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ONE-POT SYNTHESIS OF CATHEPSIN INHIBITORS: N^{α}-PROTECTED N-PEPTIDYL-O-ACETYL HYDROXYLAMINES CATALYSED BY ALCALASE FOLLOWED BY LIPASE IN ANHYDROUS t-BUTANOL.

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Abstract N^{α}-protected N-peptidyl-O-acetyl hydroxylamines [peptide= Ala-Asp(Bzl)-Phe, Pro-Phe-Leu, Ala-Phe, and Pro-Phe] have been synthesized enzymatically in one pot catalyzed by alcalase followed by lipase in t-butanol.

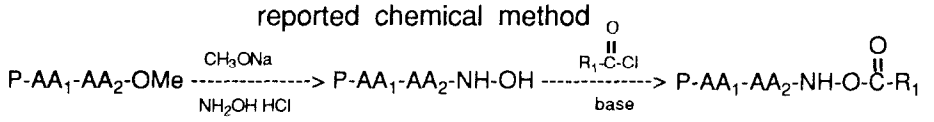
Introduction.

Enzyme inhibitors have received much attention due to their potential use in pharmaceuticals and pesticides[1-3]. N^{α}-Protected N-peptidyl-O-acyl-hydroxylamines are potential inhibitor of serine and cystein protease[4,5]. The inhibitors have been prepared from the methyl ester of N^{α}-protected peptide and hydroxylamine in sodium methanolate solution to yield the appropriate hydroxamic acid, followed by O-acylation using an acid chloride (Scheme **1-a**). We have found that the alkaline protease "alcalase" can catalyze the N-hydroxyamide bond formation of N^{α}-protected peptide ester and hydroxylamine to yield N^{α}-protected N-peptidyl hydroxamic acid. The N^{α}-protected N-peptidyl hydroxamic acid can be further O-acetylated to yield N^{α}-protected N-peptidyl- O-acetyl hydroxylamines in high yield catalyzed by a crude porcine pancrease lipase (PPL) via an irreversible trans-esterification method using vinyl acetate as an acyl-donor[6] (Scheme **1-b**). A one-pot

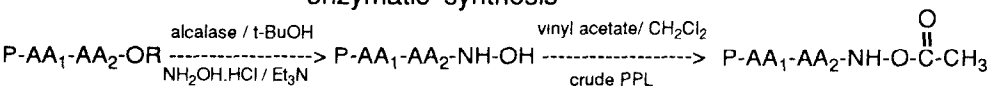
synthesis procedure has been developed by starting from esters of N^{α}-protected peptide and hydroxylamine catalyzed by alcalase in t-butanol solution, followed by crude PPL with vinyl acetate in the same solution without isolation of the intermediate of N^{α}-protected N-peptidyl hydroxamates (Scheme **1-c**).

Alcalase is a proteolytic enzyme prepared from the submerged formation of a selective strain of *Bacillus Licheniformis*. The major enzyme component in alcalase is Subtilisin Carlsberg (alkaline protease A), which is a serine protease. Alcalase is widely used as an additive in detergents as a digesting enzyme. Recently, it has been used as a catalyst for peptide bond formation in anhydrous alcohol[5]. Crude PPL is an inexpensive enzyme[8] and is widely used for its broad substrate specificity and stability in organic solvents[9-11].

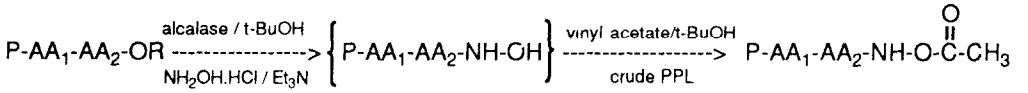
Scheme **1-a.**
reported chemical method



Scheme **1-b.**
enzymatic synthesis



Scheme **1-c.**
one-pot enzymatic synthesis



P= Cbz, Boc, Moz. R_1 = acetyl, benzoyl, p-nitrobenzoyl. R = methyl or benzyl

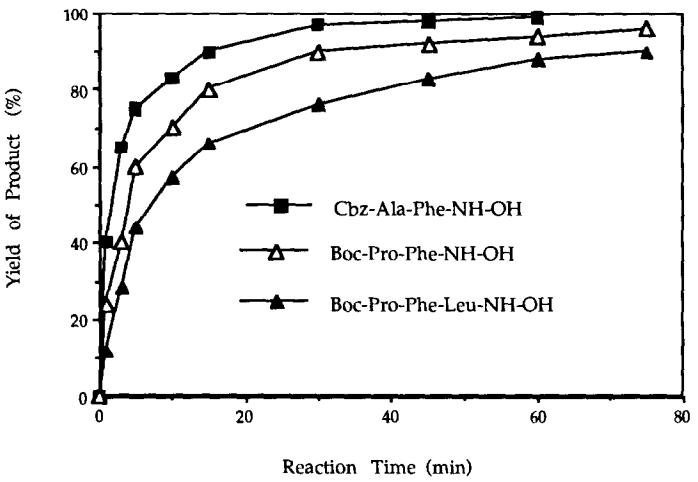
AA_1-AA_2 =Ala-Asp(Bzl)-Phe, Ala-Phe, Pro-Phe-Leu, Pro-Phe, Phe

Results and discussion.

