DOI: 10.1002/adsc.200800091

Fully Enzymatic Resolution of Chiral Amines: Acylation and Deacylation in the Presence of *Candida antarctica* Lipase B

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Received: February 12, 2008; Revised: May 9, 2008; Published online: June 23, 2008

Abstract: A fully enzymatic methodology for the resolution of chiral amines has been demonstrated. *Candida antarctica* lipase B (CaLB)-catalyzed acylation with *N*-methyl- and *N*-phenylglycine, as well as analogues having the general formula R^1–X–$CH_2CO_2R^2$ (R^1 = Me, Ph; X = O, S) afforded the corresponding enantioenriched amides, which were subsequently enzymatically hydrolyzed. Surprisingly, CaLB also proved to be the catalyst of choice for this latter step. The heteroatom in the acyl donor profoundly influences both the enzymatic acylation and deacylation; the O-substituted reagents performed best with regard to enantioselectivity as well as reaction rate in synthesis and hydrolysis.

Keywords: amines; *Candida antarctica* lipase B; enantioselective acylation; enzymatic deacylation; enzyme catalysis

Introduction

The lipase-catalyzed enantioselective acylation of chiral amines is at the basis of a highly efficient resolution process that is now performed at a multi-thousand ton per year scale.[1,2] The subsequent (chemical) deacylation step is much less developed and requires strong base at elevated temperatures,[2,3] because of the thermodynamic stability of the amide bond. Such harsh reaction conditions are not compatible with sensitive functional groups; moreover, they give rise to an undesirable salt waste stream. A completely enzymatic procedure, employing enzymes in both acylation and deacylation steps (see Figure 1) would be preferable. Enzymatic deacylation was, until recently, not practical due to the low reactivity and modest solubility of amides and a lack of suitable amidases. Amide hydrolysis in the presence of *Candida antarctica* lipase B (CaLB), for example, was slow even with large amounts of enzyme.[4] We recently reported that hydrolytic cleavage of the (*R*)-phenylglycinyl group readily takes place in the presence of the penicillin acylase from *Alcaligenes faecalis*.[5] The propensity of the phenylglycinyl group to hydrolytic cleavage has been ascribed to the zwitterionic nature of the phenylglycine hydrolysis product, which renders the hydrolysis energetically favourable.

Entirely enzymatic amine resolution procedures employing acyl reagents based on (*R*)-phenylglycine derivatives have indeed been reported[5,6] but the methodology suffers from the drawback that the recycling of the chiral acyl donor is by no means trivial. We surmised that a non-chiral amino acid would be easier to recycle[7] and have now investigated the suitability of resolution reagents based on *N*-methyl- and

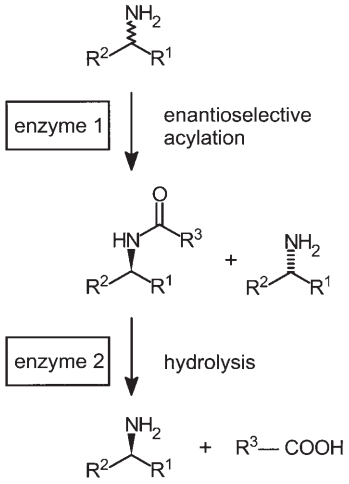

Figure 1. General scheme of fully enzymatic amine resolution.

N-phenylglycine. For comparison, we have also studied acyl donors bearing ether, thioether and methylene moieties in the β-position (see Figure 2).

