Pergamon

Tetrahedron: *Asymmetry* 13 (2002) 2389–2397

TETRAHEDRON: *ASYMMETRY*

Enantioselective lipase-catalysed kinetic resolution of acyloxymethyl and ethoxycarbonylmethyl esters of 1,4-dihydroisonicotinic acid derivatives

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Received 25 September 2002; accepted 14 October 2002

Abstract—The lipase-catalysed kinetic resolution of four derivatives of 4-[(acyloxy)methyl] and 4-ethoxycarbonylmethyl 3-methyl 5-propyl 2,6-dimethyl-1,4-dihydro-3,4,5-pyridinetricarboxylates has been investigated. Whereas the enantioselectivity of lipases towards the acyloxymethyl derivatives was rather low, the *Candida antarctica* lipase B (Novozym 435®, CAL-B)-catalysed hydrolysis of the ethoxycarbonylmethyl ester of 1,4-dihydroisonicotinic acid was enantioselective. In water-saturated diisopropyl ether at 45°C the enantioselectivity of CAL-B toward the ethoxycarbonylmethyl ester was rather moderate ($E=13.8$), but it was enhanced at rt and +4°C ($E=21.5$ and $E=28.9$, respectively). A high enantiomeric ratio ($E=45.3$) was reached at subzero temperatures, although at the expense of the reaction rate.

1. Introduction

1,4-Dihydroisonicotinic acid derivatives are important precursors for the synthesis of a novel class of amino acid-containing biologically active 1,4-dihydropyridines (1,4-DHPs). The disodium salt of 2-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido)-glutaric acid (glutapyrone) (Fig. 1), unlike classical 1,4-DHPs, lacks calcium antagonistic activity and possesses an unusually broad spectrum of biological activities at low concentrations such as neuromodulatory and neuroregulatory action. It is an anti-convulsant, stress-protective, anti-arrhythmic, cognition and memory-enhancing drug with long-term activity.[1–4]

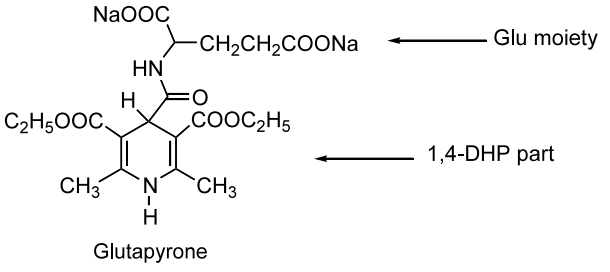

Figure 1.

The synthesis of a number of asymmetric structural analogues of nifedipine led to the development of new generations of highly selective calcium antagonist preparations.[5] Enantiomers of unsymmetrical 1,4-DHP usually differ in their biological activities[6–8] and could even have an opposite action profile.[9] Yamanouchi (Japan) was the first to introduce the most potent diastereomer of barnidipine[10,11] in Japan in 1992 and in the Netherlands in 2000, however, other calcium antagonists are marketed as racemic mixtures to this day.

Whereas the synthesis and pharmacology of enantiomerically pure 4-aryl-1,4-DHPs as novel calcium antagonists have been widely studied,[12,13] 1,4-dihydroisonicotinic acid derivatives were not synthesised until recently and even less information is available on their enantiomerically pure derivatives. The synthesis of unsymmetrical 1,4-dihydroisonicotinic acid derivatives in enantiopure form is highly necessary for the elucidation of their mechanism of action which should result in more efficient and selective preparations via rational drug design.

The stereoselective syntheses of 4-alkyl-, aryl- or pyridyl-1,4-DHP-3,5-dicarboxylates via enzyme-catalysed transformations of activated esters of 1,4-dihydro-β-carboxylic acids has been performed by several research groups.[12–16] Disregarding the fact that the reaction centre (the place of enzyme attack) was quite remote from the stereogenic carbon atom at posi-

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0957-4166/02/$ - see front matter
PII: S0957-4166(02)00655-9

tion 4, in many cases it was possible to obtain the reaction products with excellent enantioselectivities.[12,13,17] For acyloxymethyl esters of 1,4-DHP-3,5-dicarboxylates, the enantioselectivity of *Candida rugosa* lipase (CRL) and lipase AH increased together with the length or branching of the acyl chain, whereas the reverse effect was described for lipase PS in water-saturated diisopropyl ether (IPE).[16,18] Generally, CRL and *C. antarctica* lipase B (CAL-B) are very efficient catalysts and are widely used in practice. Initially, CAL-B has been designed as an additive to detergents due to its extreme resistance against deactivation by oxidising agents and temperature.[19] The usefulness of CAL-B was reported at subzero temperatures[20] as well as at 90°C.[21]

Herein, we report the synthesis of enantiomerically enriched derivatives of 1,4-dihydroisonicotinic acid as key intermediates for the synthesis of unsymmetrical potentially biologically active compounds, via lipase-catalysed kinetic resolution of acyloxymethyl and ethoxycarbonylmethyl esters of 1,4-dihydroisonicotinic acid.

