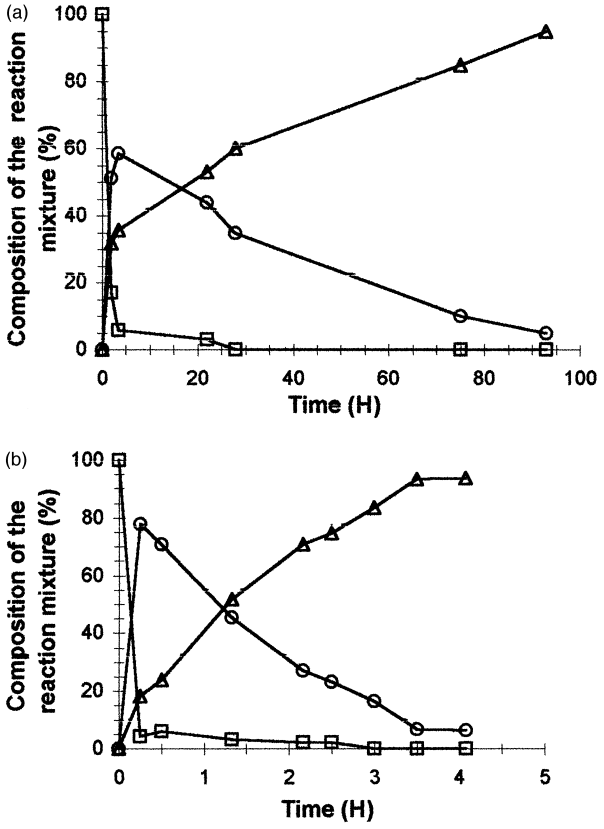


Figure 2. Time course of the reactions observed during tripeptide alcohol synthesis catalyzed by sorbed subtilisin (Acyl tripeptide esters were used as the acylating agents). Conditions applied: DMF-5%, CH_3CN-95%, (v/v), 16°C. The total volume of the reaction mixture was 600 μL, subtilisin 72–0.23 mM (a) $[\text{Z-Gly-Pro-Phe-OCH}_3]_o$ = 16.7 mM, $[\text{Leuol}]_o$ = 166.7 mM; (□) Z-Gly-Pro-Phe-OCH_3, (○) - Z-Gly-Pro-Phe-COOH; (△) - Z-Gly-Pro-Phe-Leuol. (b) $[\text{Z-Ala-Ala-Phe-OCH}_3]_o$ = 16.7 mM, $[\text{Pheol}]_o$ = 166.7 mM; (□) - Z-Ala-Ala-Leu-OCH_3, (○) - Z-Ala-Ala-Leu-COOH, (△) - Z-Ala-Ala-Leu-Pheol.

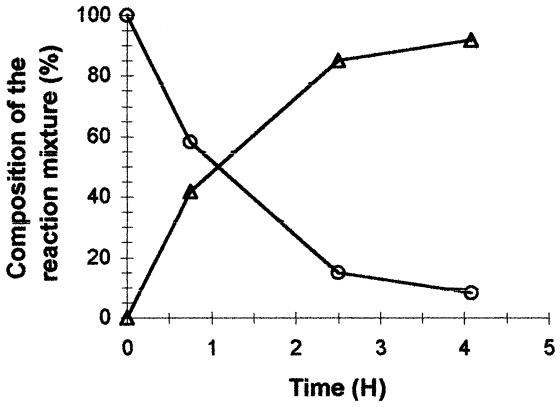


Figure 3. Time course of the reactions observed during peptide synthesis catalyzed by sorbed subtilisin (Z-Ala-Ala-Leu-COOH was used as the acylating agent). Conditions applied: DMF-5%, CH_3CN-95%, (v/v), 20°C. The total volume of the reaction mixture was 600 μL. $[\text{Z-Ala-Ala-Leu-COOH}]_o$ = 16.7 mM, $[\text{Pheol}]_o$ = 166.7 mM, subtilisin 72–0.23 mM. (○) - Z-Ala-Ala-Leu-COOH, (△) - Z-Ala-Ala-Leu-Pheol.