The kinetically controlled synthesis catalysed by serine and cysteine proteases is widely used, since only short reaction times and low enzyme

concentrations are required. This approach was used in synthesis of N^{α}-protected N-peptidyl hydroxamic acid using methyl or benzyl esters of peptide as substrates. The reaction was conducted in an anhydrous t-butanol solution. In a typical reaction, alcalase, Cbz-Ala-Phe-OBzl and hydroxylamine were stirred at 35°C. Periodically, 50 μL aliquots were taken and analysed on HPLC. The Cbz-Ala-Phe-OBzl disappeared within half an hour while Cbz-Ala-Phe-NH-OH was formed, and the concentration of Cbz-Ala-Phe-NH-OH increased constantly until the end of the reaction. In a similar manner, N^{α}-protected peptide esters were used as acyl-donors and reacted. Figure 1 shows the time-course study for the alcalase catalysed synthesis of hydroxamates from each of the correspondent peptide esters of Cbz-Ala-Phe-OBzl, Boc-Pro-Phe-OMe, and Boc-Pro-Phe-Leu-OMe, and hydroxylamine. The rate of N^{α}-protected N-peptidyl hydroxamic acid formation depended on the structure of the p-1 residue of the peptide ester and the alkyl group of the ester. The peptide with a benzyl ester yielded the N^{α}-protected N-peptidyl hydroxamic acid faster than did the other two peptides. The peptide with aromatic amino acid residue at the p-1 site reacted faster than did the peptide which had leucine at the p-1 site. In a representive preparative scale reaction, alcalase (3 mL, 7.5 AU), Cbz-Ala-Phe-OBzl (10.0 mmol), and hydroxylamine (30 mmol) in t-butanol (30 mL) were stirred at 35°C until all the acyl-donor was consumed (HPLC, about 1 h). To the reaction mixture was added ethyl acetate (250 mL), and the resulting mixture was washed with 0.1 M HCl (3x30 mL), 0.5 M $NaHCO_3$ (3x30 mL), and

Figure-1 Time course for alcalase catalyzed synthesis of peptidyl hydroxamates.



then water (3x30 mL). The organic solution was concentrated, and to the residue ether was added to precipitate the crude product. It was recrystallized with ethyl acetate/hexane (3:1) to give pure Cbz-Ala-Phe-NH-OH (3.29 g, 92% yield). The results of all other preparations are summarized in table 1. The physical properties of the products were confirmed by NMR, amino acid analysis of peptide hydrolysate or with authentic samples.

That Lipase can maintain stability and activity in non-polar solvents such as toluene, n-hexane, dichloromethane was already known. The O-acetylation of N^{α}-protected N-peptidyl hydroxamic acid was conducted in dichloromathane using vinyl acetate as an acyl-donor. From our preliminary test, only the crude PPL can catalyze the O-acetylation reaction. Other lipases, such as Lipase OF (from Amano), Lipase AP6 (from Amano), and Palatase (from Novo), were not useful. Since N^{α}-protected N-peptidyl-O-acyl hydroxylamine is an inhibitor of serine and cystein proteases, it may inhibit all the tested lipase, and only the enzyme which was contaminated in the crude PPL could catalyzed the O-acylation reaction. In a typical reaction, to N^{α}-protected N-peptidyl hydroxamic acid (1 mmol) in dichloromethane (10 mL) was added vinyl acetate (1 mL) and crude PPL (0.5 g). The resulting mixture was stirred at ambient temperature. After the N^{α}-protected N-peptidyl hydroxamic acid was consumed (monitored by TLC, about 24 h), the solution was centrifuged, and the supernatant was passed through a flash column (silica gel, eluented with CH_2Cl_2/MeOH 9:1) to yield pure N^{α}-protected N-peptidyl-O-acetyl hydroxylamines. Results are summarized in Table 1. Further investigation revealed that the crude PPL could catalyze the O-acetylation of N^{α}-protected N-peptidyl hydroxamic acid in t-butanol, and that the catalytic-activity of crude PPL in t-butanol was as fast as in dichloromethane solution. Therefore, we could synthesize the enzyme inhibitors from the esters of N^{α}-protected peptide in a one-pot procedure (see Scheme **I-c**). Because the N^{α}-protected N-peptidyl-O-acetyl hydroxylamine is an inhibitor of alcalase, once it is synthesized in the reaction solution, the N^{α}-protected N-peptidyl-acetyl hydroxylamine inhibits the proteolytic activity of alcalase in the reaction solution. In a representive preparative scale one pot reaction, alcalase (5 mL, 7.5 AU), acyl-donor (10.0 mmol),and hydroxylamine (30 mmol) in t-butanol (50 mL) were stirred at 35℃, until all the acyl-donor was consumed (hplc, about 1 h). To the reaction mixture was

added vinyl acetate (5 mL) and crude PPL (5.0 g), and the mixture was stirred for a further 24 hrs. Ethyl acetate (300 mL) was added and the resulting solution was washed with 0.1 M HCl (3x30 mL), 0.5 M $NaHCO_3$ (3x30 mL), and then water (3x30 mL). The organic solution was concentrated, n-hexane (50 mL) was added to precipitate the crude product. It was further purified either by recrystallization or flash column chromatography. The results of all other preparations are summarized in Table 1

Table 1. Enzymatic synthesis of N^{α}-protected N-peptidyl-O-acetyl hydroxylamine.