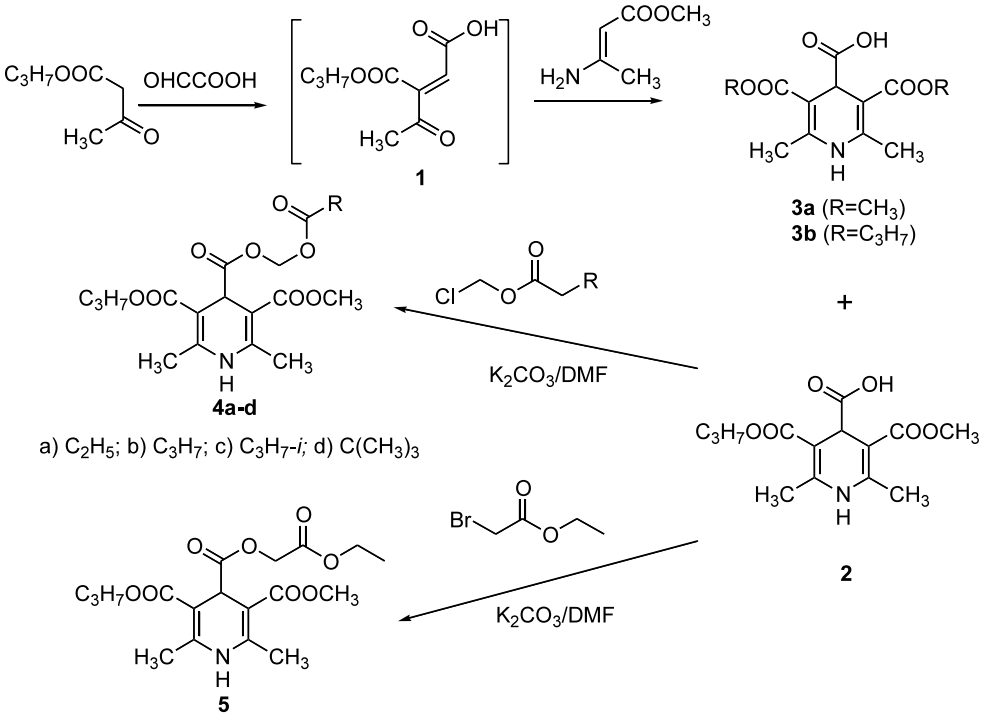
2. Results and discussion

2.1. Synthesis of 1,4-dihydroisonicotinic acid 2 and the enzymatically labile esters 4a–d and 5

The synthesis of unsymmetrical derivatives of 1,4-dihydroisonicotinic acid **2** is not very efficient. Glyoxylic acid readily forms bis-1,3-dicarbonyl derivatives upon reaction with acetoacetates, leading to symmetric 1,4-dihydropyridines **3**.[22] In the case of the three component synthesis (glyoxylic acid, alkyl acetoacetate, alkyl β-aminocrotonate) a complicated mixture is formed, wherein symmetric products prevail. Separation of mixtures of symmetrical and unsymmetrical derivatives of 1,4-dihydroisonicotinic acid (**2** and **3a**,**b**) by chromatography was found to be problematic. As a result of extensive studies, a method for the synthesis of unsymmetrical 1,4-dihydroisonicotinic acid **2** was elaborated (Scheme 1).

The formation of the undesired symmetrical by-product **3a** is due to coupling of 3-aminocrotonate to unreacted glyoxylic acid. Therefore, to prevent formation of **3a**, propyl acetoacetate was first coupled to glyoxylic acid in a 3:1 ratio, forming in situ intermediate **1**. The treatment of the reaction mixture with hydrochloric acid and the subsequent work-up with acetic anhydride markedly reduces the formation of the second by-product **3b**. The condensation of intermediate **1** with a limited amount of methyl-3-aminocrotonate gives 1,4-dihydroisonicotinic acid **2** as the major reaction product in 13% overall yield.

A chemoenzymatic approach is used for the resolution of 1,4-dihydroisonicotinic acids in the current studies. As with 3,5-dialkyl esters of 1,4-DHPs,[23] the methyl and ethyl esters of 1,4-dihydroisonicotinic acid were



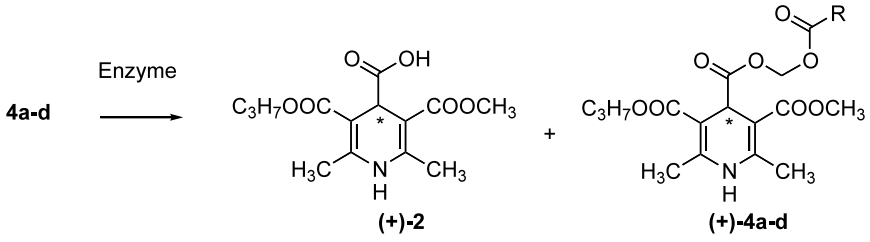
Scheme 1.

stable towards treatment with a number of commercially available hydrolases. 1,4-Dihydroisonicotinic acid **2** was adapted to make it a substrate for hydrolases by the attachment of a suitable spacer group. Since there is no literature precedent for enzymatic transformations of these compounds, a primary screening of enzymes and substrates had to be performed. For this purpose, a set of acyloxymethyl esters **4a–d** was prepared by coupling of 1,4-dihydroisonicotinic acid **2** with the corresponding acyloxymethyl chloride by reported methods[16] in 20–72% yields (Scheme 1). The corresponding ethoxycarbonylmethyl ester **5** of 1,4-dihydroisonicotinic acid **2** was synthesised in 83% yield using ethyl bromoacetate (Scheme 1).

2.2. Kinetic resolution of acyloxymethyl esters of 1,4-dihydroisonicotinic acid, 4a–d

The lipase-catalysed hydrolysis of compounds **4a–d**, which differ in size and branching of acyloxymethyl ester group, has been investigated. Several commercially available lipases exhibited hydrolytic activity toward these substrates (Scheme 2). The lipases AH, PS and Chirazym L-2 gave low E values (<2) for all derivatives of **4**. Higher selectivities were obtained for CRL and CAL-B (i.e. its immobilised form Novozym 435®). The results of the screening in organic solvents and aqueous media are listed in Table 1. CAL-B possessed an opposite stereopreference toward substrates **4a–d** compared to other tested enzymes.

Since the enantioselectivity of the enzymes was still too low for practical application, we decided to investigate the temperature dependence of the reaction. Thus, the CRL and Novozym 435® catalysed conversions of **4b** in water saturated IPE in a temperature range from −18 to +45°C were investigated. The enantioselectivities of both enzymes were rather low and slightly dependent on temperature. The enantioselectivity of CRL increased with the raise of temperature (entries 7–9),



Scheme 2.

Table 1. Lipase-catalysed hydrolysis of acyloxymethyl esters of 1,4-dihydroisonicotinic acid **4a–d**[a]