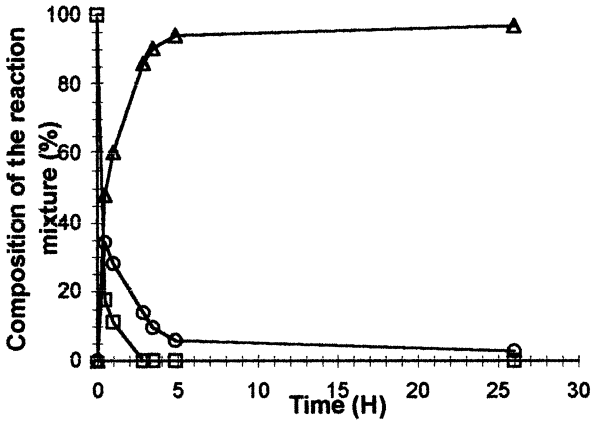
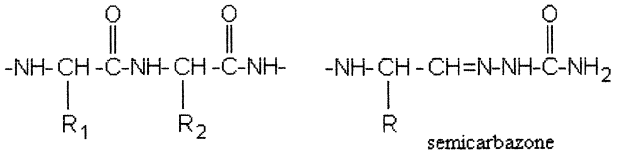


Figure 4. Time course of the reactions observed during dipeptide alcohol synthesis catalyzed by sorbed subtilisin (Acyl dipeptide methyl ester was used as the carboxyl component). Conditions applied: DMF-5%, CH_3CN-95%, (v/v), 20°C. The total volume of the reaction mixture was 600 μL. $[\text{Z-Ala-Ala-OCH}_3]_o$ = 16.7 mM, $[\text{Pheol}]_o$ = 166.7 mM, subtilisin 72–0.46 mM. (□) - Z-Ala-Ala-OCH_3, (○) - Z-Ala-Ala-OH, (△) - Z-Ala-Ala-Pheol.

is not significant. (Fig. 5). Probably this is because the N-protected dipeptide with a free COOH-group satisfies the specificity requirements of subtilisin, an enzyme that binds, preferably, acylated tri- and longer peptides, only marginally. Therefore, the direct acylation of the amino alcohol with dipeptide (reaction 3) plays only a limited role under these circumstances.

Enzymatic acylation of amino aldehyde semicarbazones was performed analogously, but there are some differences in the course of the reactions.

The structure of amino aldehyde semicarbazone resembles one of azapeptide:



It seems, that due to the presence of C=O and NH-groups, semicarbazone become rather easily bound by the S'_1 -subsite of subtilisin which assists the enzymatic acylation. There is no need for an excess of the amino component and the yields of the reactions in most cases are practically quantitative (Table 3).

Table 3. Enzymatic acylation of amino aldehyde semicarbazones (NH_2B) with acyl peptide esters ($RCOOCH_3$) catalyzed by subtilisin 72

Reagents		Reagents molar ratio $RCOOCH_3$:NH_2B	Time (H)	Yield assessed HPLC (%)
Carboxyl components	Amino components			
Z-Ala-Ala-OCH_3	Pheal-Sem	2:1	22	91
Z-Ala-Ala-OCH_3	Alaal-Sem	2:1	19	84
Z-Ala-Ala-OCH_3	Leual-Sem	2:1	8	80
Z-Ala-Ala-OCH_3	Valal-Sem	2:1	5	92
Z-Ala-Ala-Leu-OCH_3	Pheal-Sem	1:2	26	99

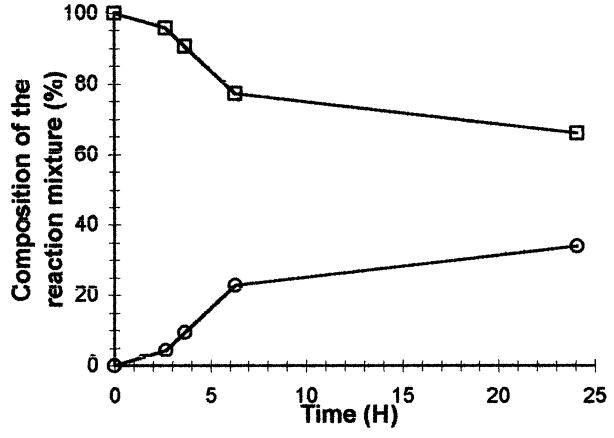


Figure 5. Time course of the reactions observed during dipeptide alcohol synthesis catalyzed by sorbed subtilisin (Z-Ala-Ala-COOH was used as the carboxyl component). Conditions applied: DMF-5%, CH_3CN-95%, (v/v), 20°C. The total volume of the reaction mixture was 600 μL. [Z-Ala-Ala-COOH]$_o$ = 16.7 mM, [Pheol]$_o$ = 166.7 mM, subtilisin 72–0.46 mM. (○) - Z-Ala-Ala-COOH, (△) - Z-Ala-Ala-Pheol.

