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Candida antarctica lipase-catalyzed hydrolysis of 4-substituted bis(ethoxycarbonylmethyl) 1,4-dihydropyridine-3,5-dicarboxylates as the key step in the synthesis of optically active dihydropyridines

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Abstract

Prochiral bis(ethoxycarbonylmethyl) substituted 4-aryl-1,4-dihydropyridine-3,5-dicarboxylates were hydrolyzed enantioselectively by *Candida antarctica* lipase B (Novozym 435). The enantiomeric excesses varied from 68 to 93%, depending on the substituent at position 4. In some cases, the e.e. could be significantly increased by changing the solvent system.

1. Introduction

4-Aryl-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (1,4-DHP) derivatives are widely used for the treatment of cardiovascular diseases (hypertension, angina pectoris, infarction).[1] 1,4-DHPs having different ester groups at the 3- and 5-positions possess a stereogenic carbon at the 4-position in the 1,4-DHP nucleus, and enantiomers often show different biological activities.[2] The first optically active DHP derivative appeared on the market in 1992; however, at present, almost all chiral DHPs are still sold as racemates.[3]

Most investigations in the field of chiral dihydropyridines have been devoted to the synthesis of calcium antagonists.[4] We are focusing on novel activities of 1,4-DHPs such as antidiabetic, nootropic, neuromodulatory, regulatory mode action and neuropeptide mimicking effects.[1,5] These activities have been found for some 4-pyridyl and 2-difluoromethoxyphenyl substituted dihydropyridines.[2,5,6] Derivatives of bis(ethoxycarbonylmethyl) substituted 1,4-dihydropyridine-3,5-dicarboxylates possess antimetastatic properties in combination with a low toxicity.[7]

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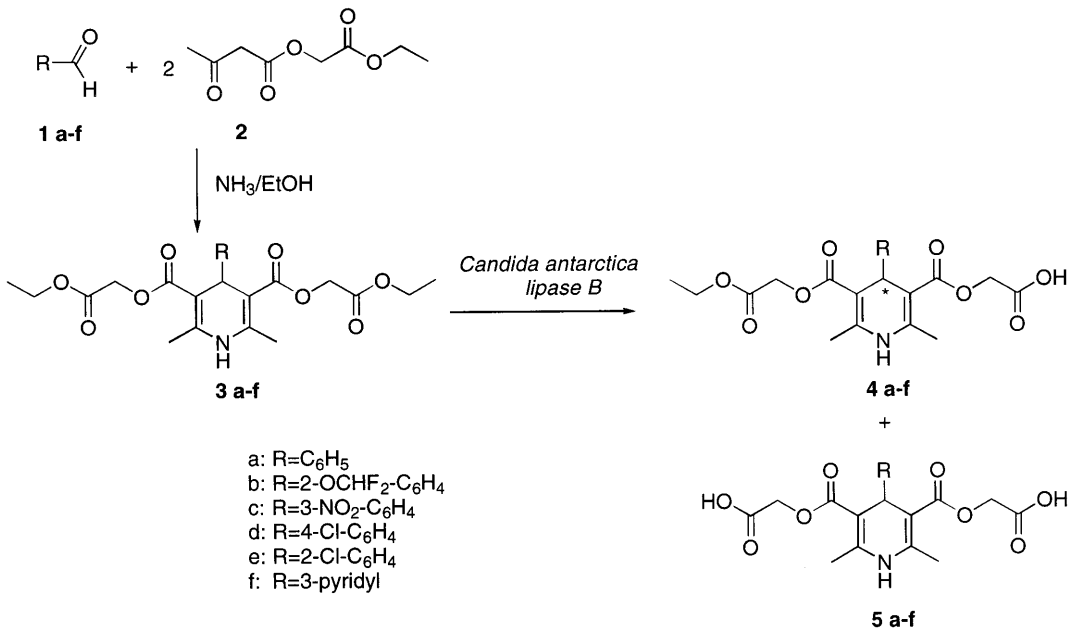
Amongst the many chemical methods for preparing enantiopure compounds, the biotechnological approach based on enzyme-catalyzed enantiomeric differentiation has become a promising approach for the synthesis of enantiopure 1,4-dihydropyridines.[8] The use of biocatalysts shows a number of distinct advantages as compared to other methods: enzymes are active under mild conditions and they often exhibit high stereoselectivity, combined with a broad substrate tolerance. In the case of 1,4-DHPs enzymes are not capable of hydrolyzing alkyl esters at the 3- and 5-positions of 4-aryl-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylates.[8] Modification of the alkyl esters to activated groups makes the hydrolysis possible, in some cases with high stereoselectivity.[9] When substituted alkyl ester chains were introduced at the 3- and/or 5-positions of the dihydropyridine ring, rabbit liver esterase showed the hydrolysis of the more distant ester group with a moderate degree of selectivity.[10] Seaprose S (*Aspergillus melleus*) catalyzed the hydrolysis or transesterification of ethoxycarbonylmethyl esters of 1,4-DHP with splitting of both 'outer' and 'inner' ester groups to give the corresponding carboxylic acid or methyl ester.[11,12] It has also been reported that the stereoselectivity of lipase AH (*Pseudomonas* sp.) toward the same 1,4-DHPs can be changed or even reversed by changing the solvent.[13]