Entry	Substrate	Lipase	Reaction medium[a]	Enzyme[d] (mg)	T (°C)	Conversion			E value[f]	Major enantiomer of **2**[e]
						Time (h)	%	e.e.$_p$ (%)		
1	**4a**	Novozym-435®	IPE/H_2O	2	Rt	3	55	52	5.0±0.4	1 (−)
2	**4a**	Novozym-435®	*t*-BuOMe/H_2O	2	Rt	3	35	57	5.8±0.7	1 (−)
3	**4a**	*C. rugosa*	IPE/H_2O	2	Rt	3	19	41	1.6±0.05	2 (+)
4	**4a**	*C. rugosa*	Buffer A[b]	1	Rt	3.2	40	61	5.0±0.4	2 (+)
5	**4a**	*C. rugosa*	Buffer B[c]	0.2	Rt	17	38	68	7.9±0.2	2 (+)
6	**4b**	*C. rugosa*	Buffer B	0.2	Rt	18	48	66	9.0±0.8	2 (+)
7	**4b**	*C. rugosa*	IPE/H_2O	4	45	5	58	35	3.2±0.3	2 (+)
8	**4b**	*C. rugosa*	IPE/H_2O	4	4	22	46	35	2.7±0.4	2 (+)
9	**4b**	*C. rugosa*	IPE/H_2O	4	−18	23	7	38	2.3±0.1	2 (+)
10	**4c**	*C. rugosa*	Buffer A	1	Rt	3	40	63	7.0±0.7	2 (+)
11	**4c**	*C. rugosa*	Buffer B	0.5	Rt	6.5	52	61	8.0±0.7 $E_{tot}=7.9$	2 (+)
12	**4d**	*C. rugosa*	Buffer B	1	Rt	12	0	–	–	–
13	**4d**	*C. rugosa*	Buffer B	1	45	120	12	9	1.2±0.03	2 (+)
14	**4d**	*C. rugosa*	IPE/H_2O	4	45	104	21	51	3.7±0.2	2 (+)

[a] Typical reaction conditions: substrate **4a–d** (0.01 mmol) was dissolved in either phosphate buffer A or phosphate buffer B (8 mL), water-saturated IPE (0.8 mL) or *t*-BuOMe (0.8 mL).
[b] Buffer A: 10% solution of acetonitrile in 20 mM K-phosphate buffer, pH 7.5.
[c] Buffer B: 15% solution of acetonitrile in 20 mM K-phosphate buffer, pH 7.5.
[d] Amount of enzyme means mg solid.
[e] The number means the order of the elution of enantiomers of **2** on the β-cyclodextrin chiral column, for the details see Section 4.
[f] $E=E_p=\{\ln[1-c(1+e.e._p)]\}/[\ln(1-c(1-e.e._p)]$; $E_{tot}=\{\ln[e.e._p(1-e.e._s)]/[e.e._p+e.e._s]\}/\{\ln[e.e._p(1+e.e._s)]/[e.e._p+e.e._s]\}$.

whereas the enantioselectivity of Novozym 435® dropped with increase of temperature from $E=2.3$ until $E=1.7$.

CRL showed better enantioselectivity toward acyloxymethyl esters **4a**–**c** in aqueous medium than the other enzymes tested, however, the E value did not exceed 9 (entries 5, 6, 10 and 11). Increasing the content of acetonitrile from 10 to 15% in phosphate buffer in order to reach better solubility of the substrates **4b**,**d** also gave better enantioselectivity of CRL (entries 4, 5 and 10, 11). The use of other co-solvents, such as *t*-BuOH and DMSO, to the phosphate buffer was not successful, as CRL was absolutely not selective towards substrate **4c** under the given reaction conditions. The influence of the bulkiness of the acyloxymethyl group on the enantioselectivity of CRL was not so clear in the examples **4a**–**d** and in all cases the enantioselectivity was moderate. However, the bulkiness of the acyloxymethyl group did affect the reactivity of the enzyme. The bulky *tert*-butyl substituted acyloxymethyl ester **4b** was not reactive at rt and became a substrate for enzymes only at higher temperatures (entries 12–14; Table 1), similarly to the corresponding bis[(pivaloyloxy)methyl] 4-[2-(difluoromethoxy)phenyl]-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate.[16]

Isobutyryloxymethyl derivative **4c** was used for the preparative synthesis of both enantiomers of 1,4-dihydroisonicotinic acid **2** using CRL in phosphate buffer pH 7.5, modified with 15% of acetonitrile. The CRL-mediated kinetic resolution of **4c** was carried out for 6.5 h with HPLC control until 50% conversion was reached. The enantiomeric excess of the reaction product (+)-**2** was 61% and for the remaining (+)-**4c**, e.e. 65%, the E value of the conversion was 8.0.

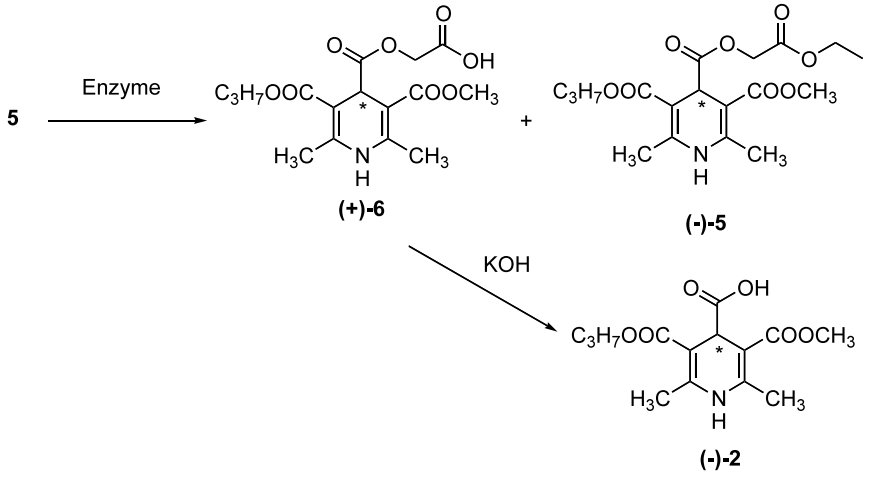
2.3. Kinetic resolution of ethoxycarbonylmethyl ester of 1,4-dihydroisonicotinic acid, 5

Preliminary screening tests revealed that lipases CRL, CAL-B, AH, *Rhizomucor miehei*, protease P6 and acylase 30,000 were capable of hydrolysing the ethoxycarbonylmethyl ester of 1,4-dihydroisonicotinic acid **5** (Scheme 3, for details see Table 2). As was observed for the lipase-catalysed hydrolysis of bis(ethoxycarbonylmethyl) substituted 4-substituted 1,4-dihydropyridine-3,5-dicarboxylates,[24] only CAL-B catalysed the hydrolysis of **5** with reasonable enantioselectivity ($E=$ 8.0 at 45°C and $E=13.7$ at rt) at the 'outer' ester group of the ethoxycarbonylmethyl ester at the 4-position. However, it is worth noting that a trace of **2** was detected. The transition from phosphate buffer pH 7.5 modified with acetonitrile to water-saturated IPE inclines CAL-B to hydrolyse **5** more stereoselectively (entries 4, 5 and 7, 8; Table 2). The effect of temperature on the CAL-B catalysed enantioselective hydrolysis was studied in detail (see Table 2). The reaction time became longer and the enantioselectivity became better at decreasing reaction temperature. At low temperatures a larger amount of enzyme was used to accelerate the reaction. The preparative experiments were carried out in water-saturated IPE. At 45°C the enantioselectivity of the relatively fast reaction was moderate ($E=$ 13.8), whereas acceptable enantioselectivity of enzyme was reached at rt ($E=21.5$), albeit at the expense of the reaction rate. The highest enantiomeric ratio ($E=45.3$) was reached at −12°C, however, after 2 months of incubation only 35% of the substrate was converted.

