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ENZYMATIC SYNTHESIS OF PEPTIDYL AMINO ALCOHOLS AND PEPTIDYL AMINO ALDEHYDES - SERINE PROTEINASE INHIBITORS

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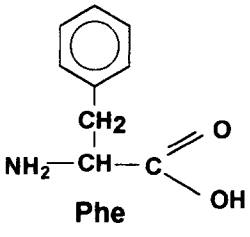
Abstract: An enzymatic synthesis of peptidyl amino alcohols is described. The reactions were performed in organic solvents using as a catalyst subtilisin distributed on the surface of macroporous silica. Subsequent mild oxidation of peptidyl amino alcohols results in peptide aldehydes - the potent specific inhibitors of serine proteinases.

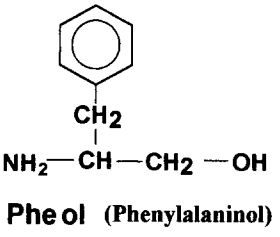
Currently, there is much interest in the peptidyl amino alcohols. These compounds are involved in the biochemical processes of a cell (e.g. ethanolamine is a structural unit of gramicidin A and of phosphatidylinositol-anchored proteins). The chemical synthesis of peptidyl amino alcohols is a rather complex process, which includes many tedious stages.[1,2]

We propose a convenient method for the preparation of peptidyl amino alcohols by an enzymatically catalyzed acylation of amino alcohols with N-protected peptide esters in a mixture of organic solvents with low water content. Subtilisin 72 (serine proteinase from *Bacillus subtilis*, st. 72) distributed over the surface of macroporous silica (silochrom) is used as the catalyst.

This enzyme, which is closely related to the well characterized subtilisin Carlsberg, has a rather broad specificity. A number of reactions of peptide bond synthesis catalyzed by subtilisin 72 were described.[3,4] Moreover, its S_1'-subsite has been shown by us to be capable of accepting different amino components, not only amino acids and peptides (data not shown). For example, amino alcohols (ethanolamine or phenylalaninol structural analog of Phe) [5]



Phe

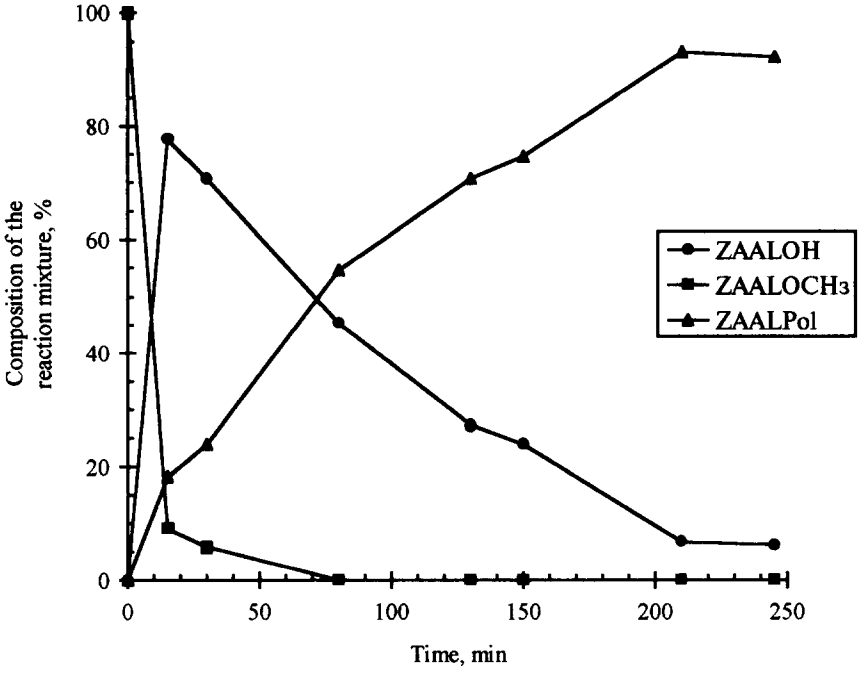


Phe ol (Phenylalaninol)

can occupy the S_1'-subsite of subtilisin and therefore may serve as a nucleophile in peptide bond formation.

The N-protected peptide Z-Ala-Ala-Leu-OCH_3 served as the carboxyl component in the enzymatic acylation of phenylalaninol.