In this paper, the *Candida antarctica* B lipase-catalyzed enantioselective hydrolysis of prochiral bis(ethoxycarbonylmethyl) 1,4-dihydropyridine-3,5-dicarboxylates **3a–f** with different substituents at the position 4 is described.

2. Results and discussion

Bis(ethoxycarbonylmethyl) substituted 1,4-dihydropyridine-3,5-dicarboxylates **3a–f** were prepared by Hantzsch cyclization of ethoxycarbonylmethyl acetoacetate **2** with aromatic aldehydes **1a–f** in ethanol with 42–67% yields (Scheme 1). In the case of aryl substituted 1,4-DHPs **3a–e**,



Scheme 1.

gradual addition of ammonia to the reaction mixture increases the yield by compensating for the loss of ammonia due to evaporation.

The first enzymatic asymmetrizations of the substrates **3a–f** were performed in phosphate buffer pH 7.5, modified with 15% of acetonitrile, at 45°C using protease P6 (*Aspergillus melleus*) and acylase 30,000 (*Aspergillus* sp.). Both enzymes readily hydrolyzed the 'outer' ester group on both sides of the substrates, eventually leading to the diacids **5a–f**. This contrasts the earlier reports where these enzymes and Seaprose S hydrolyzed the 'inner' ester.[11,12] Furthermore, alkaline hydrolysis only takes place at the 'outer' ester groups[7] because of steric and electronic factors.

Long reaction times and low enantioselectivity of protease P6 (*Aspergillus melleus*) and acylase 30,000 (*Aspergillus* sp.) in the given reaction conditions led us to investigate other possibilities of conversion of substrates **3a–f**. Initial studies of the enantioselectivity of *Candida rugosa* lipase and *Rhizomucor miehei* lipase towards the substrates **3a–f** showed that these enzymes have also low enantioselectivity under the given reaction conditions.

Candida antarctica lipase B (CAL B) is a very efficient catalyst for the enantioselective transformations of different kinds of substrates and is widely used in practice.[14] When *Candida antarctica* B lipase was used for the asymmetrization of **3a–f** in phosphate buffer pH 7.5 (modified with acetonitrile, at 45°C; Table 1), a rather high enantioselectivity was reached together with shorter reaction times, so this enzyme was used for all subsequent experiments. The reaction should be carefully monitored by HPLC because of the subsequent hydrolysis to the diacid. The limiting factor of the hydrolysis of the aryl substituted **3a–e** is their insolubility in aqueous medium. Mixing the phosphate buffer with acetonitrile enhances the solubility of the substrates **3a–e**, but the reaction mixture remains heterogenous at the start. The fact that the substrates dissolve during the reaction makes it difficult to directly compare reaction rates for the different substrates. Only in the case of 4-pyridyl substituted 1,4-DHP **3f** was good solubility in buffer with just 5% of acetonitrile observed.