On Fig. 6 the HPLC typical chromatogram of the reaction mixture (Z-Ala-Ala-Leual-Sem synthesis) is presented. The amino component—leucinal semicacarbazone (Leual-Sem)—being very hydrophilic, was eluted first and was masked by the broad peak of DMSO, so the yield of the peptide product could be evaluated through the conversion of the carboxyl component, taken in a double excess. Therefore the yield of the peptide assessed by the HPLC method could not exceed 50% (Fig. 7). The twofold excess of the carboxyl component leads to the accumulation of Z-Ala-Ala-COOH in the course of the reaction, as the result of enzymatic hydrolysis of the starting ester. It seems that dipeptide Z-Ala-Ala-COOH does not take part in the enzymatic acylation of the semicarbazone.

In the Z-Ala-Ala-Leu-Pheal-Sem synthesis, the double excess of the amino component was used. The enzy-

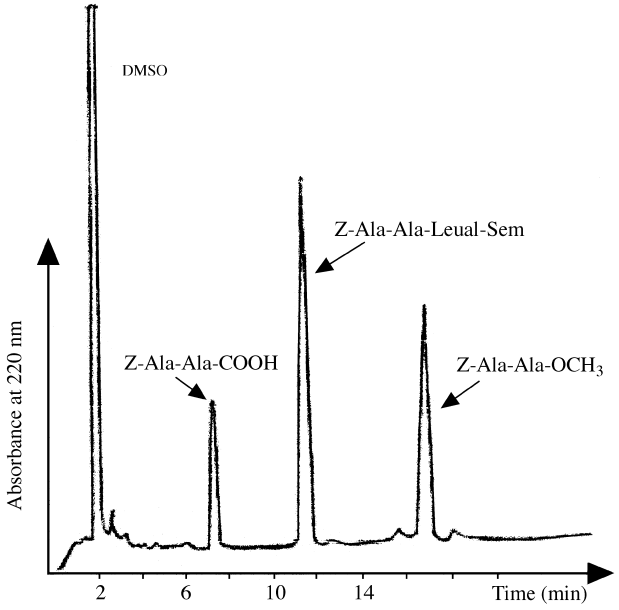


Figure 6. Chromatogram of the reaction mixture during Z-Ala-Ala-Leual-Sem synthesis. HPLC in System B. Condition applied see Figure 7.

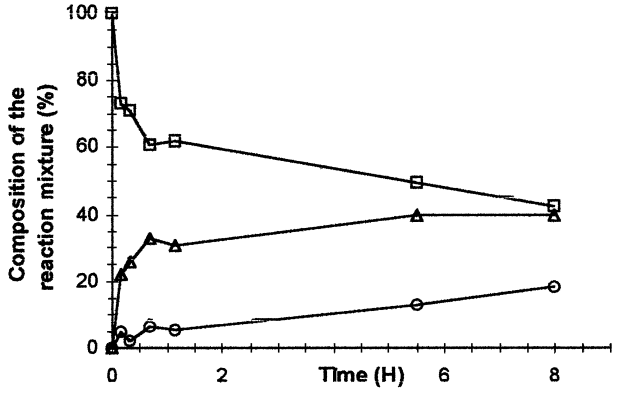


Figure 7. Time course of the reactions observed during enzymatic acylation of amino aldehyde semicarbazone with acyl dipeptide ester. Conditions applied: DMSO-8%, CH_3CN-92%, (v/v), 20°C. The total volume of the reaction mixture was 600 μL. [Z-Ala-Ala-OCH_3]$_o$ = 50 mM, [Leual-Sem]$_o$ = 26 mM, subtilisin 72–0.46 mM. (□) - Z-Ala-Ala-OCH_3, (○) - Z-Ala-Ala-COOH, (△) - Z-Ala-Ala-Leual-Sem.

