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Chemo-Enzymatic Dynamic Kinetic Resolution of Amino Acid Thioesters

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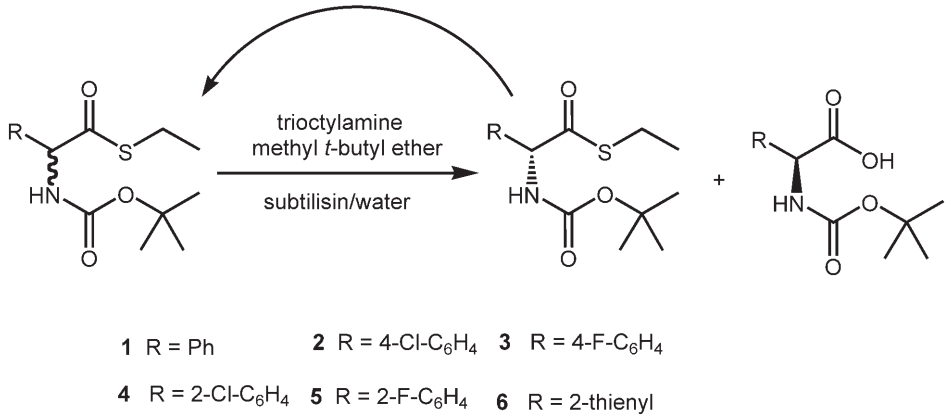
Abstract: The L-forms of racemic-*N*-protected-β,γ-unsaturated α-amino acid thioesters were found to be substrates for the subtilisin-catalysed hydrolysis to the corresponding acids. The D-enantiomer was continuously racemised in the presence of an organic base. The combined reactions in a biphasic system allowed the deracemisation of the amino acid derivatives based on a dynamic kinetic resolution. Excellent yields and enantioselectivities were achieved.

Keywords: amino acids; biotransformations; dynamic kinetic resolution; enzyme catalysis; racemization

Hydrolytic enzymes are the most readily available biocatalysts for synthetic applications, particularly in the kinetic resolution of racemic mixtures.[1] Like in any resolution method, a maximum of 50% yield of enantiomerically pure product can be obtained, based on racemic starting material. When kinetic resolution is coupled with an *in situ* racemisation of the substrate either chemical or enzymatic, the yield limitation can be overcome leading to a much more efficient process: a deracemisation based on a dynamic kinetic resolution (DKR).[2] A well known example in amino acid chemistry is the industrial process D-*hydantoinase-carbamoylase* which allows the large scale preparation of relevant D-amino acids.[3] In this process a microbial catalyst containing the two enzymatic activities transforms the *R*-starting material into the D-amino acids, while the remaining hydantoin is continuously racemised at slightly basic pH. Requisites for a successful DKR are an enzyme selective for one of the enantiomers, a racemising system (chemical or enzymatic) acting on the substrate but not on the product, and a rate of racemisation of the substrate higher than the rate of conversion to product. These conditions require the design of suitable substrates. In thioesters, the acidity of the hydrogen in the α-position is higher in comparison to the corresponding oxo esters, amides or acids. The enzymatic transformation of a thioester into the corresponding carboxylate with a higher pK_a of the α-proton is the basis for a successful DKR, provided that the enzymatic systems resist the basic conditions required for substrate racemisation. This concept has been applied in the DKR of α-alkyl thioesters.[4] Our interest in the synthesis and deracemisation of non-natural amino acids[5] prompted us to design racemic compounds suitable for this novel application. From previous data[6] aryllycines and analogues were considered as candidates. In fact not only *p*-hydroxyphenylglycine esters have been shown to be the substrate for subtilisin, but also phenylglycine (Phg) has been partly deracemised in the presence of chiral copper catalysts indicating a suitable p*K*a value for the α-proton.[7]

We initially checked the ability of subtilisin Carlsberg to transform amino acid thioesters **1–6** (Scheme 1) and found a good activity on these *N*-Boc amino acid derivatives with reaction rates comparable although inferior to the one observed with the corresponding oxoesters.[8]

Racemisation conditions were studied by observing the exchange rate of the α-proton of the thioester with deuterium from CD_3OD by ^1H NMR. In a typical experiment to 20 mg of substrate dissolved in 500 μL of DMSO-d_6 and 100 μL of CD_3OD, 0.5 equivs. of trioctylamine were added at 31 °C. The 50% exchange rate varied from a few minutes (for compound **6**) to several hours for the 2-substituted phenylglycines. In all cases, no exchange was observed in the absence of trioctylamine. Control experiments with the corresponding oxo esters and acids showed no measureable proton exchange under similar condi-



Scheme 1.

tions. This indicated that the thioester, but not the oxo ester, was suitable for the *in situ* racemisation method and that the product acid was configurationally stable under thioester-racemising conditions. Further experiments showed that thioesters were not prone to spontaneous hydrolysis. The deuterium exchange rate was highest for **6** ($t_{1/2}$ of 10 min) and lowest for the 2-substituted arylglycines **1–5** ($t_{1/2}$ of several h).