Table 1
CAL B-catalyzed hydrolysis of **3a–f** in phosphate buffer, pH 7.5 modified with acetonitrile at 45°C

Entry	Substrate	Reaction medium[a]	Time (h)	Chemical yield (%)	Enantiomeric excess (e.e., %)	Optical rotation, $[\alpha]_D^{20}$
1	**3a**	A	19	87	93	+49.9
2	**3b**	A	48	44	79	+44.7
3	**3c**	A	20	66	77	+52.5
4	**3d**	A	96	31	68	+5.5
5	**3e**	A	48	29	72	+15.1
6	**3f**	B	18	58	82	+23.7

[a] (A) 15% solution of acetonitrile in 20 mM K-phosphate buffer, pH 7.5; (B) 5% solution of acetonitrile in 20 mM K-phosphate buffer, pH 7.5.

The above mentioned solubility difficulties of the substrates such as **3d**,**e** and insufficient enantioselectivity of CAL B under the given reaction conditions towards substrates **3b–f** forced us to search for more suitable reaction conditions. It is known that the stereoselectivity of an enzyme can change considerably and sometimes even can reverse on transition from one solvent to another.[13,15]

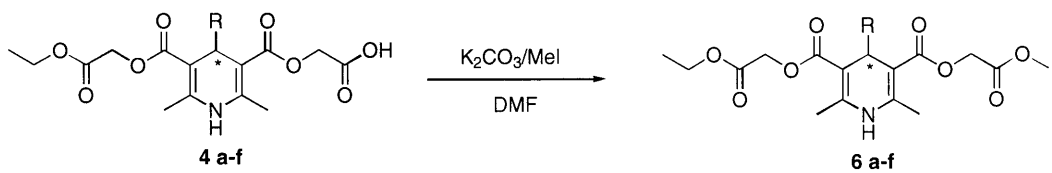
Altering the reaction media led in some cases to a higher enantioselectivity of CAL B towards substrates **3**; some examples are given in Table 2. From this table it is clear that the e.e. of **4b** was remarkably improved when water-saturated isopropylether (IPE) was used, albeit at the expense of the reaction rate. However, in other cases changing the reaction medium to IPE did not have a significant influence on the enantioselectivity of enzyme. Other organic solvents such as acetone, tetrahydrofuran, *t*-butanol and dimethylsulfoxide were used instead of acetonitrile, but in all cases (including acetonitrile) an increase of the amount of organic solvent in reaction mixture led to longer reaction times and a decreased e.e. of the product.

Table 2
Some examples of CAL B-catalyzed hydrolysis of **3a–f** in different reaction media at 45°C

Entry	Substrate	Reaction medium[a]	Time (h)	Chemical yield of **4** (%)	Enantiomeric excess (e.e., %)
1	**3a**	C	48	21[b]	88
2	**3a**	D	22	49[b]	55
3	**3b**	C	282	47	97
4	**3c**	C	168	15[b]	67
5	**3d**	D	48	27[b]	68
6	**3d**	E	48	35[b]	69
7	**3e**	D	200	40	1
8	**3e**	E	24	39[b]	70
9	**3e**	F	28	55	92
10	**3e**	G	138	39	93

[a] Reaction medium: (C) IPE/water; (D) 15% solution of *t*-butyl alcohol in 20 mM phosphate buffer pH 7.5; (E) 15% solution of acetone in 20 mM phosphate buffer pH 7.5; (F) 1% DMSO solution in 20 mM phosphate buffer pH 7.5; (G) 20% of DMSO in 20 mM phosphate buffer pH 7.5.
[b] Chemical yields were determined by HPLC.

The structure of the products was proven by mass spectrometry. Since mass spectra of the enzymatic products **4a–f** did not show molecular ions, the acids were converted into the corresponding esters **6a–f** by esterification with MeI in DMF (Scheme 2). Other ester derivatives of compound **3b** which could be suitable for the determination of the absolute configuration by X-ray analysis were synthesized using different approaches. Attempts to obtain suitable crystals failed, despite the fact that racemic analogues of the synthesized esters are often crystalline materials, as for example in the case of **6d**,**e**. It is already known[16] that problems with crystallization of stereoisomers may occur even if the corresponding racemate is a crystalline substance.