During the crystallisation of (+)-**6** with an e.e. of 75%, it was found that the e.e. of the crystals was much lower than the e.e. of the mother liquor.

It is possible to obtain both enantiomers of **2** from (+)-**6** and (−)-**5** by chemical hydrolysis in low yields. The reaction was not studied in detail, but the possible cause of the low yields is decarboxylation during the hydrolysis and work-up.[25] The complexity of the reaction mixture obtained also hampers the isolation of the product. The acid (+)-**6** with 85% e.e. gave (−)-**2** with 67% e.e.; the loss of the e.e. occurred during the crystallisation.

To estimate the enantiomeric ratio (E value)[26] of the enzymatic kinetic resolutions, the e.e.s of the products



Scheme 3.

Table 2. Enzyme-catalysed hydrolysis of ethoxycarbonylmethyl esters of 1,4-dihydroisonicotinic acid **5**[a]

Entry	Lipase	Reaction medium	Enzyme[b] (mg)	T (°C)	Conversion			E value[e]	Major enantiomer of **6**[d]
					Time	%	e.e.$_p$ (%)		
1	Protease P6	Buffer[a]	2	Rt	24 h	8	10	1.3 ± 0.04	**2** (−)
2	Acylase 30,000	Buffer[a]	2	Rt	24 h	5	0	1	–
3	CRL	Buffer[a]	2	Rt	24 h	5	0	1	–
4	Novozym 435®	Buffer[a]	7.5	45	28 h	49	62	8.0 ± 1.1	**1** (+)
5	Novozym 435®	Buffer[a]	7.5	Rt	28 h	33	79	13.7 ± 2.1	**1** (+)
6	CRL	IPE/H_2O	3.5	Rt	15 days	7	0	1	–
7	Novozym 435®	IPE/H_2O	7.5	45	46 h	47 calcd	75 (67[c])	14.0 ± 2.0 $E_{tot}=13.9$	**1** (+)
8	Novozym 435®	IPE/H_2O	7.5	Rt	27 days	51 calcd	79 (81[c])	23 ± 5.8 $E_{tot}=21.1$	**1** (+)
9	Novozym 435®	IPE/H_2O	4.5	4	15 days	46	87	28.9 ± 4.1	**1** (+)
10	Novozym 435®	IPE/H_2O	7.5	−12	63 days	35 calcd	93 (50[c])	$E_{tot}=45.3$	**1** (+)

[a] Typical reaction conditions: **5** (0.01 mmol) was dissolved in 15% solution of acetonitrile in 20 mM K-phosphate buffer, pH 7.5 (8 mL) or in water-saturated IPE (usually 0.5 mL, but for entries 8 and 10: 0.75 mL).
[b] Amount enzyme means mg solid.
[c] The e.e. of remaining substrate **5**.
[d] The number of the enantiomer means the order of the elution of enantiomers of **6** on a β-cyclodextrin chiral column, for details see Section 4.
[e] $E=E_p=\{\ln[1-c(1+\text{e.e.}_p)]\}/\{\ln[1-c(1-\text{e.e.}_p)]\}$; $E_{tot}=\{\ln[\text{e.e.}_p(1-\text{e.e.}_s)]/[\text{e.e.}_p+\text{e.e.}_s]\}/\{\ln[\text{e.e.}_p(1+\text{e.e.}_s)]/[\text{e.e.}_p+\text{e.e.}_s]\}$.

of reactions **2** and **6** were measured during the reaction. Enantiomeric ratios were calculated using the Chen equations[26,27] or the computer programs 'EIVFIT'[28] and 'Selectivity-Win-1.0'.[19] Monitoring the stereochemical course of the reactions in IPE was difficult, as the available normal-phase chiral columns were found to be not enantioselective toward the 1,4-dihydroisonicotinic acid derivatives **2** and **6**. Only a reversed-phase β-cyclodextrin chiral column was suitable for the analysis of 1,4-dihydroisonicotinic acids **2** and **6**, however, a trace of IPE or *t*-BuOMe dramatically decreased the separations on β-cyclodextrin CSP. On the other hand, preparation of samples for analysis without IPE, declined the accuracy of the measurement of the conversion, and curve deviations were significant. Reliable conclusions about the conversion and enantioselectivity of the enzymes in IPE should be based on comparison of the e.e.s of both the reaction product **2** or **6** and the remaining substrate **5**.

3. Conclusions

Two different strategies for lipase-catalysed resolution of unsymmetrical 1,4-dihydroisonicotinic acid derivatives have been developed. The first method consists of resolution of acyloxymethyl esters of 1,4-dihydroisonicotinic acid **4a**–**d**, the second is based upon the resolution of the ethoxycarbonylmethyl ester of 1,4-dihydroisonicotinic acid **5**. The lipase-catalysed kinetic resolutions of four acyloxymethyl derivatives **4a**–**d** have been studied with variation of the enzyme, the structure of the acyloxymethyl group and the reaction conditions. *C. rugosa* lipase showed better enantioselectivity towards acyloxymethyl esters **4a**–**c** than the other enzymes tested, however, the E value did not exceed 9 in aqueous medium. The influence of the steric bulk of the acyl group of the acyloxymethyl ester on the enantioselectivity of the enzymes was not so clear. *C. antarctica* lipase B-catalysed hydrolysis of the ethoxycarbonylmethyl ester of 1,4-dihydroisonicotinic acid **5** at the 'outer' ester group of the ethoxycarbonylmethyl substituent was enantioselective. The transition from aqueous medium to water-saturated IPE led to a better stereoselectivity of CAL-B in the hydrolysis of **5**. The reaction time was longer and the enantioselectivity was better at lower reaction temperature. In water-saturated diisopropyl ether at 45°C, the enantioselectivity of CAL-B toward the ethoxycarbonylmethyl ester was moderate ($E=13.8$), but was enhanced at rt and +4°C ($E=21.5$ and $E=28.9$, respectively). A high enantiomeric ratio ($E=45.3$) was obtained at sub-zero temperatures, although at the expense of the reaction rate. Research on the synthesis and enantioselective resolutions of other 1,4-dihydroisonicotinic acid derivatives is currently in progress and will be reported in due course.