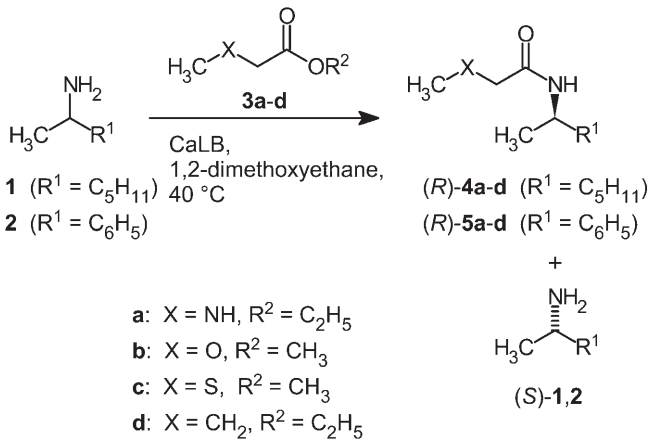

Figure 2. CaLB-catalyzed amine resolution with acyl reagents CH_3–X–$CH_2CO_2R^2$.

The acylation biocatalyst that we employed was CaLB, which has an excellent record as amine resolution catalyst[8,9] and readily tolerates non-natural reactants and reaction conditions.[10] The funnel-like active site of CaLB is known to be sterically restricted;[11] hence, it may be expected that the substituent in the acyl donor, methyl or phenyl, will affect the enantiorecognition.[12,13] Selecting a biocatalyst for the hydrolysis step proved to be much less straightforward, due to the strict selectivity of penicillin acylase,[14] as well as other amide-hydrolysing enzymes. We will describe how this problem was satisfactorily solved eventually.

Results and Discussion

Amine Acylation

We selected 2-heptylamine (**1**) and 1-phenylethylamine (**2**) as suitable models for aliphatic and arylalkyl amines. These were subjected to enantioselective acylation by acyl donor reagents with the general formula CH_3–X–$CH_2CO_2R^2$ (R^2 = methyl or ethyl; X = NH, O, S or CH_2, **3a–d**, see Figure 2) in the presence of Novozyme 435 (immobilized CaLB). All reactions were performed with strict exclusion of water; zeolite KA and CaA were added to absorb any traces of moisture as well as the liberated alcohol.

The nature of the moiety in the β position profoundly affected the course of the acylation, as would be expected.[15,16] The acylation rate increased in the order X = CH_2 < NH < S < O, as is observed, for example, in the resolution of **2** (Figure 3), which reconfirms the accelerating effect of a β-oxygen atom in the acyl donor.[15] This rate increase has originally been attributed to an inductive effect of the β-oxygen atom,[15] but Cammenberg et al. recently concluded that it should rather be ascribed to hydrogen bonding of the amino group in the nucleophile to the acyl-chain oxygen.[16] The lower reactivity of the S-substituted acyl donor **3c**, compared with **3b**, supports the hydrogen bond hypothesis as S is a weaker H-bond acceptor than O.[17] It also refutes the electronegativity hypothesis, which would predict **3c** to be more reactive than **3b** because S is slightly more electronegative than O.[18]

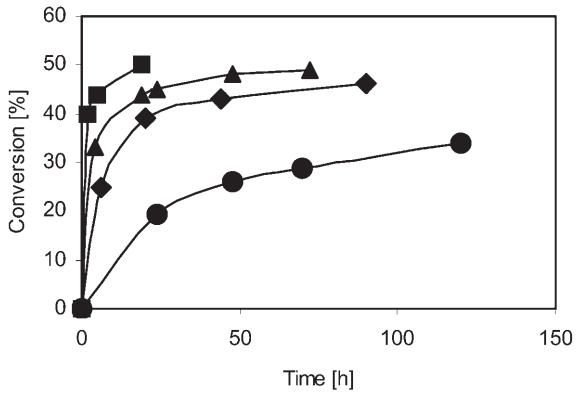

Figure 3. Effects of the acyl donor on the acylation of **2**; (◆) **3a**, X = NH; (■), **3b**, X = O); (▲), **3c**, X = S; (●), **3d**, X = CH_2.