4 a-f **6 a-f**

Scheme 2.

3. Conclusions

In conclusion, the *Candida antarctica* lipase catalyzed asymmetrization of 4-substituted bis(ethoxycarbonylmethyl) 1,4-dihydropyridine-3,5-dicarboxylates has been developed. Although complete stereoselectivity of *Candida antarctica* lipase towards all substrates was not achieved in all cases, the results obtained have shown that a change of the reaction conditions can improve the stereoselectivity of the process. Our investigations of lipase-catalyzed hydrolysis of 1,4-DHPs show the way for the synthesis of various chiral biologically active dihydropyridines containing different aryl and heterocyclic substituents in position 4.

4. Experimental

4.1. General

Flash column chromatography was performed on Merck silica gel 60 (230–400 or 70–230 mesh). ^{1}H NMR spectra were recorded on a Bruker WH 90/DC (90 MHz) or a Bruker AC-E 200 (200 MHz) or a Bruker Avance DPX 400 (400 MHz) spectrometer. ^{13}C NMR spectra were recorded on a Bruker AC-E 200 (50 MHz) or a Bruker Avance DPX 400 (100 MHz) spectrometer. 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 241 digital polarimeter. Elemental analyses were determined on a Carlo Erba elemental analyzer. The reaction mixtures were analyzed by HPLC on a 4.6×250 mm column packed with 5 μm Spherisorb ODS-2 (Phase Separations) using a Gynkotek 480 pump and Applied Biosystems 758A programmable absorbance detector at 254 nm. The solvent system acetonitrile/water/acetic acid (60:40:0.1) was used as mobile phase at a flow rate of 1.0 ml/min. Determination of enantiomeric excesses of the products **4a**–**f** was performed by direct analysis on a chiral column Chirex 3011, 4.6×250 mm, 5 μm (Phenomenex) using a Ginkotek 580A pump and an Applied Biosystems 759A absorbance detector at 254 nm. Other Pirkle and cavity-type columns were useful for the analysis of e.e. of products of the reactions but this was the most universal one. The eluent was 0.05 M ammonium acetate in methanol at a flow rate of 1.0 ml/min for **4a**–**c**,**d**,**f** or dichloromethane/methanol/acetic acid (80:20:0.5) for compound **4e**. Peak areas were determined electronically with the Chromeleon chromatography data system, Dionex Softron GmbH (Germering, Germany). Enzymatic reactions were carried out in a New Brunswick Scientific Innova 4080 incubatory orbital shaker. Immobilized *Candida antarctica* lipase B, Novozym 435, was a gift from Novo Nordisk A/S (Bagsvaerd, Denmark).

4.2. General procedure of the synthesis of bis(ethoxycarbonylmethyl) 4-aryl-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylates *3a*–*e*

Ethoxycarbonylmethyl acetoacetate **2** (29.0 g, 0.15 mol), 0.077 mol of benzaldehyde **1a** or the corresponding substituted benzaldehyde **1b**–**e** and 5 ml (0.065 mol) of 25% aqueous ammonia were dissolved in 50 ml of ethanol and were heated under reflux. After 2 h of refluxing, 5 ml of 25% aqueous ammonia was added in two to three portions with 0.5 h intervals. The usual

quantity of added ammonia was 10 ml (0.13 mol). Usual time of refluxing was 6 h. After cooling until −5°C the precipitated product was filtered and recrystallized from ethanol.

4.2.1. Bis(ethoxycarbonylmethyl) 1,4-dihydro-2,6-dimethyl-4-phenylpyridine-3,5-dicarboxylate ***3a***

Yield: 42%, mp 84–85°C; ^{1}H NMR ($CDCl_3$, 90 MHz): δ 1.23 (6H, t, $J=7.0$ Hz, CH_2CH_3), 2.33 (6H, s, 2×CH_3), 4.15 (4H, q, $J=7.0$ Hz, CH_2CH_3), 4.55 (4H, s, 2×$COOCH_2COO$), 5.09 (1H, s, CH), 6.00 (1H, br s, NH), 7.05–7.30 (5H, m, C_6H_5); anal. calcd for $C_{23}H_{27}NO_8$: C, 62.01; H, 6.11; N, 3.14; found: C, 62.06; H, 6.12; N, 3.14.