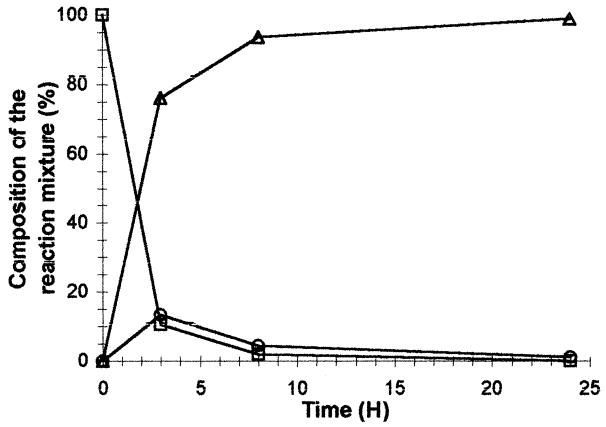


Figure 8. Time course of the reactions observed during enzymatic acylation of amino aldehyde semicarbazone with acyl tripeptide ester. Conditions applied: DMF-5%, CH_3CN-95%, (v/v), 20°C. The total volume of the reaction mixture was 600 μL. $[Z\text{-}Ala\text{-}Ala\text{-}Leu\text{-}OCH3]_o = 16.7$ mM, $[Pheal\text{-}Sem]_o = 33.4$ mM, subtilisin 72–0.23 mM. (□) - Z-Ala-Ala-Leu-OCH_3, (○) - Z-Ala-Ala-Leu-COOH, (△) - Z-Ala-Ala-Leu-Pheal-Sem.

Table 4. Inhibition of subtilisin and α-chymotrypsin with peptide aldehydes

Enzyme (substrate)	K_i(M)		
	Z-Ala-Ala-Leual	Z-Ala-Ala-Pheal	Z-Ala-Ala-Valal
Subtilisin 72 (Z-Ala-Ala-Leu-pNA)	2.23×10^{-7}	4.0×10^{-7}	2.9×10^{-6}
α-Chymotrypsin (Pyr-Phe-pNA)	9.05×10^{-5}	2.2×10^{-6}	4.9×10^{-4}

matic hydrolysis of starting tripeptide ester was negligible and conversion of the reaction to the equilibrium synthesis in this case was not observed (Fig. 8).

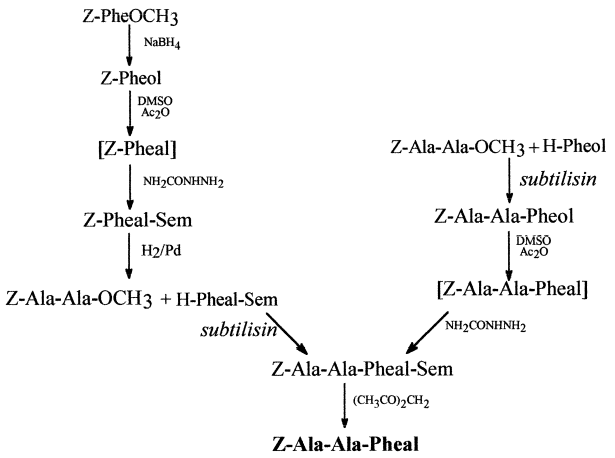
After removal of the protecting group with acetylacetone,[22] the peptides with a free aldehyde group were tested as proteinase inhibitors.

Inhibitory tests

Peptide aldehydes obtained in a such way was shown to be potent inhibitors of subtilisin and α-chymotrypsin. Kinetic parameters of inhibition were determined in the hydrolysis of the corresponding chromogenic substrates for every enzyme (Table 4)

Discussion

There are two ways of semi-enzymatic synthesis of the peptide aldehydes following our scheme Scheme 1. The first one—the enzymatic acylation of amino alcohols and subsequent oxidation of the resulted peptide alcohols. Synthesis of peptide aldehydes via peptide alcohols oxidation have some inconveniences, which are mentioned above (large excess of amino alcohol, freshly distilled reagents for oxidation, loss of the peptide product, possibility of the racemization during purification of the peptide aldehyde after oxidation). However, it is



Scheme 1. General scheme of semi-enzymatic peptide aldehyde synthesis.

rather fast and could be useful for preliminary investigation of inhibition of the enzymes.