With X = NH (**3a**) the issue becomes confused, however, as the accelerating effect is much less than that of oxygen or sulfur, although nitrogen is a much stronger H-bond acceptor than oxygen, as judged from solvatochromic effects[17] and Kamlet–Taft β parameters.[19] The electronegativity hypothesis, on the other hand, would predict **3a** to react slower than **3d**, because nitrogen is electron-releasing relative to carbon.[18] The subject obviously requires further study but it would seem, on the basis of the present results, that the overall effects of β-hetero atoms cannot be attributed to one single cause but rather are a combination of H-bond formation, inductive effects and, presumably, steric interactions.

Good to excellent enantioselectivities were obtained with all acyl donors. Amine **2** was resolved with an enantiomeric ratio (E) > 100 with all acyl donors, while with **1** E ranged from 51 (X = O) to > 100 (X = CH_2). Remarkably, the enantiomeric ratio decreased in the order X = CH_2 > NH > S > O with this latter amine.

Kinetic resolution using the sterically more demanding acyl donors **6a–d**, with the general formula C_6H_5–X–$CH_2CO_2R^2$ (X = NH, O, S, CH_2; R^2 = CH_3, C_2H_5; see Figure 4) was investigated to assess any steric effects of the donor. These acyl reagents were much less active in the acylation of **1** and **2** than their

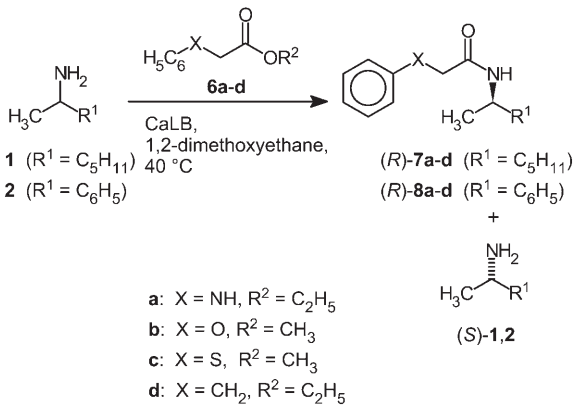

Figure 4. CaLB-catalyzed resolution with acyl reagents C_6H_5–X–$CH_2CO_2R^2$.

methyl counterparts, which could be explained by steric hindrance and/or their lower hydrogen bond-accepting capability due to the electron-withdrawing phenyl ring.[17] Acyl donor **6b** (X = O, see Table 2) was an exception as it reacted hardly slower than **3b**. It is worth noting here that a phenyl-substituted oxygen is a much weaker hydrogen-bond acceptor than a methyl-substituted one but also much more electron-withdrawing.[18] Finally, it is remarkable that the amino-substituted donor **6a** was much less active than the isomeric (*R*)-phenylglycine esters[6] under comparable conditions, contrary to what would be expected on account of branching effects.

The enantiomer discrimination by **6** was unsatisfactory in general, with the exception of oxygen-substituted **6b**, which gave excellent results. In conclusion, so far, the acyl donors of choice with regards to rate and enantioselectivity are the O-substituted acyl donors **3b** and **6b**.

Enzymatic Deacylation

Efficient enzymatic deacylation of the enantiomerically enriched amides **4**, **5**, **7**, and **8** (see Figure 5) proved to be far from trivial. Hydrolysis in the presence of *A. faecalis* penicillin G acylase, the favoured catalyst for removing the (*R*)-phenylglycyl group,[6] was quite sluggish (<10% conversion in 4 d), as would be expected in view of the enzyme's specificity for phenylacetic acid derivatives. Other amide hydrolyzing enzymes (thermolysin, amino acylase I from *Aspergillus melleus* or porcine kidney) also failed to hydrolyze these amides at an appreciable rate.

Table 1. Kinetic resolution[a] of **1** and **2** with acyl donors CH_3–X–$CH_2CO_2R^2$.

