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N-Octanoyldimethylglycine Trifluoroethyl Ester, an Acyl Donor Leading to Highly Enantioselective Protease-Catalysed Kinetic Resolution of Amines

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Abstract: The use of *N*-octanoyldimethylglycine trifluoroethyl ester as acyl donor in the kinetic resolution of aliphatic amines catalysed by proteases led to enantiomeric ratios >200 in most cases. The resolutions mediated by Protex 6L were shown to be much faster than the resolutions achieved with the most efficient commercially available serine proteases, i.e., alkaline protease, Properase 1600L, and Subtilisin

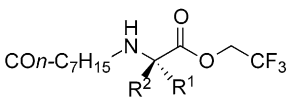
Keywords: amines; enantioselectivity; enzyme catalysis; kinetic resolution

Introduction

The importance of amines in the agrochemical and pharmaceutical industries has stimulated an enormous interest for their production in optically pure form. Biocatalysis ranks now among the most important of these processes.[1] A panel of enzymatic routes is available including the use of hydrolases, transaminases, amine oxidases or amine reductases. Whereas enzymatic resolution of amines by lipases has been extensively investigated and excellent results have been reported, literature data concerning their kinetic resolution (KR) by proteases are by far less numerous.[2,3]

During the course of our efforts to devise protocols for the chemoenzymatic dynamic kinetic resolution of amines through the association of a radical racemisation procedure with an enzymatic kinetic resolution,[4] we have had to optimise the kinetic resolution of amines by both lipase CAL-B[5] and a series of serine proteases.[6] This led us to report the first switchable DKR process allowing the synthesis of either (*R*)- or (*S*)-amides from racemic amines depending on the nature of the enzyme.[4c,d] In order to reach this goal, we have had to investigate the reactivity and the enantioselectivity of different serine proteases in the resolution of amines performed with different acyl donors.[6b]

In preliminary investigations,[6b] in which the amine acylation was achieved with a large series of peptide mimics, Protex 6L turned out to be a non-selective catalyst. However, it reacted much faster than any other tested enzyme. Our attention was focused on the ability of Protex 6L to become as selective as the most selective proteases, i.e., alkaline protease, Properase 1600L, and Subtilisin.[7] This goal was reached by using **3d** (Figure 1), a peptide mimetic derived from dimethylglycine, as acyl donor. The results of this study are reported herein.



	R^1	R^2
3a	H	H
3b	Me	H
3c	$PhCH_2$	H
3d	Me	Me

Figure 1. Acyl donors.

A computational approach undertaken in order to rationalise the specific behaviour of acyl donor **3d** is detailed in the Supporting Information.

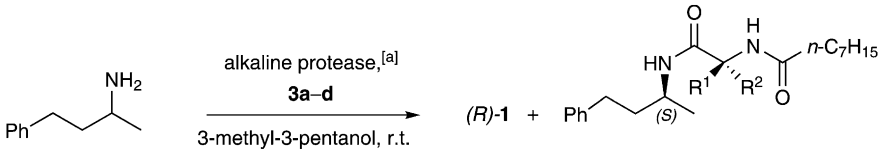
Results and Discussion

The most commonly used acyl donors[8] (e.g., ethyl α-methoxyacetate[9]), known to be particularly efficient in lipase KR, were tested first. As already reported they gave disappointing results with Subtilisin as compared to peptide mimetics which are close to the natural substrates of proteases.[6a]

A series of peptide mimetics[10] was tested with alkaline protease, i.e., the commercially available protease that was shown to be the best suited to DKR purposes, owing to its compatibility with the radical racemisation conditions at room temperature.[11] The results obtained with 4-phenyl-2-butyl amine (**1a**, see Figure 3) that was used as model amine all along our study are reported in Table 1. As shown in entry 4, *N*-octanoyldimethylglycine trifluoroethyl ester (**3d**) was far more selective than the corresponding trifluoroethyl esters derived from either glycine (**3a**, entry 1), L-alanine (**3b**, entry 2), or L-phenylalanine (**3c**, entry 3). The time of reaction was too long for the purpose of using it as reagent in a DKR reaction. It was totally incompatible with the very short time needed for sulfanyl radical-mediated racemisation procedure (commonly 60 to 90 min). Nevertheless, the enantiomeric ratio was exceptionally high for a protease-catalysed amine KR.[2,3] Such an increase in itself, obtained with a commercially available enzyme, deserved further investigations.