4. Experimental

4.1. General

All reagents were purchased from Aldrich, Acros or Merck and used without further purification. HPLC grade solvents were from Labscan. Flash column chromatography was performed on Merck silica gel 60 (230–400 mesh or 70–230 mesh). Preparative TLC was performed on 20×20 cm silica gel TLC-PET F_{254} foils (Fluka). *C. rugosa* lipase (lipase (EC 3.1.1.3) Type VII from *C. rugosa*, activity 875 U/mg, contains lactose as an extender) was purchased from Sigma. Acylase 30,000 (*Aspergillus* sp., activity ≥30,000 U/g), Protease P6 (*Aspergillus melleus*, activity ≥60,000 U/g), Lipase AH (*Pseudomonas* sp., activity unknown), Lipase PS

(*Pseudomonas cepacia*, activity ≥30,000 U/g) and Lipase PS 800 (activity 839,000 U/g) were gifts from Amano Pharmaceutical Co., Ltd. (Japan). Immobilised *C. antarctica* lipase B (Novozym 435®, activity 5600 or 7200 P.L.U. (propyl laureate units)/g) was a gift from Novo Nordisk A/S (Bagsvaerd, Denmark). *C. antarctica* lipase fraction B (CHIRAZYME L-2, lyo., activity >173,000 U/g), *Rhizomucor miehei* lipase (CHIRAZYME L-9, c.-f, lyo., activity ≥15.2 U/g) were gifts from Boehringer-Mannheim (Mannheim, Germany). Enzymatic reactions were carried out in a New Brunswick Scientific Innova 4080 incubatory orbital shaker (250 rpm). ^{1}H NMR spectra were recorded on a Bruker AC-E 200 (200 MHz) spectrometer. ^{13}C NMR spectra were recorded on a Bruker AC-E 200 (50 MHz). Chemical shifts are reported in parts per million (ppm) relative to trimethylsilane (δ 0.00). Mass spectral data and accurate mass measurements were determined on a Finnigan MAT 95 mass spectrometer. Melting points were determined on a Boetius apparatus and are uncorrected. Optical rotation values were measured with a Perkin Elmer 141 or 241 digital polarimeter. Elemental analyses were determined on a Carlo-Erba elemental analyser.

The analysis of the reaction mixtures and the determination of enantiomeric excesses of **2** and **6** were performed by analysis on an enantioselective column LiChroCART 250-4 ChiraDex, 5 μm (Merck) using a Ginkotek 580A pump and an Applied Biosystems 759A absorbance detector at 254 nm. The e.e. of **6** was determined using a 15% solution of acetonitrile in 0.01 M (NaH_2PO_4/Na_2HPO_4) phosphate buffer, pH 4.1, as eluent at a flow rate of 0.8 mL/min. The solvent system acetonitrile/water/acetic acid (10/90/0.1) at a flow rate of 0.8 mL/min was used for the determination of e.e. of **2**. Peak areas were determined electronically with the Chromeleon chromatography data system, Dionex Softron GmbH (Germering, Germany).

4.2. 3-(Methoxycarbonyl)-2,6-dimethyl-5-(propoxycarbonyl)-1,4-dihydro-4-pyridinecarboxylic acid, 2

A mixture of propyl acetoacetate (18 mL, 0.12 mol), 50% aqueous glyoxylic acid (4.5 mL, 0.04 mol) and morpholine (0.2 mL, 0.002 mol) in ethanol (5 mL) was stirred at room temperature for 16 h. Then concentrated HCl (0.5 mL) was added and the reaction mixture was evaporated under reduced pressure. The residue was treated with acetic anhydride (4 mL, 0.042 mol) and after standing for 30 min, the acetic anhydride was removed in vacuum. The residue was cooled and methyl 3-aminocrotonate (1 g, 0.0087 mol) was added under stirring. The reaction mixture was dissolved in ethyl acetate (5 mL) and stored in a refrigerator for 16 h. The precipitate was filtered, washed with ethyl acetate and dried to give **2** (1.5 g, 12.6%) as a white powder, mp 187–190°C. ^{1}H NMR (DMSO-d_6, 200 MHz): δ 0.88 (t, 3H, J=7.5 Hz, CH_3), 1.58 (sextet, 2H, J=7.5 Hz, CH_2), 2.21 (s, 3H, CH_3), 2.22 (s, 3H, CH_3), 3.60 (s, 3H, CH_3), 3.90–4.10 (m, 2H, CH_2), 4.59 (s, 1H, CH), 8.89 (br s, 1H, NH), 11.92 (br s, 1H, COOH); ^{13}C (DMSO-d_6, 50 MHz): δ 10.47 (CH_3), 17.91 (2×CH_3), 21.75 (CH_2), 38.24–40.75 (CH, overlap with DMSO-d_6), 50.81 (CH_3), 64.74 (CH_2), 96.97 (C), 97.31 (C), 145.81 (C), 146.15 (C), 166.85 (C), 167.32 (C), 174.48 (C); MS: m/z (rel. abund.) 297 (M^+, 0.7), 253 (19), 252 (100), 238 (10), 211 (8), 210 (73), 209 (8), 206 (12), 178 (11), 165 (8), 150 (8); HRMS calcd for $C_{14}H_{19}NO_6$: 297.1212, found: 297.1210. Anal. calcd for $C_{14}H_{19}NO_6$: C, 56.56; H, 6.44, N, 4.71; found: C, 56.51; H, 6.29; N, 4.62%.