Amine	Acyl donor	X	Amide	v_{init} [%][b]	Time [h]	Conv. [%]	ee_{amine} [%]	*E*
1	**3a**	NH	**4a**	100	63	51	97	94
2	**3a**	NH	**5a**	54	128	45	80	>99
1	**3b**	O	**4b**	>390	5	50	91	51
2	**3b**	O	**5b**	>350	19	50	99	>99
1	**3c**	S	**4c**	167	24	50	91	80
2	**3c**	S	**5c**	141	48	48	91	>99
1	**3d**	CH_2	**4d**	11	216	42	76	>99
2	**3d**	CH_2	**5d**	6	216	40	73	>99

[a] *Reaction conditions:* amine (5 mmol), acyl donor (3 mmol), Novozym 435 (100 mg) in 1,2-dimethoxyethane (5 mL), zeolite KA and CaA (150 mg each) at 40 °C.
[b] Initial rate (conv./time) relative to the acylation of **1** by **3a**.

Table 2. Kinetic resolution[a] of **1** and **2** with acyl donors C_6H_5-X-$CH_2CO_2R^2$.

Amine	Acyl donor	X	Amide	v_{init} [%][b]	Time [h]	Conv. [%]	ee_{amine} [%]	*E*
1	**6a**	NH	**7a**	16	216	48	63	11
2	**6a**	NH	**8a**	12	118	51	54	12
1	**6b**	O	**7b**	>120	7	50	99	>99
2	**6b**	O	**8b**	>120	24	50	98	>99
1	**6c**	S	**7c**	22	200	48	68	14
2	**6c**	S	**8c**	19	200	43	68	23
1	**6d**	CH_2	**7d**	3	312	8	n.d.	n.d.
2	**6d**	CH_2	**8d**	1	312	7	n.d.	n.d.

[a] *Reaction conditions:* amine (5 mmol), acyl donor (3 mmol), Novozym 435 (100 mg) in 1,2-dimethoxyethane (5 mL), zeolite KA and CaA (150 mg each) at 40 °C.
[b] Initial rate (conv./time) relative to the acylation of **1** by **3a**.

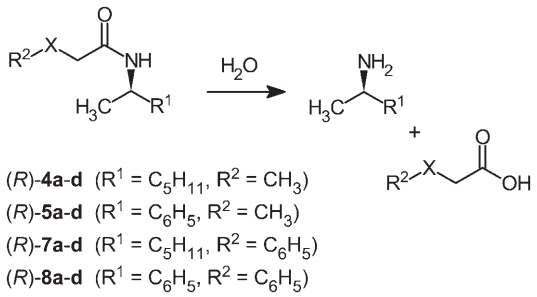

Figure 5. Hydrolysis of the enantiomerically pure amides **4**, **5**, **7** and **8** (full structures are given in Figure 2 and Figure 4).

Table 3. Deacylation of amides **4**, **5**, **7** and **8** in the presence of CaLB.[a]

Amide	X	R^1	Time [h]	Conv. [%]
(*R*)-**4a**	NH	C_5H_{11}	147	24
(*R*)-**5a**	NH	C_6H_5	120	14
(*R*)-**4b**	O	C_5H_{11}	120	74
(*R*)-**5b**	O	C_6H_5	120	100
(*R*)-**4c**	S	C_5H_{11}	120	36
(*R*)-**5c**	S	C_6H_5	120	85
(*R*)-**7a**	NH	C_5H_{11}	90	30
(*R*)-**8a**	NH	C_6H_5	120	3
(*R*)-**7b**	O	C_5H_{11}	120	21
(*R*)-**8b**	O	C_6H_5	120	42
(*R*)-**7c**	S	C_5H_{11}	120	50
(*R*)-**8c**	S	C_6H_5	144	39

[a] *Reaction conditions:* amide (5 mg, 20–27 μmol) in 0.1 M phosphate buffer pH 7.0 (3.5 mL), Novozym 435 (30 mg, 135 LU), 70 °C.