As shown in Table 2, the resolution of **1a** performed with Protex 6L in the presence of **3d** in 3-

Table 1. Alkaline protease-catalysed KR of **1a** at 21 °C in the presence of **3a–d**.

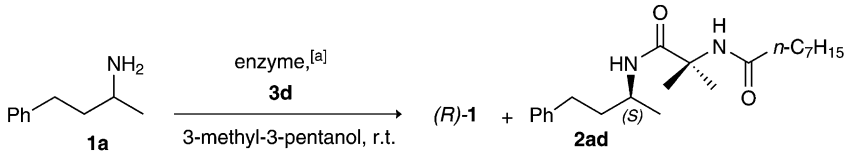


1a **2aa–2ad**

Entry	Acyl Donor	Time	**1a** *ee* [%][b]	**2aa–2ad** *ee* [%][c]	Conversion (C) [%][d]	*E*[e]
1	**3a**	35 min	83	91	48	57[6b]
2	**3b**	90 min	87	94	48	99[6b]
3	**3c**	40 min	75	94	44	72[6b]
4	**3d**	24 h	73	>99.5	42	>200 (this paper)

[a] *Standard procedure:* reactions were performed on a 0.5 M solution of **1a** (0.125 mmol) in 3-methyl-3-pentanol at room temperature, with 6 mg of coated enzyme (co-lyophilised in different buffers corresponding to optimal pH with Brij®56 and *n*-octyl α,β-D-glucopyranoside; 4/1/1, w/w/w) and 1 equiv. of acyl donor.
[b] Determined by GC after derivatisation.
[c] Determined by HPLC.
[d] Calculated according to $C = ee_S/(ee_S + ee_P)$ unless otherwise stated.
[e] Enantiomeric ratios were calculated according to $E = \ln[(1-C)(1-ee_S)]/\ln[(1-C)(1+ee_S)]$.[12]

Table 2. KR of **1a**, achieved with different enzymes at 21 °C.



1a **2ad**

Entry	Enzyme	Time	**1a** *ee* [%][b]	**2ad** *ee* [%][c]	Conversion (C) [%]	*E*
1	alkaline protease	24 h	73	>99.5	42	>200
2	Properase 1600L	96 h	53	>99.5	35	>200
3	Subtilisin Novo	7 days	33	>99.5	25	>200
4	Protex 6L	10 h	97	>99.5	49	>200

[a] Standard procedure given in Table 1 unless otherwise stated.
[b] Determined by GC after derivatisation.
[c] Determined by HPLC.

Table 3. Solvent effect on the KR of **1a**, mediated with Protex 6L and **3d**.

Entry	Solvent/Concentration	Time	**1a** *ee* [%][b]	**2ad** *ee* [%][c]	Conversion (C) [%]	*E*
1	3-MP/0.5 M	10 h	97	>99.5	49	>200
2	*t*-BuOH/0.5 M	72 h	29	>99.5	22	>200
3	3-MP/THF (1/1)/0.5 M	48 h	60	68	47	9
4	3-MP/THF (1/1)/0.1 M	48 h	83	62	57	11
5	3-MP/THF (2/1)/0.1 M	40 h	71	87	45	29
6	3-MP/hexane (2/1)/0.1 M	40 h	54	96	36	82
7	3-MP/toluene (2/1)/0.1 M	40 h	73	93	44	61

[a] Standard procedure given in Table 1 unless otherwise stated.
[b] Determined by GC after derivatisation.
[c] Determined by HPLC.

methyl-3-pentanol (3-MP) was completed in 10 h. It was much faster than the resolutions carried out with any of the other selected proteases (entries 1–4), under the reported conditions. It can be remembered that, for the resolution of **1a**, Protex 6L led to 46% conversion in 10 min ($E=46$) in the presence of **3a**; it led to 46% conversion in 50 min ($E=67$) in the presence of **3b**; and to 54% conversion in 54 min ($E=61$) with **3c**.[6b]

Further improvement of the KR was achieved by testing the efficacy of a series of organic solvents (Table 3).