The second way—enzymatic acylation of amino aldehyde semicarbazones which were preliminarily obtained by the chemical route. The latter way is longer than the first one, but all chemical stages were carried out with amino acid derivatives which are more simple and less valuable than peptide ones. If the reaction is scaled-up, the loss of the product could be diminished. Amino aldehyde semicarbazones are very stable and could be stored for a long time without any loss of their optical purity. Moreover, this way allows us to obtain a family of peptide derivatives containing the same amino aldehyde residue but with different peptide moieties which would be useful when specificity requirements of the enzymes must be examined.

Experimental

General

Amino acid and peptide esters have been prepared by conventional routes. Phenylalaninol was obtained as described.[23] L-Leuol and α-chymotrypsin were purchased from Sigma.

Subtilisin 72, purified in this laboratory has been distributed over the surface of the silochrome as describe earlier.[24]

Reverse-phase HPLC was performed on a Gilson 704 chromatograph using Becman Ultrasphere ODS column (4.6×250 mm). Solutions A and B contained 0.05% CF_3COOH and 0.05% $(C_2H_5)_3N$ respectively. Solutions A—5% CH_3CN, solution B—90% CH_3CN. A linear gradient of B from 30 to 78% in 40 min (System A) and linear gradient of B from 25 to 40% in 25 min (System B) with 1.5 mL min^{-1} was used. Detection was performed at 220 nm.

Optical rotation was measured with Jasco DIP-360 polarimeter. ^1H NMR Spectra were recorded on Bruker AC-200 spectrometer.

Reduction of the amino acid esters

Z-Leuol. To the solution of Z-Leu-ONSu (60 mg; 160 μmol) in 700 μl of dioxane sodium borohydride (50 mg; 1.4 mmol) and methyl alcohol (110 μl; 2.75 mmol) subsequently were added, then the solution of acetic acid (80 μl) in dioxane (500 μl) was added dropwise at 12°C. The mixture was stirred at 75°C for 1.5 h. The solvent was evaporated and the residue was distributed between water (10 mL) and chloroform (20 mL). The water layer was washed with chloroform (3×5 mL). The combined chloroform fractions were washed with water (1×5 mL), and with saturated aqueous NaCl. Then the chloroform solution was dried over Na_2SO_4 and was evaporated. Flash chromatography on silica gel using chloroform:ethyl acetate (6:1) v/v gave 350 mg (88% yield) of Z-Leuol. $[\alpha]_D^{20} = -28$ (c = 1, ethanol) HPLC retention time of Z-Leuol was 13.0 min (System A); conversion of Z-Leu-ONSu to Z-Leuol was 96%.

^{1}H NMR ($CDCl_3$, 200 MHz, δ ppm): 0.88(d, 6H, -CH($C\underline{H}_3$)$_2$); 1.28(m, 2H, -$C\underline{H}_2$CH(CH_3)$_2$); 1.6(m, 1H, -CH_2 $C\underline{H}$(CH_3)$_2$; 3.44(m, 2H, -$C\underline{H}_2$OH); 3.6(q, 1H, CH_2 O$\underline{H}$); 3.74(m, 1H, > $C\underline{H}CH_2CH(CH_3)_2$; 5.04(q, 2H, C_6 $H_5C\underline{H}_2$O-); 5.19(t, 1H, -CON$\underline{H}$-); 7.29(s, 5H, $C_6\underline{H}_5$-). Z-Pheol and Z-Valol were obtained analogously.

Z-Valol. ($CDCl_3$, 200 MHz, δ ppm): 0.91(t, 6H, -CH ($C\underline{H}_3$)$_2$); 1.82(m, 1H, -$C\underline{H}$(CH_3)$_2$); 2.55(s, 1H, -CH_2 O$\underline{H}$); 3.44(m, 1H, > $C\underline{H}C\underline{H}$($CH_3$)$_2$); 3.62(m, 2H, -$C\underline{H}_2$ OH); 5.0(s, 1H, -CON$\underline{H}$-); 5.04(q, 2H, $C_6H_5C\underline{H}_2$-); 7.32 (m, 5H, $C_6\underline{H}_5$-)