Persistent literature reports on amidase activity of CaLB[4,20–23] prompted us to apply this latter enzyme to the hydrolysis of these amides. The highest rate was observed at 70 °C with Novozym 435 but even then **4d**, **5d**, **7d** and **8d**, as well as **8a**, reacted with extreme sluggishness. All of the other amides could be hydrolyzed at an appreciable rate (see Table 3).

A considerable effect of the molecule size on the reaction rate was observed as the phenyl-substituted amides **7** and **8** were hydrolyzed much slower than the amides derived from $CH_3{-}X{-}CH_2CO_2H$ (**4** and **5**). A somewhat similar chain length effect was recently reported by Torres-Gavilán et al.[24] The hydrolysis rate of **4** and **5** increased in the order of X = NH < S < O, as was observed in the acylation step, strongly indicating that similar substituent effects are apparent here. Amides bearing an oxygen (**4b** and **5b**) were hydrolyzed the fastest, but still 700 times slower than synthesis, resulting in 100% conversion of **5b** within 120 h.

In conclusion, it would seem that the hydrogen bond-accepting and electron demanding character of the oxygen in **4b** and **5b** has a much more profound effect on the hydrolysis than the formation of a zwitterionic hydrolysis product from the N-analogues **4a** and **5a** (Table 4). Hence, acyl donors with X = O are the reactants of choice with regards to acylation as well as deacylation.

Table 4. Preparative-scale hydrolysis of **5b** and **5c**.[a]

Amide	Time [h]	Conversion [%]	
		40 °C	60 °C
(*R*)-**5b**	48	100	87
	72	100	90
(*R*)-**5c**	48	94	48
	72	100	67

[a] *Reaction conditions:* amide (0.3 mmol) in 0.1 M phosphate buffer pH 7.0 (3 mL), CaLB CLEA (90 mg, 600 LU) at 40 °C.

Encouraged by these results, we next performed the hydrolysis of **5b** and **5c** at a synthetically relevant concentration. A cross-linked enzyme aggregate (CLEA) of CaLB[25] was employed as the biocatalyst, for easy recovering and recycling. At 60 °C the reaction unexpectedly came to a standstill at incomplete conversion. It would seem that thermal deactivation of the CLEA is significant at 60 °C, which is presumably caused by dissociation of the cross-links as CaLB is intrinsically stable up to 70 °C. Complete hydrolysis could be accomplished, in contrast, at 40 °C. The rather high catalyst usage is still an obstacle to practical application, but it is relevant to note here that the biocatalyst is recyclable. Presumably, improved hydrolysis rates could result from appropriate mutagenesis.[26]

Hydrolysis of racemic **5b** in the presence of CaLB showed negligible enantiodiscrimination, which indicates that the rate-determining steps in synthesis and hydrolysis are different,[27] and the issue was not pursued any further. Enantiomerically pure (*R*)-**1** and (*R*)-**2** were obtained, nevertheless, on account of the good enantioselectivities in the acylations.

Furthermore, the hydrolysis of the phenoxy-substituted amides (**7b** and **8b**) using penicillin V acylase was investigated, as this latter enzyme is known to have a high affinity for the phenoxyacetyl moiety.[28] From tests with three penicillin V acylases from different sources and formulations (Table 5) we found Semacylase (a commercial preparation from Novozymes) to be the best catalyst, as almost 100% amide was hydrolyzed within 120 h.

Conclusions

Proof of principle of fully enzymatic resolution of two chiral amines, 2-heptylamine and 2-phenylethylamine,

Table 5. Hydrolysis of **7b** and **8b** catalyzed by penicillin V acylase.[a]

Amide	Pen V acylase	PVU[b]	Time [h]	Conv. [%]
(*R*)-**7b**	Semacylase	6	120	98
	Cryptocossus sp.	4	120	17
	Biocat V	6	120	14
(*R*)-**8b**	Semacylase	6	75	95
	Cryptocossus sp.	4	120	21
	Biocat V	6	120	52

[a] *Reaction conditions:* amide (20 μmol) in 0.1 M phosphate buffer pH 7.0 (3.5 mL), penicillin V acylase at 40 °C.
[b] Penicillin V acylase units, see Experimental Section.

has been delivered, using acyl donor reagents with NH, O and S moieties in the β-position and CaLB as acylation and deacylation catalyst. The preferred acyl reagent was methyl 2-methoxyacetate, on account of its high rate in acylation and deacylation. Efficient resolution of these amines was also accomplished upon acylation with methyl 2-phenoxyacetate in the presence of CaLB and deacylation catalyzed by penicillin V acylase.