Proteases are reputedly less stable in organic solvents than lipases, and coating of the enzyme was shown to significantly affect both the rate of reaction and the enantiomeric ratio.[13] These experiments confirmed the pioneering results of Klibanov,[3a] that is, tertiary alcohols led to the highest enantiomeric ratios. However, 3-methyl-3-pentanol (entry 1) was far superior to *tert*-butanol (entry 2) regarding the reaction rate. The mixtures of solvents that were investigated led to very low enantiomeric ratios (examples are given in entries 3–7).

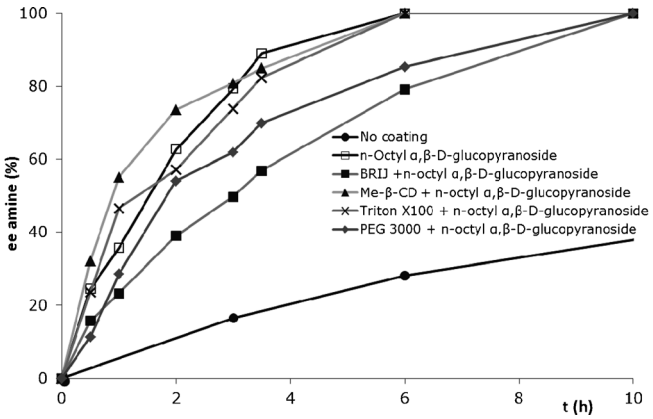


Figure 2. Influence of Protex 6L coating on the rate of resolution of amine **1a** in 3-methyl-3-pentanol. a) In the case of only one coating agent: Protex 6L/*n*-octyl α,β-D-glucopyranoside = 4/1. b) In the case of two coating agents: Protex 6L/coating agent 1/coating agent 2 = 4/1/1.

The nature of the coating agents also influenced the reactivity (Figure 2). The enantiomeric excess of the residual (*R*)-amine reached 28% in 6 h, and did not exceed 40% after 10 h of reaction in the absence of coating. It reached 79 or 85% in 6 h when the enzyme was coated with Brij 56 + *n*-octyl α,β-D-glucopyranoside, or PEG3000 + *n*-octyl α,β-D-glucopyranoside, respectively. After the same period (6 h) the *ee* was very close to 100% with either *n*-octyl α,β-D-glucopyranoside alone, or with Me-β-cyclodextrin (Me-β-CD) + *n*-octyl α,β-D-glucopyranoside, or even with Triton B + *n*-octyl α,β-D-glucopyranoside.

The 1:1 ratio of Me-β-cyclodextrin/*n*-octyl α,β-D-glucopyranoside was selected.[14] The ratio of enzyme to the binary coating was also further optimized.

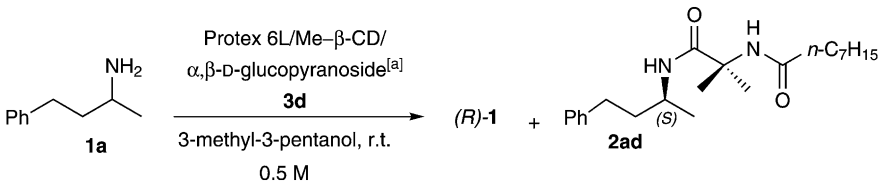
According to the data reported in Table 4. the fastest resolution was observed for an 8:1:1 ratio of Protex 6L/Me-β-cyclodextrin/*n*-octyl α,β-D-glucopyranoside, with which the rate was found to be slightly superior to that registered for the 4:1:1 ratio (entries 3 and 5). The conditions described in entry 6 were therefore selected for all further experiments.

The reaction profile was confirmed by repeating the resolution of amine **1a** on a 5-mL scale (the plot of amine *ee*/time is given in the Supporting Information). The yields and the *ee* were similar (Table 5).

The optimised experimental conditions were then applied to a large series of amines of various structural features shown in Figure 3 to investigate which structural parameters were determining for the discrimination of the two enantiomers. The results are given in Table 5.

The non-catalysed reaction of amines **1a**, **1d**, and **1e**, with **3d**, were followed by ^{19}F NMR (the conversion was determined from the signals of trifluoromethyl groups of the donor and of released trifluoroethanol, respectively). After 7 days the amount of non-catalysed acylation was 0.8%, 1.3%, and 0.7%, respectively. It could therefore be neglected.