4.3. 3-Methyl 4-[(propionyloxy)methyl] 5-propyl 2,6-dimethyl-1,4-dihydro-3,4,5-pyridinetricarboxylate, 4a

To a solution of **2** (1.188 g, 4.0 mmol) in dry DMF (2 mL), K_2CO_3 (0.828 g, 6 mmol) was added at rt and the reaction mixture was stirred for 2 h, after which propionyloxymethyl chloride (0.735 g, 6 mmol) was added. The mixture was stirred overnight, diluted with $CHCl_3$ and washed with water (three times) and brine, dried over $MgSO_4$ and evaporated. The remaining residue was flash chromatographed on silica gel with chloroform/petroleum ether (bp 40–60°C)/isopropyl alcohol (10:3:1→10:10:1) to give **4a** (0.31 g, 20.2%) as a white precipitate from hexane/ethyl acetate, mp 70–72°C and unreacted **2** (0.39 g, 32.8%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.94 (t, 3H, J=7.5 Hz, CH_3), 1.10 (t, 3H, J=7.5 Hz, CH_3), 1.66 (sextet, 2H, J=7.5 Hz, CH_2), 2.28 (s, 6H, 2×CH_3), 2.31 (q, 2H, J=7.5 Hz, CH_2), 3.70 (s, 3H, CH_3), 3.91–4.17 (m, 2H, CH_2), 4.86 (s, 1H, CH), 5.68 (s, 2H, CH_2) 6.19 (br s, 1H, NH); ^{13}C ($CDCl_3$, 50 MHz): δ 8.75 (CH_3), 10.58 (CH_3), 19.16 (2×CH_3), 22.08 (CH_2), 27.28 (CH_2), 40.50 (CH), 51.35 (CH_3), 65.85 (CH_2), 79.37 (CH_2), 97.61 (C), 97.96 (C), 146.11 (C), 146.39 (C), 166.95 (C), 167.39 (C), 172.61 (C), 172.95 (C); MS: m/z (rel. abund.) 383 (M^+, 0.06), 253 (14), 252 (100), 238 (2), 211 (3), 210 (25), 204 (3), 178 (3), 165 (4), 150 (2), 57 (5); HRMS calcd for $C_{18}H_{25}NO_8$ 383.1580, found 383.1575. Anal. calcd for $C_{18}H_{25}NO_8$: C, 56.39; H, 6.57; N, 3.65; found: C, 56.40; H, 6.61; N, 3.67%.

4.4. 4-[(Butyryloxy)methyl] 3-methyl 5-propyl 2,6-dimethyl-1,4-dihydro-3,4,5-pyridinetricarboxylate, 4b

This compound was prepared using the same method as used for compound **4a**, starting from 0.297 g (1 mmol) of **2**, 1.5 mL of DMF, 0.242 g (1.75 mmol) of K_2CO_3 and 0.273 g (2 mmol) of butyryloxymethyl chloride. Flash chromatography on silica gel with petroleum ether (bp 40–60°C)/chloroform/isopropyl alcohol (10:3:1) followed by crystallisation from ether/hexane gave **4b** (0.285 g, 72%) as a white precipitate, mp 71–73°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.87 (t, 3H, J=7.4 Hz, CH_3), 0.89 (t, 3H, J=7.4 Hz, CH_3), 1.56 (sextet, 2H, J=7.4 Hz, CH_2), 1.62 (sextet, 2H, J=7.4 Hz, CH_2), 2.22 (t, 2H, J=7.4 Hz, CH_2), 2.23 (s, 6H, 2×CH_3), 3.65 (s, 3H, CH_3), 3.92–4.13 (m, 2H, CH_2), 4.82 (s, 1H, CH), 5.64 (s, 2H, CH_2) 6.14 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 10.57 (CH_3), 13.55 (CH_3), 18.11 (CH_2), 19.16 (2×CH_3), 22.09 (CH_2), 35.79 (CH_2), 40.46 (CH), 51.33 (CH_3), 65.85 (CH_2), 79.35 (CH_2), 97.64 (C), 98.00 (C), 146.08 (C), 146.37 (C), 166.93 (C), 167.37 (C), 172.09 (C), 172.53 (C); MS: m/z (rel. abund.) 397 (M^+, 0.06), 253 (14), 252 (100), 211 (2), 210 (22), 204 (2), 178 (2), 165 (3), 150 (3), 71 (3), 43

(3); HRMS calcd for $C_{19}H_{27}NO_8$: 397.1737, found: 397.1736.

4.5. 4-[(Isobutyryloxy)methyl] 3-methyl 5-propyl 2,6-dimethyl-1,4-dihydro-3,4,5-pyridinetricarboxylate, 4c

This compound was prepared via the same method as used for compound **4a**, starting from **2** (0.346 g, 1.16 mmol), DMF (1.5 mL), K_2CO_3 (0.242 g, 1.75 mmol) and isobutyryloxymethyl chloride (0.318 g, 2.3 mmol). Flash chromatography on silica gel with petroleum ether (bp 40–60°C)/chloroform/isopropyl alcohol (10:3:1) followed by crystallisation from ether/hexane gave **4c** (0.286 g, 62%) as a white solid, mp 64–65°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.89 (t, 3H, $J=7.4$ Hz, CH_3), 1.08 (d, 6H, $J=7.0$ Hz, 2×CH_3), 1.62 (sextet, 2H, $J=7.4$ Hz, CH_2), 2.24 (s, 6H, 2×CH_3), 2.51 (septet, 1H, $J=7.0$ Hz, CH), 3.66 (s, 3H, CH_3), 3.92–4.13 (m, 2H, CH_2), 4.82 (s, 1H, CH), 5.64 (s, 2H, CH_2) 5.95 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 10.51 (CH_3), 18.55 (2×CH_3), 19.05 (2×CH_3), 22.04 (CH_2), 33.76 (CH), 40.41 (CH), 51.28 (CH_3), 65.80 (CH_2), 79.29 (CH_2), 97.52 (C), 97.89 (C), 146.15 (C), 146.45 (C), 166.87 (C), 167.29 (C), 172.48 (C), 175.48 (C); MS: m/z (rel. abund.): 338 ((M−$COOCH_3$)$^+$, 8), 280 (59), 254 (32), 253 (100), 252 (85), 238 (28), 211 (52), 210 (95), 178 (47), 165 (50), 150 (45), 71 (71), 43 (50). Anal. calcd for $C_{19}H_{27}NO_8$: C, 57.42; H, 6.85; N, 3.52. Found: C, 57.42; H, 6.84; N, 3.42%.