Experimental Section

Materials

Novozym 435 (immobilized *Candida antarctica* lipase B, CaLB, 4500 LU g^{-1}) was kindly donated by Novozymes (Bagsvaerd, Denmark). A cross-linked enzyme aggregate (CLEA) of CaLB was donated by CLEA Technologies (Delft, The Netherlands). Penicillin G acylase from *E. coli* (PGA-300) was obtained from Roche Diagnostics (Penzberg, Germany) as a gift. Penicillin G acylase from *Alcaligenes faecalis* was obtained from the fermentation of recombinant *E. coli* cells.[29] Semacylase (190 PVU g^{-1}) was kindly donated by Novozymes (Bagsværd, Denmark), Biocat V (190 PVU g^{-1}) was purchased from Biochemie, penicillin V acylase from *Cryptococcus* sp. (126 PVU g^{-1}) was received from the Academy of Sciences of the Czech Republic (Prague, The Czech Republic) as a gift. All other compounds were purchased from ACROS and Sigma or synthesized as described below.

Enzyme Assays

One unit (LU) of lipase will liberate one μmol per min of butyric acid from 8% tributyrin at pH 7.5 and 40 °C. One unit (PVU) of penicillin V acylase will liberate one μmol per min of phenoxyacetic acid from 2% penicillin V at pH 8 and 40 °C.

Enzymatic Reactions

Lipase-catalyzed acylation of amines: Acyl donor (3 mmol) and amine (5 mmol) were dissolved in 5 mL of 1,2-dimethoxyethane. Novozym 435 (100 mg) and zeolite NaA and CaA (powder, 150 mg each) and 1,3-dimethoxybenzene (150 μL, internal standard) were added. The reaction mixture was incubated at 40 °C; samples (100 μL) were withdrawn at regular intervals to monitor the reaction over time. Initial rates (conversion/time) relative to the acylation of **1** by **3a** are given in Table 1 and Table 2.

The reaction was stopped at 50% amine conversion by removal of the enzyme and the molecular sieves. The unreacted amine was isolated by extraction with dilute sodium hydroxide solution (pH 8.5). The organic fraction was concentrated under vacuum and pure amide was obtained by recrystallization from *n*-hexane.

Enzymatic hydrolysis of the amides: Preliminary hydrolysis experiments in the presence of CaLB (see Table 3) were performed with amide (5 mg, 20–27 μmol) in 3.5 mL 0.1 M phosphate buffer pH 7, in the presence of immobilized CaLB (Novozym 435, 30 mg, 135 LU) at 70 °C. The preparative experiments were performed with **5b** or **5c** (0.1 M) and CaLB CLEA (90 mg, 600 LU) in 3 mL 0.1 M phosphate buffer pH 7 at 40 °C.

Amides **7b** and **8b** (5 mg, 20 μmol) were subjected to hydrolysis in the presence of penicillin acylase V (30 mg) in 0.1 M phosphate buffer pH 7 (3.5 mL) at 40 °C.