Z-Pheol. ($CDCl_3$, 200 MHz, δ ppm): 2.9(d, 2H, $J = 7$ Hz, $C_6H_5C\underline{H}_2$CH-); 3.5–3.8(m, 3H, $C_6H_5CH_2C\underline{H}$- and -$C\underline{H}_2$ OH); 3.95(1H, broad, -O$\underline{H}$); 5.0(1H, broad, -N$\underline{H}$); 5.2(s, 2H, $C_6H_5C\underline{H}_2$O-); 7.12–7.45(m, 10H, 2$C_6\underline{H}_5$-)

Oxidation of amino alcohols with CrO_3 *Py complex in the presence of Ac_2O

Z-Valal. Py×CrO_3 (805 mg; 4.5 mmol) was suspended in dichloromethane (6.5 ml) and then Z-Valol (483 mg; 2 mmol) and acetic anhydride (1.63 ml; 17.4 mmol) were added. The mixture was stirred for a period of 18 min at 16°C. Conversion of Z-Valol to Z-Valal according to the HPLC data was 80%, retention time of Z-Valal was 12.9 min (System A). The reaction mixture was supplied on silica column (3×3.5sm) and eluted with ethyl acetate (20 ml). The solvent was evaporated. The resulted aldehyde was converted into its semicarbazone without purification. (See below). The yield of Z-Valal-Sem after purification by flash-chromatography in chloroform: ethyl acetate (7:3) was 375 mg (63%)

Preparation of semicarbazones

Z-Pheal-Sem. Z-Pheol (855 mg, 3 mmol) was dissolved in DMSO (25 mL), then freshly distilled $(CH_3CO)_2O$ (2.8 mL, 30 mmol) was added. The reaction mixture was shaken for 3.5 h at 37°C, then diluted with 50 ml of water and freeze-dried. The resulting acyl amino aldehyde was converted into its semicarbazone without purification. Yellow oil of Z-Pheal was dissolved in C_2H_5OH (6.3 mL) and H_2O (2.7 mL), then semicarbazide hydrochloride (330 mg) and CH_3COONa (270 mg) was added. The reaction mixture was stirred for 5 min at 80°C, then the solvent was evaporated. Z-Pheal-Sem was purified by flash-chromatography in chloroform: ethyl acetate (7:3) and in ethyl acetate on silica. The yield of the product—670 mg (66%), HPLC retention time—11.7 min (System A). Semicarbazones of other amino and peptide aldehydes were prepared analogously.

Pheal-Sem. Z-Pheal-Sem (210 mg, 0.618 mmol) was dissolved in CH_3OH (20.8 mL) and H_2O (5.2 mL), then 0.1 M $HCOONH_4$ (6.5 mL) and Pd-black was added, pH of reaction mixture was be 4. After stirring for 2 h at 20°C the catalyst was filtered off, the filtrate evaporated in vacuo. The yield of Pheal-Sem was 150 mg (93%). $[\alpha]_D^{20} = -51$ (c = 1, MeOH), HPLC retention time - 2.79 min (System A)

^{1}H NMR ($CDCl_3$, 200 MHz, δ ppm): 2.85(d, 2H, $J = 7$ Hz, -$C\underline{H}_2C_6H_5$); 4.08–4.13(m, 1H, ($C\underline{H}CH_2$-); 6.15 (2H, broad, -NHCON$\underline{H}_2$); 7.1–7.38(m, 6H, -$C_6\underline{H}_5$ and -C$\underline{H}$ =); 8.3(d, 2H, $J = 7$ Hz, N$\underline{H}_2$CH <); 9.85(1H, broad, -N$\underline{H}$CO-)

Valal-Sem. This was obtained analogously. The yield was 198 mg (98%). $[\alpha]_D^{20} = 20$ (c = 1, MeOH), HPLC retention time — 2.01 min (System A).

^{1}H NMR ($CDCl_3$, 200 MHz, δ ppm): 0.9(t, 6H, -CH ($C\underline{H}_3$)$_2$); 1.85(m, 1H, -$C\underline{H}$(CH_3)$_2$; 3.43(m, 1H, ($C\underline{H}$CH (CH_3)$_2$; 6.3(s, 2H, -CON$\underline{H}_2$); 7.15(d, 1H, -C$\underline{H}$ = N-); 8.35(s, 2H, N$\underline{H}_2$CH <); 9.9(s, 1H, = N-N$\underline{H}$CO-).