4.2.2. Bis(ethoxycarbonylmethyl) 4-(2′-difluoromethoxyphenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate ***3b***

Yield: 55%, mp 101–102°C; ^{1}H NMR ($CDCl_3$, 90 MHz): δ 1.20 (6H, t, $J=7.0$ Hz, CH_2CH_3), 2.30 (6H, s, 2×CH_3), 4.10 (4H, q, $J=7.0$ Hz, CH_2CH_3), 4.50 (4H, s, 2×$COOCH_2COO$), 5.33 (1H, s, CH), 6.25 (1H, br s, NH), 6.45 (1H, t, $J=75$ Hz, $OCHF_2$), 6.87–7.40 (4H, m, C_6H_4); anal. calcd for $C_{24}H_{27}F_2NO_9$: C, 56.36; H, 5.32; N, 2.74; found: C, 56.42; H, 5.33; N, 2.71.

4.2.3. Bis(ethoxycarbonylmethyl) 1,4-dihydro-2,6-dimethyl-4-(3′-nitrophenyl)-pyridine-3,5-dicarboxylate ***3c***

Yield: 67%, mp 157–159°C; ^{1}H NMR ($CDCl_3$, 90 MHz): δ 1.18 (6H, t, $J=7.0$ Hz, CH_2CH_3), 2.33 (6H, s, 2×CH_3), 4.09 (4H, q, $J=7.0$ Hz, CH_2CH_3), 4.50 (4H, s, 2×$COOCH_2COO$), 5.77 (1H, s, CH), 6.57 (1H, br s, NH), 7.10–7.67 (4H, m, C_6H_4); anal. calcd for $C_{23}H_{26}N_2O_{10}$: C, 56.32; H, 5.34; N, 5.71; found: C, 56.38; H, 5.32; N, 5.70.

4.2.4. Bis(ethoxycarbonylmethyl) 4-(4′-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate ***3d***

Yield: 69%, mp 130–132°C; ^{1}H NMR ($CDCl_3$, 90 MHz): δ 1.22 (6H, t, $J=7.0$ Hz, CH_2CH_3), 2.33 (6H, s, 2×CH_3), 4.15 (4H, q, $J=7.0$ Hz, CH_2CH_3), 4.54 (4H, s, $COOCH_2COO$), 5.05 (1H, s, CH), 6.07 (1H, br s, NH), 7.08 and 7.23 (4H, two d, $J=9$ Hz, C_6H_4); anal. calcd for $C_{23}H_{26}ClNO_8$: C, 57.56; H, 5.46; N, 2.92; found: C, 57.56; H, 5.47; N, 2.90.

4.2.5. Bis(ethoxycarbonylmethyl) 4-(2′-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate ***3e***

Yield: 38%, mp 115–117°C; ^{1}H NMR ($CDCl_3$, 90 MHz): δ 1.16 (6H, t, $J=7.0$ Hz, CH_2CH_3), 2.30 (6H, s, 2×CH_3), 4.10 (4H, q, $J=7.0$ Hz, CH_2CH_3), 4.50 (4H, s, $COOCH_2COO$), 5.44 (1H, s, CH), 6.41 (1H, br s, NH), 6.88–7.41 (4H, m, C_6H_4); anal. calcd for $C_{23}H_{26}ClNO_8$: C, 57.56; H, 5.46; N, 2.92; found: C, 57.56; H, 5.45; N, 2.91.