Since β-amino alcohol has no carbonyl group, its interaction with the enzyme S_1'-subsite is less efficient than that of corresponding amino acid residue. To compensate this shortcoming the 10-fold excess of the amino component was used to ensure a good yield of the reaction product. To prepare the catalyst, subtilisin was sorbed on the support in an amount corresponding to the maximal value according to its adsorbtion isoterm on this silica derivative.[6] The time course of the reaction (data obtained by the HPLC method) is plotted below.



It should be noted that in the course of the reaction the tripeptide ester, Z-Ala-Ala-Leu-OCH_3, becomes exhausted rather early. Although the 5% DMF in acetonitrile was used as a solvent and no water was introduced in it, the hydrolysis of the starting tripeptide ester occurs, which led to the formation of Z-Ala-Ala-Leu-OH. Perhaps this is to be attributed to the water traces present in the solvent or bound by this hydrophilic silica support. The peptide, thus formed, is still capable to act as an acylating agent. Thus, the yield of the tripeptidyl-β-amino alcohol is the result of the following reactions:

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

$$\text{Z-Ala-Ala-Leu-OH} + \text{H-Pheol} \xrightarrow{\text{subtilisin}} \text{Z-Ala-Ala-Leu-Pheol} + H_2O \quad (2)$$

It follows that acylpeptides with free COOH-group can be used as carboxyl components in subtilisin-catalyzed peptide synthesis. Similarly proceeds amino alcohol acylation with N-protected dipeptide esters, but in this case the reaction

$$\text{Z-Ala-Ala-OH} + \text{H-Pheol} \xrightarrow{\text{subtilisin}} \text{Z-Ala-Ala-Pheol} + H_2O \quad (3)$$

proceeds very slowly. This is probably because the N-protected dipeptide with a free COOH- group only marginally corresponds to the specificity requirements of subtilisin, an enzyme that binds preferably tri- and longer peptides. The application of the enzyme distributed over the solid-phase support and the high yield of the product simplified its isolation. After the catalyst was filtered off, the excess of Pheol was washed off with 5% citric acid from the mixture taken in ethyl acetate. The structure of peptidyl alcohol was confirmed by ^1H NMR spectra and by the investigation of hydrochloric acid hydrolyzate by means of TLC as well as amino acid analysis.[7]

Analysis of the enzymatic synthesis kinetic curves allowed us to select the optimal conditions that were successfully applied for the synthesis of some peptidyl-β-amino alcohols (Table 1).

Table 1. Synthesis of peptidyl-β-amino alcohols catalyzed by subtilisin 72.

(The derivatives given in italic were oxidized to respective aldehydes).

peptidyl-β-amino alcohols	time (h) of the reaction	YIELD (%)	
		assessed HPLC	preparative
Z-Ala-Ala-Leu-Pheol	4	94	77
Z-Ala-Ala-Leu-Leuol	3	65	-
Z-Ala-Ala-Leu-Trpol	24	99	-
Z-Ala-Ala-Pheol	4	83	68
Z-Ala-Ala-Leuol	24	94	37
Z-Gly-Pro-Phe-Leuol	53	95	70

Subsequent oxidation of peptidyl amino alcohols results in the peptide aldehydes, which are the potent specific inhibitors of serine proteinases. For example, aldehyde derivative Z-Gly-Pro-Phe-Leual was proposed by Orlowsky *et al.* as a very potent inhibitor for multicatalytic proteinases - proteasomes.[8]

We accomplished mild oxidation of three peptidyl amino alcohols and Z-Pheol by anhydrous DMSO and 20-fold excess of acetic anhydride that gave satisfactory yields. The corresponding aldehyde derivatives were purified by flash-chromatography in the mixture chloroform : ethyl acetate over silica. The structures of the resulted peptide aldehydes were confirmed by ^{1}H NMR spectra.

One of the peptide aldehydes thus obtained, Z-Ala-Ala-Pheal, was shown to be a potent inhibitor of subtilisin and α-chymotrypsin (Table 2); whereas, the corresponding peptidyl amino alcohol, Z-Ala-Ala-Pheol, was only a weak inhibitor for subtilisin with K_i about $1*10^{-3}$ M.