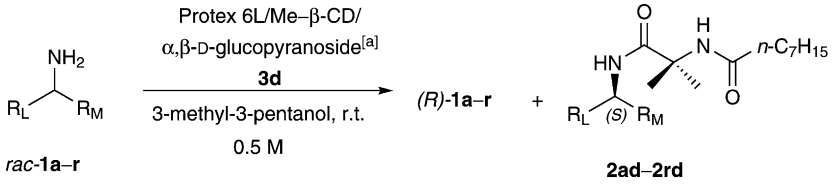
Table 4. Influence of the enzyme/coating agents ratio on the resolution rate.



Entry	Protex 6L/Me-β-CD/α,β-D-glucopyranoside	Time [h]	**1a** *ee* [%][b]
1	2/1/1	3	54
2	2/1/1	6	81
3	4/1/1	3	78
4	4/1/1	6	>99.5
5	8/1/1	3	81
6	8/1/1	6	>99.5
7	16/1/1	3	40
8	16/1/1	6	70

[a] *Standard procedure.* as given in Table 1 except for the coating agents.
[b] Determined by GC after derivatisation.

Table 5. Resolution of amines **1a–r**.



Amine	Time [h]	Amine		Amide		Conversion (C) [%]	*E*
		ee [%][b]	Yield	*ee* [%][c]	Yield		
1a	6	96	48%	>99.5	48%	49	>200
1a[d]	7	>99.5	49%	>99.5	49%	49	>200
1b	72	38	63%	>99.5	21%	27	>200
1c	7	90	43%	68	54%	57	14
1d	11 days	61	47%	63	44%	49	8
1e	72	60	60%	96	35%	38	200
1f	6 days	63	59%	>99.5	33%	39	>200
1g	18 days	58	61%	>99.5	34%	37	>200
1h	7 days	91	51%	>99.5	43%	48	>200
1i	7 days	76	55%	>99.5	39%	43	>200
1j	72	83	46%	78	51%	52	21
1k	8 days	95	53%	99	46%	49	>200
1l	48 h	97	49%	99	46%	49	>200
1m	48 h	87	43%	74	51%	54	18
1n	19 days	68	54%	96	38%	41	103
1o	19 days	38	39%	27	55%	59	2.4
1p	6 days	54	39%	39	53%	58	4
1q	72	97	47%	98	45%	50	>200
1r	7 days	88	50%	96	44%	48	153

[a] See general procedure.
[b] Determined by GC after derivatisation.
[c] Determined by HPLC.
[d] 20-fold scale-up.

Unusually high enantiomeric ratios were observed in most cases. The lowest *E* factors were noted for benzylic amines (with the exception of the hindered *ortho*-substituted amine **1n**). The saturated equivalent of amine **1c**, that is, amine **1d** behaved similarly. There is clearly an effect of the bulk of the substitu-

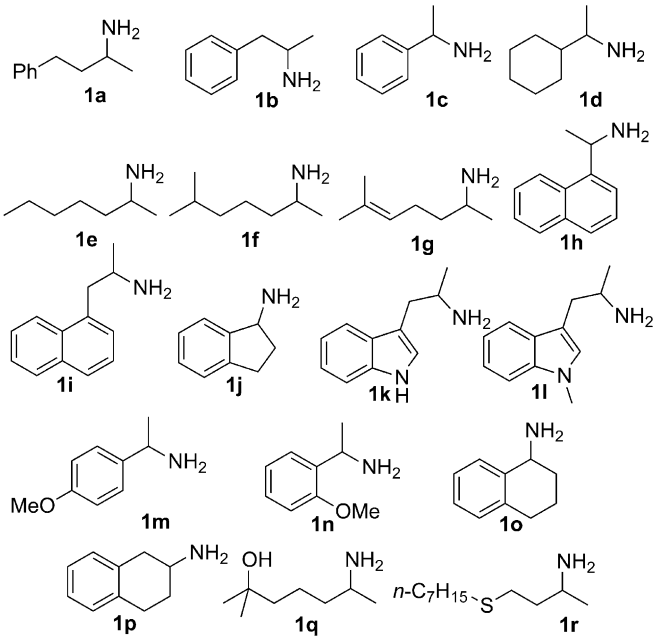


Figure 3. Amine structures.

ent in position beta relative to nitrogen which is likely to hinder the approach of the acyl enzyme.