4.3. Bis(ethoxycarbonylmethyl) 1,4-dihydro-2,6-dimethyl-4-(3′-pyridyl)-pyridine-3,5-dicarboxylate ***3f***

Ethoxycarbonylmethyl acetoacetate **2** (37.6 g, 90.2 mol), 9.4 ml (10.7 g, 0.1 mol) of pyridine-3-carboxaldehyde **1f** and 10 ml (0.13 mol) of 25% aqueous ammonia solution were dissolved in 50 ml of ethanol and were heated under reflux for 3 h. After cooling until −5°C the precipitate was filtered off and recrystallized from ethanol to give 25.4 g (57%) of crystalline product, mp 143–145°C; ^{1}H NMR ($CDCl_3$, 90 MHz): δ 1.21 (6H, t, $J=7.0$ Hz, CH_2CH_3), 2.34 (6H, s, 2×CH_3), 4.17 (4H, q, $J=7.0$ Hz, CH_2CH_3), 4.54 (4H, s, 2×$COOCH_2COO$), 5.09 (1H, s,

CH), 7.06 (1H, br s, NH), 7.17 (1H, dd, $J_{5,4}=8$ Hz, $J_{5,6}=4$ Hz, H_5 Py), 7.65 (1H, dt, $J_{4,5}=8$ Hz, $J_{4,2}=J_{4,6}=2$ Hz, H_4 Py), 8.32 (1H, dd, $J_{6,5}=4$ Hz, $J_{6,4}=2$ Hz, H_6 Py), 8.50 (1H, d, $J_{2,4}=2$ Hz, H_2 Py); anal. calcd for $C_{22}H_{26}N_2O_8$: C, 59.19; H, 5.87; N, 6.27; found: C, 59.04; H, 5.86; N, 6.17.

*4.4. General procedure for enzymatic hydrolysis of 1,4-dihydropyridine-3,5-dicarboxylates **3a–e***

A solution of 0.5 mmol of **3a**,**c**,**d** or 0.4 mmol of **3b**,**e** in 60 ml of acetonitrile was added to 340 ml of 20 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5) and heated to 45°C, after which 600 mg of Novozym 435 was added. The resulting mixture was shaken at 350 rpm and heated at 45°C. Reactions were monitored by HPLC (see Tables 1 and 2 for further details about reaction times). After removal of the enzyme by filtration, the filtrate was adjusted to pH 5.0 by adding 1 M HCl and extracted with chloroform (3×100 ml). The combined organic layers were concentrated under reduced pressure. The residue was flash chromatographed on silica gel using solvent system chloroform/isopropyl alcohol/acetic acid (100:20:0.1) to give the following monoacids.

*4.4.1. (+)-1,4-Dihydro-2,6-dimethyl-5-ethoxycarbonylmethoxycarbonyl-4-phenyl-3-pyridinecarboxylic acid **4a***

Yield: 181 mg (87%) as a precipitate from hexane, mp 169–170°C; $[\alpha]_D^{20}$ +49.9 (*c* 1.0, MeOH); ^{1}H NMR (DMSO-d_6, 200 MHz): δ 1.17 (3H, t, $J=7.1$ Hz, CH_2CH_3), 2.31 (6H, s, 2×CH_3), 4.10 (2H, q, $J=8.0$ Hz, CH_2CH_3), 4.32 (2H, ABq, $COOCH_2COO$), 4.60 (2H, s, $COOCH_2COO$), 4.98 (1H, s, CH), 7.12–7.21 (5H, m, C_6H_5), 9.03 (1H, s, NH); ^{13}C NMR (CD_3OD, 50 MHz): δ 14.41 (CH_3), 18.86 (CH_3), 18.91 (CH_3), 40.20 (CH), 60.41 (CH_2), 62.17 (CH_2), 63.26 (CH_2), 102.97, 104.10, 127.04 (CH), 128.69 (CH), 128.90 (CH), 147.51, 148.47, 148.92, 168.78, 169.48, 170.10, 176.51.