Table 2. Inhibition of hydrolysis of chromogenic substrates with Z-Ala-Ala-Pheal

ENZYME (SUBSTRATE)	IC_{50}(M)	K_i(M)
Subtilisin 72 (Z-Ala-Ala-Leu-*p*NA)	$9.0*10^{-7}$	$1.8*10^{-7}$
α-Chymotrypsin (Pyr-Phe-*p*NA)	$9.5*10^{-7}$	$2.2*10^{-6}$

EXPERIMENTAL

Starting peptides and derivatives have been prepared by conventional routes. The amino alcohols L-Pheol and L-Trpol were obtained as described in [9], L-Leuol was purchased from Sigma.

α-Chymotrypsin (Sigma) and subtilisin 72, purified in this laboratory, have been distributed over the surface of the silochrome as described earlier.[4]

Reverse-phase HPLC was performed on Gilson 704 Chromatograph using Beckman Ultrasphere ODS column (4.6x250 mm). Solution A and B contained 0.05% CF_3COOH and 0.05% $(C_2H_5)_3N$. Solution A - 5% CH_3CN, solution B - 90% CH_3CN. Detection was performed at 220 nm.

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

Z-L-Pheol. To 1.6 g of $NaHCO_3$ suspended in 9 ml of water and 11 ml of ethyl acetate, Pheol (0.75 g, 5 mmol) was added followed by 2 ml of benzyl chloroformate. The reaction mixture was

vigorously stirred over night at 20° C. The organic layer was separated, washed subsequently with 5% citric acid and water, then ethylacetate was evaporated giving 1.2 g of crude Z-Pheol (84%). Optical rotation after recrystallization of the amino alcohol from ethylacetate-hexane ($[\alpha]_D^{20}$ -41.5° (c = 1, MeOH) was identical to the literature data.[10]

Z-Gly-Pro-Phe-Leuol (*analytical experiment*). Z-Gly-Pro-Phe-OCH_3 (5 mg, 0.01 mmol) and H-Leuol (6.4 μl, 0.05 mmol) were dissolved in DMF (30 μl), then CH_3CN (370 μl) and silochrom (50 mg) containing 4 mg of subtilisin 72 were added. The mixture was gently shaken at 20° C. Periodically 5 μl aliquots of the reaction mixture were taken, diluted with 150 μl of CH_3CN and applied onto the HPLC column.

Z-Ala-Ala-Leu-Pheol (*preparative experiment*). Z-Ala-Ala-Leu-OCH_3 (254 mg, 0.6 mmol) and H-Pheol (906 mg, 6 mmol) were dissolved in DMF (1.8 ml) and CH_3CN (34.2 ml) , then silochrom (3 g) containing subtilisin 72 (240 mg) was added. After shaking for 4 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 residue was washed with 5% citric acid and water, then dried *in vacuo* over P_2O_5. The yield of the product - 254 mg (77%) , $[\alpha]_D^{20}$ -47.2° (c = 1, MeOH).

Oxidation of amino alcohols. Z-Pheol (99 mg, 0.35 mmol) was dissolved in DMSO (3.1 ml), then freshly distilled $(CH_3CO)_2O$ (0.33 ml, 3.5 mmol) was added. The reaction mixture was shaken for 6 h at 37°, then diluted with 30 ml of water and freeze-dried that gave 112 mg of the crude product, which contained 76% of the acyl amino aldehyde. After purification by flash-chromatography in chloroform : ethyl acetate (10:1) on silica $[\alpha]_D^{20}$ -53° (c = 1, MeOH) was found for Z-Pheal thus obtained that agreed with the literature data.[11]

Peptidyl amino alcohols were oxidized analogously.

Kinetical measurements.

The kinetic parameters for subtilisin- or α-chymotrypsin-catalyzed hydrolysis of 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 15-20 min. Each experimental set was performed in triplicate.

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5. Standard three or one letter abbreviations are used for amino acid residues, which all belong to L-series; Pol :phenylalaninol; *p*NA : *p*-nitroanilide.

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7. ^{1}H NMR ($CDCl_3$, 200 MHz),δ 3,52-3,76 (*m* 3H, NC*H* CH_2O), the amino alcohols can not be detected under the standard conditions of amino acid analysis of peptide hydrolyzate, but their spots were visualized with ninhydrin after TLC on Silufol plates (Czech Republic) in butanol:pyridin:acetic acid:water (10:15:3:12).

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