The resolution of **1** was initially studied under non-racemising conditions: 2 g of **1** in 150 mL of a 2:1 mixture of water:methyl *tert*-butyl ether (approximately 50 mM) at pH 8.0 and 37 °C were treated with 500 U of subtilisin and the pH controlled in a pHstat apparatus. The base addition stopped sharply at 50% conversion (24 h). The hydrolysis was restored by addition of trioctylamine (1.5 mL, 3.38 mmol, 0.5 equivs.) and stoichiometric base addition was complete after 48 h. The profile of the reaction as registered on a pHstat before and after trioctylamine addition is reported in Figure 1.

Due to the hydrophobic nature of the amino acids and to the presence of the *N*-protecting group, the L-*N*-Boc-Phg-OH was recovered by extraction of the water phase with ethyl acetate (3×50 mL) at pH 3 after separation from the organic layer containing the trioctylamine and further extraction at pH 8. Drying and evaporation of the solvent gave a quantitative amount of L-*N*-Boc-Phg-OH as a yellow oil. The enantiomeric excess evaluated with chiral HPLC analysis was >99%.

L-*N*-Boc-Phg-OH was deprotected with dioxane saturated with HCl at 60 °C to give 1.106 g of L-Phg hydrochloride as a white solid (87% yield from **1**, *ee*> 99%), $[\alpha]_D^{25}$: +147.4 (*c* 0.8 on free amino acids ; HCl 1 N). A procedure similar to the one described was applied to thioesters **2–6** and the results are summarised in Table 1.

The efficiency of the deracemisation process depends on the rate of enzymatic hydrolysis and the proton extraction rate. The latter can be modulated by the trioctylamine concentration which influences the racemisation rate with pseudo-first order kinetics. Figure 2 shows the correlation between TOA equivalent and time course (arbitrary units) as measured on compound **1** in DMSO-d_6 from ^{1}H NMR studies.

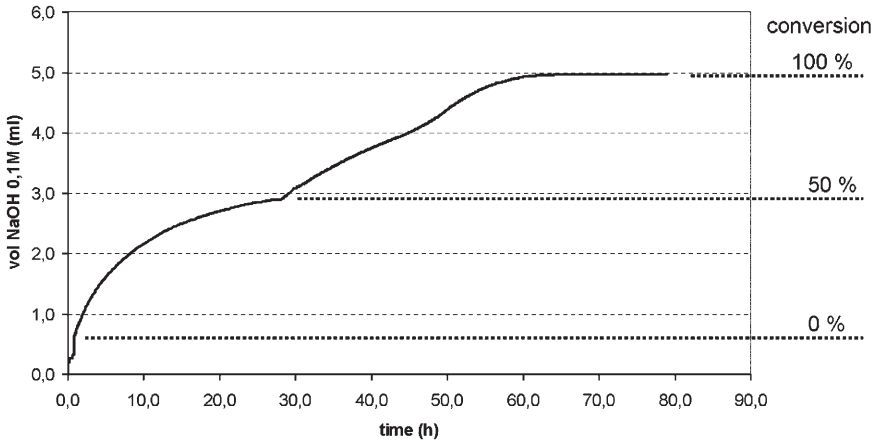


Figure 1. Time course of the DKR of compound **1** before and after trioctylamine addition.

Table 1. Subtilisin-catalysed DKR of compounds **1–6** in the presence of trioctylamine.

Substrate	Conversion [%]	Base/Substrate	Time [h]	*ee* AA
1 (R = Ph)	> 95 %	0.5	48	> 99 %
2 (R = 4-Cl-C_6H_4)	> 95 %	0.5	36	> 99 %
3 (R = 4-F-C_6H_4)	> 95 %	0.5	20	> 99 %
4 (R = 2-Cl-C_6H_4)	> 95 %	1	96	> 99 %
5 (R = 2-F-C_6H_4)	> 95 %	1	12	> 99 %
6 (R = Th)	> 95 %	0.5	5	> 99 %

The data reported in Table 1 reflect the combined effect of these two parameters. Due to the complete and well known specificity of subtilisin for L-amino acid esters, the enantiomeric excesses of all products were higher than 99 %.

Conditions for the effective chemo-enzymatic dynamic kinetic resolution of racemic compounds are being continuously sought after.[9] The present method of DKR of *N*-protected β,γ-unsaturated α-amino acid thioesters is highly efficient allowing the complete deracemisation of the amino acid derivative in high yield and selectivity. The enzyme selectivity parallels the one observed with the oxoesters[5c,d] and assures the enantioselectivity of the hydrolytic process. However, the presence of the sulphur atom is essential in providing a useful racemisation rate. The latter can also be dependent on the nature of the thioester, the R group and the nitrogen protecting group. The method should therefore be of general applicability to acyclic and cyclic α-substituted carboxylic acid thioesters, and not limited to β,γ-unsaturated α-amino or amino acid derivatives. Moreover, the opposite enantiomers are expected from hyrolysis catalysed by enzymes of opposite stereochemical preferences. Computational studies are in progress in order to define the influence of the groups affecting the acidity of the α-proton thus identifying new substrates for the DKR.