4.6. 4-[(Pivaloyloxy)methyl] 3-methyl 5-propyl 2,6-dimethyl-1,4-dihydro-3,4,5-pyridinetricarboxylate, 4d

This compound was prepared via the same method as used for compound **4a**, starting from **2** (0.297 g, 1.0 mmol), DMF (0.5 mL), K_2CO_3 (0.138 g, 1.0 mmol) and pivaloyloxymethyl chloride (0.301 g, 2 mmol). Flash chromatography on silica gel with petroleum ether (bp 40–60°C)/ethyl acetate (1:1) gave **4d** (0.249 g, 60.6%) as a white powder from hexane–ether, mp 52–54°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.94 (t, 3H, $J=7.4$ Hz, CH_3), 1.15 (s, 9H, 3×CH_3), 1.67 (sextet, 2H, $J=7.4$ Hz, CH_2), 2.28 (s, 6H, 2×CH_3), 3.70 (s, 3H, CH_3), 3.97–4.17 (m, 2H, CH_2), 4.87 (s, 1H, CH), 5.68 (s, 2H, CH_2), 6.11 (br s, 1H, NH); ^{13}C ($CDCl_3$, 50 MHz): δ 10.56 (CH_3), 19.22 (2×CH_3), 22.09 (CH_2), 26.79 (3×CH_3), 38.65 (C), 40.32 (CH), 51.35 (CH_3), 65.84 (CH_2), 79.44 (CH_2), 97.73 (C), 98.09 (C), 146.01 (C), 146.34 (C), 166.86 (C), 167.28 (C), 172.17 (C), 175.89 (C); MS: m/z (rel. abund.) 411 (M^+, 0.07), 294 (1) 280 (2) 254 (1) 253 (11), 252 (100), 211 (1), 210 (12), 178 (1), 165 (2), 57 (2); HRMS calcd for $C_{20}H_{29}NO_8$ 411.1893, found 411.1888. Anal. calcd for $C_{20}H_{29}NO_8$: C, 58.38; H, 7.11; N, 3.40; found: C, 58.38; H, 7.07; N, 3.41%.

4.7. 4-Ethoxycarbonylmethyl 3-methyl 5-propyl 2,6-dimethyl-1,4-dihydro-3,4,5-pyridinetricarboxylate, 5

This compound was prepared via the same method as used for compound **4a**, starting from **2** (0.595 g, 2 mmol), dry DMF (1.0 mL), K_2CO_3 (0.276 g, 2 mmol) and ethyl bromoacetate (0.334 mL, 3 mmol). The reaction time after addition of ethyl bromoacetate was 2 h. Flash chromatography on silica gel with petroleum ether (bp 40–60°C)/ethyl acetate (1:1) gave **5** (0.67 g, 83%) as a white precipitate from ether/hexane, mp 70–72°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.94 (t, 3H, $J=7.4$ Hz, CH_3), 1.22 (t, 3H, $J=7.2$ Hz, CH_3), 1.69 (sextet, 2H, $J=7.4$ Hz, CH_2), 2.28 (s, 6H, 2×CH_3), 3.73 (s, 3H, CH_3), 3.99–4.15 (m, 2H, CH_2), 4.14 (q, 2H, $J=7.2$ Hz, CH_2), 4.53 (s, 2H, CH_2), 4.97 (s, 1H, CH), 6.43 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 10.58 (CH_3), 14.08 (CH_3), 19.07 (2×CH_3), 22.12 (CH_2), 40.08 (CH), 51.34 (CH_3), 61.06 (CH_2), 61.29 (CH_2), 65.80 (CH_2), 97.71 (C), 98.06 (C), 146.21 (C), 146.52 (C), 167.11 (C), 167.53 (C), 167.62 (C), 173.56 (C); MS: m/z (rel. abund.) 382 ((M−H)$^+$, 0.05), 338 (2), 324 (2), 296 (3), 280 (2), 254 (2), 253 (14), 252 (100), 211 (2), 210 (16), 165 (3), 150 (2). Anal. calcd for $C_{18}H_{25}NO_8$: C, 56.39; H, 6.57; N, 3.65. Found: C, 56.47; H, 6.63; N, 3.61%.

4.8. *Candida rugosa* lipase-catalysed kinetic resolution of (±)-4c

A solution of (±)-**4c** (135 mg, 0.34 mmol) in acetonitrile (40 mL) was added to K_2HPO_4/KH_2PO_4 buffer (20 mM, pH 7.5, 230 mL) after which *C. rugosa* lipase (17 mg) was added. The resulting mixture was shaken at 250 rpm for 7.5 h at 25°C until the conversion reached 50% according to HPLC. The remaining (+)-**4c** was extracted from the reaction mixture with CH_2Cl_2 (3× 100 mL), washed with water, dried over $MgSO_4$ and concentrated under reduced pressure to give (+)-**4c** (66 mg, 49%) as an amorphous triturate from ether–hexane; $[\alpha]_D^{20}$ +2.8 (*c* 1, $CHCl_3$) or $[\alpha]_D^{20}$ +4.4 (*c* 1, MeOH); 65% e.e. The water layer was adjusted to pH 5.0 by adding dilute aqueous HCl and extracted with ethyl acetate (5×200 mL). The extract was concentrated under reduced pressure. The residue was triturated from ether-hexane to give (+)-**2** (42.0 mg, 42%) of as a white powder from hexane–ether, mp 161–163°C; $[\alpha]_D^{20}$ +2.0 (*c* 2, MeOH); 61% e.e. The ^{1}H NMR spectra were identical to those described for the racemic precursors **2** and **4c**. Anal. calcd for (+)-**4c** ($C_{19}H_{27}NO_8$): C, 57.42; H, 6.85; N, 3.52; found: C, 57.29; H, 6.85; N, 3.55. Anal. calcd for (+)-**2** ($C_{14}H_{19}NO_6$): C, 56.56; H, 6.44, N, 4.71; found: C, 56.80; H, 6.37; N, 4.81%.

4.9. Novozym 435®-catalysed kinetic resolution of (±)-5 at 45°C

To a solution of (±)-**5** (0.383 g, 1 mmol) in water-saturated IPE (50 mL) was added Novozym 435® (0.750 g) and the resulting mixture was shaken at 250 rpm for 48 h at 45°C until the conversion reached 50% according to HPLC. After removal of the enzyme by filtration, the filtrate was concentrated under reduced pressure. The residue was flash chromatographed on silica gel with petroleum ether (bp 40–60°C)/ethyl acetate (1:1) to give unreacted (−)-**5** (0.195 g, 51%). After changing of the mobile phase to dichloromethane/petroleum ether (bp 40–60°C)/isopropyl alcohol/acetic acid (7:3:0.1) (+)-**6** (0.170 g, 48%) was obtained.