$P\text{-}AA_1\text{-}AA_2\text{-}OR$ ----------> $P\text{-}AA_1\text{-}AA_2\text{-}NH\text{-}OH$ --------> $P\text{-}AA_1\text{-}AA_2\text{-}NH\text{-}O\text{-}CO\text{-}CH_3$

	rxn time (h)	yield (%)	rxn time (h)	yield (%)	yield (one pot) (%)
Cbz-Ala-Asp(Bzl)-Phe-OBzl	2.0	85[1]	24	37[2]	41
Cbz-Ala-Phe-OBzl	2.0	92[3]	24	66[4]	64
Boc-Pro-Phe-Leu-OMe	2.5	86[5]	28	45[6]	-
Boc-Pro-Phe-OMe	2.5	80[7]	20	52[8]	42
Moz-Phe-OMe	1.5	91[9]	15	83[10]	72

1). mp:158-159, $[\alpha]^{25}_D$:-36.67 (c 0.60, MeOH); 2).mp:94-96, $[\alpha]^{25}_D$:-10.00 (c 0.40, MeOH);
3). mp:150-152, $[\alpha]^{25}_D$:-32.73 (c 0.55, MeOH); 4).mp:145-146, $[\alpha]^{25}_D$: +8.0 (c 0.50, MeOH);
5). mp:166-168, $[\alpha]^{25}_D$:-51.43 (c 0.35, MeOH); 6).mp:72-74, $[\alpha]^{25}_D$:-27.27 (c 1.10, MeOH);
7). mp:159-160, $[\alpha]^{25}_D$:-65.71 (c 0.70, MeOH); 8).mp:55-57, $[\alpha]^{25}_D$:-22.22 (c 0.45, MeOH),
9). mp:137-140, $[\alpha]^{25}_D$:- 5.00 (c 0.80, MeOH);10).mp:112-114, $[\alpha]^{25}_D$:+25.45 (c 1.10, MeOH)

N^{α}-protected N-peptidyl-O-acetyl hydroxylamines can be prepared by reaction of the corresponding hydroxylamine and acyl chloride in alkaline condition. Nevertheless, we think that this enzymatic synthesis possesses advantages over other conventional methods. It is experimentally very simple. The chance of racemization of amino acids in sodium methoxide solution, can be avoided by using this enzymatic process. The procedure illustrated here describes a practical application of alcalase for N^{α}-protected N-peptidyl-O-acetyl hydroxamic acid synthesis.

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References and Notes:

1. Sham, H. L. Betebenner, D. A. Widegburg, N. E., Saldivar, A. C. Kohlbrenner, W. E. Vasavanonda, S. Kempf, D. J. Norbeck, D. W. Zhao, C. Clement, J. J. Erickson, J. E. Plattner, J. J., Biochem. Biophy. Res. Commu. **1991**, 175, 914-919.
2. Wang, K. K. W. TIPS, 1990, 11, 139-142.
3. Zollner, H. Handbook of Enzyme Inhibitors, Ed H. Zollner. VCH. Verlagsgesellschaft mbH, D-6940, Weinheim. **1990**, p 51.
4. Demuth, H. U. , C. Schonlein, A. Barth Biochem. Biophy Acta, **1989**, 996, 19-22.
5. Smith, R. A. P. J. Coles, R. W. Spencer, L. J. Copp. C. S. Jones, A. Krantz.Biochem. Biophy. Res. Commu. **1988,** 155. 1201-1205.
6. Chen, S. T., Hsiao, S. H., Wang, K. T., Bioorg. & Med. Chem. Letters **1991**, 1(9), 445-450.
7. Wang, Y. F., Lalonde,J. J., Momongan, M.,Bergbreiter, D.E., Wong, C. H., J. Amer. Chem. Soc. **1988**, 110, 7200-7206.
8. Semeriva, M. & Desnuelle, P., In Advances in Enzymology, Eds: Meister, A. **1978** Vol 48, p. 319-370. Interscience Publishers, John Wiley & Sons, New York.
9. Kasel, W., P. G. Hultin, J. B. Jones, Chem. Commu. **1985**, 1563-1565.
10. West, J. B.,Wong, C. H., Tetrahedron Lett. **1987** 28(15), 1629-1632.
11. Matos, J. R., West, J. B., Wong, C. H.,, Biotech. Lett, **1987,** 9(4), 233-236.