Experimental Section

Subtilisin Carlsberg from *B. subtilis* from Fluka and Alcalase 2.5L DX from Novozymes were used in all experiments and gave similar results.

General Procedure for D,L-*N*-Boc-amino Acids SEt Synthesis

D,L-N-*Boc-Phg-SEt:* To a solution of D,L-*N*-Boc-Phg-OH (3 g, 11,9 mmol) in 100 mL of dichloromethane, DCC (2.71 g, 13.1 mmol, 1.1 equivs.) and DMAP (150 mg, 1.2 mmol, 0.1 equiv.) were added and then ethanethiol (1.1 mL, 14.3 mmol, 1.2 equiv.) was added at room temperature. After a short induction period, the temperature rises to about 30–35 °C and a crystalline white precipitate was formed. The reaction was brought to completion by further stirring overnight at room temperature. At this time, a yellow suspension was obtained and the reaction was finished. The reaction mixture was filtered to eliminate a portion of white solid (*N*,*N*′-dyclohexylurea) and the filtrate was evaporated under reduced pressure using a rotary evaporator. The crude product was purified by column chromatography on silica gel using hexane/ethyl acetate (95:5) as eluent to give a white solid; yield: 2.9 g (82 %). R_f: 0.33 (hexane-ethyl acetate, 9/1). ^{1}H NMR (DMSO-d_6): δ = 1.13 (t, 3H), 1.41 (s, 9H), 2.80 (2H), 5.27 (br s, 1H), 7.35 (m, 3H), 7.42 (m, 2H), 7.99 (br s, 1H); ^{13}C NMR ($CDCl_3$): δ = 198.65

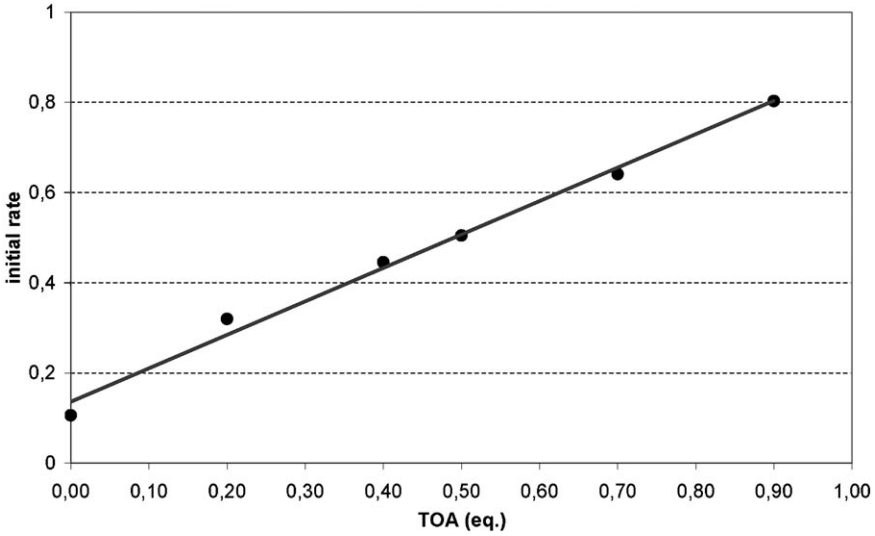


Figure 2. Rate of exchange of α-H in compound **1** as a function of base concentration.

(CHCOS), 154.65 (NHCOO), 136.82 (*C* ar.), 128.89 (*C* ar), 128.50 (*C* ar), 127.44 (*C* ar), 80.23 [$C(CH_3)_3$], 64.50 (NHCHCOS), 28.22 [$C(CH_3)_3$], 23.46 (SCH_2CH_3), 14.25 (SCH_2CH_3).

General Procedure for the Dynamic Kinetic Resolution on D,L-*N*-Boc-amino Acids SEt

Dynamic Kinetic Resolution of* D,L-N-*Boc-Phg-SEt: To a solution of D,L-*N*-Boc-Phg-SEt (2 g, 6.67 mmol) in 50 mL of MTBE, water (100 mL) and trioctylamine (1.5 mL, 3.38 mmol, 0.5 equivs.) were added. The pH was adjusted to 8.0 with NaOH (0.5 M) and 510 U of subtilisin was added. The reaction was kept at pH 8.0 by the automatic addition of NaOH (0.5 M), at 37 °C, under vigorous mechanical stirrer. After 2 days, the consumption of NaOH 0.5 N reached 100 %. The water phase was separated from organic layer and extracted (at pH 8) with 30 mL of diethyl ether. L-*N*-Boc-Phg-OH was recovered by extraction of the water phase with ethyl acetate (3×50 mL) at pH 3. Drying and evaporation of the solvent gave a quantitative amount of L-*N*-Boc-Phg-OH as a yellow oil. The enantiomeric excess analysed by chiral HPLC was > 99 %.

L-*N*-Boc-Phg-OH was deprotected with dioxane saturated with HCl at 60 °C to give L-Phg-OH·HCl as a white solid; yield: 1.106 g (87 %; *ee* > 99 %); $[\alpha]_D^{25}$: +149.4 (*c* 0.8 1N HCl, lit[10]: +156 (*c*1, 1 N HCl).

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