Oxidation of peptidyl amino alcohols with DMSO:Ac_2O mixture

Z-Ala-Ala-Pheal. Peptide alcohol Z-Ala-Ala-Pheol (114.5 mg, 269 μmol) was dissolved in DMSO (4.5 mL), then freshly distilled $(CH_3CO)_2O$ (508 μL 5.38 mmol) was added. The reaction mixture was shaken for 3 h at 37 °C, then diluted with 8 ml of water and freeze-dried. The total yield of Z-Ala-Ala-Pheal according to the HPLC data was equal to 83%. HPLC retention time 9.82 min (SystemA). The resulted peptide aldehyde was converted into its semicarbazone without purification. The yield of Z-Ala-Ala-Pheal-Sem after purification by flash-chromatography in chloroform:ethyl acetate (7:3) was 62 mg (48%).

Enzymatic synthesis

Z-Ala-Ala-Pheal-Sem. Z-Ala-Ala-OCH_3 (154 mg, 0.5 mmol) and Pheal-Sem (51.5 mg, 0.25 mmol) were dissolved in DMF (0.75 mL) and CH_3CN (14.25 mL), then silochrom (2.5 g) containing subtilisin 72 (200 mg) was added. After shaking for 20 h at 20°C the catalyst was filtered off, rinsed with a mixture of DMF and CH_3CN, the filtrates combined and evaporated in vacuo. The yield of the product according to the HPLC data was 91% (System B, retention time—16.8 min). The resulted peptidyl amino aldehyde semicarbazone

was purified by flash-chromatography in chloroform: ethyl acetate (7:3), then eluted with C_2H_5OH. The yield of Z-Ala-Ala-Pheal-Sem was 90 mg (75%), $[\alpha]_D^{20} = -40$ (c = 1, methanol).

^{1}H NMR ($CDCl_3$, 200 MHz, δ ppm): 1.25(d, 9H, $J = 7$ Hz, >CHC$\underline{H}_3$); 4.02–4.12(m, 1H, >C$\underline{H}$CH=); 4.18–4.32(m, 2H, >C$\underline{H}$CO-); 5.05(s, 2H, -OC$\underline{H}_2C_6H_5$); 6.2(2H, broad, -CON$\underline{H}_2$); 7.12(s, 1H, -C$\underline{H}$=); 7.32(s, 5H, -$C_6\underline{H}_5$); 7.75–8.0(m, 3H, -CON$\underline{H}$CH>); 9.9(1H, broad, -N$\underline{H}$CO-)

Removal of protecting group

Z-Ala-Ala-Pheal-Sem (12 mg, 25 μmol) was dissolved in CH_3OH (1.6 mL) and) 0.5 N HCl (850 μL), then $(CH_3CO)CH_2$ (51 μL, 0.5 mmol) was added. The reaction mixture was shaken for 4.5 h at 20°C. The solvent was evaporated and the resulted peptide aldehyde was purified by flash-chromatography in chloroform:ethyl acetate (7:3) on silica. The yield of Z-Ala-Ala-Pheal was 8 mg (76%). HPLC retention time 10.43 min (System A). $[\alpha]_D^{20} = -63$ (c = 1, methanol).

^{1}H NMR ($CDCl_3$, 200 MHz, δ ppm): 1,98 (d, 6H, $J = 7$ Hz, (CHC$\underline{H}_3$); 3,42 (d, 2H, $J = 6$ Hz, $C_6H_5C\underline{H}_2CH$>); 4.08–4.25 (m, 2H, >C$\underline{H}CH_3$); 4.32–4.62 (m, 1H, $C_6H_5CH_2$ C$\underline{H}$<); 5.12 (s, 2H, $C_6H_5C\underline{H}_2$CO-); 6.45 (2H, broad, N$\underline{H}$); 7.08–7.5(m, 11H, 2$C_6\underline{H}_5$- and N$\underline{H}$); 9.6(s 1H, -C$\underline{H}$O).

Inhibitory tests

The kinetic parameters for subtilisin-or α-chymotrypsin-catalyzed hydrolysis of the corresponding chromogenic substrates were determined under the condition of initial rate measurements from double reciprocal plots of v^{-1} versus $[S]^{-1}$ both in the presence and in the absence of peptide aldehydes. Conversion of the substrates did not exceed 10% for 10–15 min. Each experimental set was performed in triplicate.

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