4.9.1. (+)-4-Carboxymethyl 3-methyl 5-propyl 2,6-dimethyl-1,4-dihydro-3,4,5-pyridinetricarboxylate, (+)-6. (+)-**6** was obtained as a viscous oil, e.e. 75%, $[\alpha]_D^{20}$ +5.3 (*c* 1, MeOH) or $[\alpha]_D^{20}$ +5.0 (*c* 1, acetone), and was crystallised from ether to give (+)-**6** (11.2 mg), mp 82–84°C, e.e. 46%. Repeated crystallisation of mother liquor containing (+)-**6** from ethyl acetate gave (+)-**6** (4.1 mg), e.e. 35%, mp 109–111°C, the e.e. of the mother liquor was 85%. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.88 (t, 3H, $J=7.4$ Hz, CH_3), 1.58 (sextet, 2H, $J=7.4$ Hz, CH_2), 2.23 (s, 6H, 2×CH_3), 3.60 (s, 3H, CH_3), 3.87–4.10 (m, 2H, CH_2), 4.42 (s, 1H, CH), 4.78 (s, 2H, CH_2), 9.01 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz): δ 10.46 (CH_3), 18.70 (2×CH_3), 21.70 (CH_2), 38.20–40.75 (CH, overlap with DMSO-d_6), 50.91 (CH_3), 60.82 (CH_2), 64.84 (CH_2), 96.06 (C), 96.33 (C), 146.86 (C), 147.05 (C), 166.51 (C), 166.95 (C), 168.83 (C), 172.55 (C); MS: *m*/*z* (rel. abund.): 355 (M^+, 0.04), 324 (0.06), 296 (3), 268 (5), 253 (14), 252 (100), 211 (4), 210 (33), 192 (2), 178 (4), 165 (4), 150 (4); HRMS calcd for $(M-OCH_3)^+$, 324.1447, found 324.1443.

4.9.2. (−)-4-Ethoxycarbonylmethyl 3-methyl 5-propyl 2,6-dimethyl-1,4-dihydro-3,4,5-pyridinetricarboxylate, (−)-5. (−)-**5** was obtained as a viscous oil, e.e. 67% (determined after conversion to (−)-**6**); $[\alpha]_D^{20}$ −4.4 (*c* 1, $CHCl_3$) or $[\alpha]_D^{20}$ −4.1 (*c* 1, MeOH). The ^{1}H NMR spectrum was identical to that described for its racemic precursor **5**. MS: *m*/*z* (rel. abund.): 382 ($(M-H)^+$, 0.09), 338 (1), 324 (3), 296 (4), 253 (14), 252 (100), 211 (2), 210 (23), 185 (3), 165 (3), 150 (3); HRMS calcd for $(M-H)^+$ 382.1502 and for $(M-C_2H_5O)^+$ 338.1240, found for $(M-H)^+$ 382.1500 and for $(M-C_2H_5O)^+$ 338.1239.

4.10. Novozym 435®-catalysed kinetic resolution of (±)-5 at 25°C

The kinetic resolution of (±)-**5** was performed via the same procedure as used for the resolution of **5** at 45°C, but in 75 mL of water-saturated IPE. The incubation with Novozym 435® for 27 days gave (+)-**6** (0.167 g, 47%) as a viscous oil, 79% e.e., $[\alpha]_D^{20}$ +6.4 (*c* 1, MeOH) and (−)-**5** (0.191 g, 49.8%) as a viscous oil, 81% e.e. (determined after conversion to (−)-**6**); $[\alpha]_D^{20}$ −5.3 (*c* 1, $CHCl_3$). The ^{1}H NMR spectra were identical to those described for the racemic precursors (±)-**5** and (±)-**6**.

4.11. Novozym 435®-catalysed kinetic resolution of (±)-5 at −12°C

The kinetic resolution of (±)-**5** was performed via the same procedure as used for the resolution of **5** at 45°C, but in 75 mL of water-saturated IPE. The incubation with Novozym 435® for 63 days in the refrigerator at −12°C gave (+)-**6** (0.120 g, 34%) as a viscous oil, 93% e.e., $[\alpha]_D^{20}$ +7.0 (*c* 1, MeOH) and (−)-**5** (0.225 g, 59%) as a viscous oil, 50% e.e. (determined after conversion to (−)-**6**); $[\alpha]_D^{20}$ −3.2 (*c* 2, $CHCl_3$). The ^{1}H NMR spectra were identical to those described for the racemic precursors **5** and (+)-**6**.

4.12. (−)-3-(Methoxycarbonyl)-2,6-dimethyl-5-(propoxycarbonyl)-1,4-dihydro-4-pyridinecarboxylic acid, (−)-2

To a solution of (+)-**6** (0.105 g, 0.294 mmol) with an e.e. of 85% in ethanol (3 mL) was added a solution of KOH (0.042 g, 0.75 mmol) in ethanol (1 mL). After being stirred under reflux for 1.5 h, the reaction mixture was evaporated, diluted with water, acidified with dilute aqueous HCl until pH 5.0, and extracted four times with ethyl acetate. The organic layer was evaporated and chromatographed on silica gel with petroleum ether (bp 40–60°C)/ethyl acetate (1:1) to give a product (0.037 g) that was purified again on TLC-PET foil with petroleum ether (bp 40–60°C)/chloroform/isopropyl alcohol (10:10:1) as eluent to give (−)-**2** (0.007 g, 8%) as a white precipitate from ether: mp 162–164°C; 67% e.e., $[\alpha]_D^{20}$ −4.77 (*c* 0.524, MeOH). The ^{1}H NMR spectrum was identical to that described for its racemic precursor **2**.

Acknowledgements

Financial support by the Copernicus programme (ERB-CIPA-CT 94 0121), NATO (Linkage grant LST.CLG 974948), the PhD programme of Wageningen University, Grant 173, LZ-12, and the PhD Grant of the Latvian Council of Science is gratefully acknowledged. We are indebted to Mr. A. van Veldhuizen for recording the NMR spectra, to Mr. C. J. Teunis and Mr. H. Jongejan for the mass spectral analyses and to Mr. E. J. C. van der Klift and Emma Sarule for the elemental analyses.

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