Table 2 shows that very high enantiomeric factors can be obtained with different proteases, sharing the same catalytic triad. Subtilisin Novo (entry 3) was used as a model enzyme, to approach by docking calculations the affinity of this protease for acyl donors **3a–d**. Such an approach of a multistep process leading to chiral amides from racemic amines should be taken cautiously but it could give interesting information about which step is likely to determine the rate of the resolution process.

Subtilisin Novo had already been used as model in previous studies.[4b] In the latter, several structures of Subtilisin Novo were tested, but their docking results were not significatively different. This is the reason why only the 2 sni structure of the PDB database was selected.

The different acyl donors were docked into the enzyme using Autodock 4.0.[15]

The computational details are reported in the Supporting Information. They suggest that the four acyl donors have rather close affinities (**3c** exhibits the strongest one).

The dynamic conformational behaviour of acyl-enzyme complexes formed from **3a–d** was sampled. Their conformational flexibility was examined by means of systematic torsion around the different rotatable bonds of the acyl moiety. The partition of conformations with a significant contribution in each series was analysed. In the case of **3d** the contribution of conformations in which the chain is folded is much more important than in the cases of the other acyl donors. The *gem*-dimethyl effect seems to facilitate the folding of the lipophilic octanoyl chain. This makes for a more difficult access to the hindered reactive carbonyl group. Therefore, the *gem*-dimethyl group promotes the formation of non-productive conformations of the acyl-enzyme complex in which the apolar chain lies out of the enzyme channel. This conformational peculiarity might be responsible for the slowing down of the reactions performed in the presence of **3d**.

Conclusions

Simple acyl donor engineering, i.e., the use of *N*-octanoyldimethylglycine trifluoroethyl ester, was found to have a remarkable influence on the enantioselectivity of protease-catalysed resolution of aliphatic amines. Enantiomeric ratios >200 are viable and can have practical application for a large series of amines using a commercially available enzyme, Protex 6L.

Experimental Section

General Experimental Methods and Characterisation of New Products

See the Supporting Information

Protease Coating Procedure

Octyl α,β-D-glucopyranoside (60 mg) and methyl-β-cyclodextrin (60 mg) were diluted at 60 °C in 50 mL of buffer (phosphate 0.1 M, pH 7), the solution was then cooled to room temperature. The Protex 6L liquid extract (500 mg) was added to 25 mL of this solution. The mixture was rapidly frozen in liquid N_2 and lyophilised for 20 h.

General Procedure for the Kinetic Resolution of 1a–r

To a solution of acyl donor (0.187 mmol, 1.5 equiv.) in 3-methyl-3-pentanol (250 μL), 10 mg of coated protease and amine (**1a–r**) (0.125 mmol, 1 equiv.) were added. The resulting mixture was stirred at 21 °C. The kinetic resolution was monitored by analysing aliquots at different time intervals. The amide *ee* was determined by HPLC after dilution in the eluting solvent. The amine *ee* was determined by GC after dilution in diethyl ether and derivatisation in trifluoroacetamide using 1.5 equiv. of *N*-methyl-bis-trifluoroacetamide.

Supporting Information

Computational studies are detailed in the Supporting Information as are also the synthesis of **3d** and characterisation data of all new kinetic resolution products [**2ad–2rd** and Boc-(*R*)-**1l**, Boc-(*R*)-**1m**, Boc-(*R*)-**1n**, Boc-(*R*)-**1o**, Boc-(*R*)-**1p**, Boc-(*R*)-**1r**].

Acknowledgements

Gifts of alkaline protease, from Valley Research, of Subtilisin Novo from Novozymes, and gifts of Protex 6L, and Properase 1600L from Genencor, are gratefully acknowledged. This work was supported by the Agence Nationale de la Recherche (ANR 06-Blan-0137). The authors thank Dr Hugues-Olivier Bertrand (Accelrys) for fruitful discussions and Dr Anne-Lise Bottalla for her contribution. All of the computations were performed with thanks to the large-scale facilities of the Centre de Calcul et de Recherche Technologique (CCRT) of the French Nuclear Agency (CEA) and at the Centre Regional de Competences en Modelisation Moleculaire (CRCMM) in Marseille.

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