Synthesis of Acyl Donors

Phenylpropionate ethyl ester: A solution of 3-phenylpropionyl chloride (10 g, 60 mmol) in 50 mL of absolute ethanol and 4.6 g (60 mmol) of pyridine was stirred overnight at room temperature. The excess of ethanol was removed under vacuum. Ethyl acetate (20 mL) was added and the resulting solution was washed with cold water. The organic layer was dried over Na_2SO_4. Ethyl 3-phenylpropionate was obtained as a colorless oil; yield: 6.136 g (57.4%); GC-MS: MW = 178; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.21$ (t, 3H, OCH_2CH_3), 2.62 (t, 2H, CH_2CH_2CO), 2.93 (t, 2H, CCH_2CH_2), 4.12 (q, 2H, OCH_2CH_3), 7.29–7.15 (m, 5H, aromatic).

Phenylthioacetate methyl ester: Thiophenol (10 g, 0.09 mol) was dissolved in a mixture of 50 mL dry methanol and 10 mL solution of sodium methoxide in methanol [30%, (wt)]. Methyl chloroacetate (16.6 g, 0.15 mol) was added slowly and the resulting mixture was stirred overnight at room temperature. The excess of methanol was removed under vacuum and dichloromethane (50 mL) was added. The resulting solution was washed with cold water and dried over Na_2SO_4. Kugelrohr distillation afforded an unpleasantly smelling oil; yield: 7.5 g; GC-MS: MW = 182; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.64$ (s, 3H, OCH_3), 3.7 (s, 2H, SCH_2CO), 7.42–7.21 (m, 5H, aromatic).

Analytical Methods

The progress of the acylation reactions was monitored by gas chromatography using a CP Sil 5 CB column (50 m × 0.53 mm) at programmed temperature from 50–250 °C. The chiral analysis of the unconverted amine was performed by derivatization with trifluoroacetic anhydride at 80 °C (2-heptylamine) or 100 °C (1-phenylethylamine) followed by chiral GC on an Astec β-PH GC column (0.35 mm × 30 m).

The hydrolysis of **5** and **8** was monitored by HPLC on a Waters 8 × 100 mm, 6 μ Symmetry C18 cartridge contained in a Waters RCM 8 × 10 compression module, eluant

MeOH-H_2O [65:35 (v/v), SDS 1 gL^{-1} at pH 3.5 (KH_2PO_4 1 gL^{-1}], 1 mLmin^{-1}, UV detection at 215 nm.

The analysis of the aliphatic amines liberated in the hydrolysis of **4** and **7** was performed by allowing a precise amount of reaction mixture and a solution of 2-pentylamine to react for 1 min with a commercial solution of *o*-phthaldialdehyde in the presence of 2-mercaptoethanol (1 mL). Formation of **1** was quantified by HPLC on a Waters 8× 100 mm, 6 μ Symmetry C18 cartridge contained in a Waters RCM 8×10 compression module, eluant MeOH-H_2O 80:20 (v/v) at 1 mLmin^{-1}, UV detection at 340 nm.

The enantiomeric excess of **1** was measured by extracting the amine at pH 11 into hexane, derivatization with trifluoroacetic anhydride and GC on a Chiraldex 0.25 mm× 30 m β-PH GC column. The enantiomeric purity of **2** was determined by chiral HPLC on a Daicel 4×150 mm, 5 μm Crownpack CR+ column, eluant aqueous 0.1 M $HClO_4$, pH 1.5 at 0.6 mLmin^{-1}, UV detection at 215 nm.

Acknowledgements

Generous donations of enzymes by Novozymes, CLEA Technologies, Roche Diagnostics and the Academy of Sciences of The Czech Republic are gratefully acknowledged. Thanks are due to Prof. Dr. Kasche (Technische Universität Harburg, Germany) for help with the recombinant expression of the penicillin acylase from A. faecalis. Experimental assistance by Dr. M. H. A. Janssen (CLEA Technologies) is gratefully acknowledged. Dr. Ismail wishes to thank the Gadjah Mada University (Yogyakarta, Indonesia) for leave of absence. Financial support for H. Ismail by the QUE Project of the Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia and by the Faculty for the Future Program of the Schlumberger Foundation is gratefully acknowledged.

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