*4.4.2. (+)-4-(2′-Difluoromethoxyphenyl)-1,4-dihydro-2,6-dimethyl-5-ethoxycarbonylmethoxycarbonyl-3-pyridinecarboxylic acid **4b***

Yield: 85 mg (44%) as a precipitate from hexane, mp 151–153°C; $[\alpha]_D^{20}$ +44.7 (*c* 0.855, MeOH); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.13 (3H, t, $J=7.1$ Hz, CH_2CH_3), 2.26 (3H, s, CH_3), 2.29 (3H, s, CH_3), 4.08 (2H, q, $J=7.1$ Hz, CH_2CH_3), 4.24 (2H, ABq, $COOCH_2COO$), 4.50 (2H, ABq, $COOCH_2COO$), 5.25 (1H, s, CH), 6.95–7.13 (3H, m, C_6H_4), 7.00 (1H, t, $J=74.9$ Hz, $OCHF_2$), 7.29 (1H, dd, C_6H_4), 9.03 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 14.76 (CH_3), 19.10 (CH_3), 19.19 (CH_3), 34.91 (CH), 60.82 (CH_2), 61.33 (CH_2), 63.35 (CH_2), 100.73, 103.16, 117.94 (CH, t, $J=252.9$ Hz, $OCHF_2$), 118.29 (CH), 125.89 (CH), 128.26 (CH), 131.70 (CH), 139.81, 145.71, 148.10, 148.98, 167.27, 167.60, 169.00, 173.06.

*4.4.3. (+)-1,4-Dihydro-2,6-dimethyl-5-ethoxycarbonylmethoxycarbonyl-4-(3′-nitrophenyl)-3-pyridinecarboxylic acid **4c***

Yield: 153 mg (66.0%) as crystals from ether/petroleum ether, mp 172–174°C; $[\alpha]_D^{20}$ +52.5 (*c* 1.0, MeOH); ^{1}H NMR (DMSO-d_6, 200 MHz): δ 1.10 (3H, t, $J=7.0$ Hz, CH_2CH_3), 2.32 (6H, s, 2×CH_3), 4.04 (2H, q, $J=7.1$ Hz, CH_2CH_3), 4.25 (2H, ABq, $COOCH_2COO$), 4.59 (2H, s, $COOCH_2COO$), 5.07 (1H, s, CH), 7.50 (1H, t, C_6H_4), 7.73 (1H, d, C_6H_4), 7.96 (2H, d+s, C_6H_4), 9.33 (1H, s, NH); ^{13}C NMR (CD_3OD, 50 MHz): δ 14.37 (CH_3), 18.85 (CH_3), 18.93 (CH_3), 40.55 (CH), 61.44 (CH_2), 62.20 (CH_2), 63.47 (CH_2), 102.21, 103.35, 122.06 (CH), 123.53 (CH), 130.08 (CH), 135.51 (CH), 148.36, 149.34, 149.38, 151.21, 168.18, 168.93, 169.89, 176.83.

4.4.4. (+)-4-(4′-Chlorophenyl)-1,4-dihydro-2,6-dimethyl-5-ethoxycarbonylmethoxycarbonyl-3-pyridinecarboxylic acid ***4d***

Yield: 70 mg (31%) as a viscous oil; $[\alpha]_D^{20}$ +5.5 (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.24 (3H, t, $J=7.1$ Hz, CH_2CH_3), 2.33 (6H, s, 2×CH_3), 4.19 (2H, q, $J=7.1$ Hz, CH_2CH_3), 4.59 (2H, ABq, $COOCH_2COO$), 4.60 (2H, s, $COOCH_2COO$), 5.06 (1H, s, CH), 6.45 (1H, s, NH), 7.16 and 7.25 (4H, two d, $J=9$ Hz, C_6H_4); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 14.07 (CH_3), 19.10 (CH_3), 38.61 (CH), 60.06 (CH_2), 60.53 (CH_2), 61.49 (CH_2), 102.50, 102.59, 128.14 (CH), 129.24 (CH), 131.92, 145.58, 146.19, 146.39, 166.68, 168.70, 173.10.

4.4.5. (+)-4-(2′-Chlorophenyl)-1,4-dihydro-2,6-dimethyl-5-ethoxycarbonylmethoxycarbonyl-3-pyridinecarboxylic acid ***4e***

Yield: 53 mg (29%) as a precipitate from ether/petroleum ether, mp 175–177°C; $[\alpha]_D^{20}$ +15.1 (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.22 (3H, t, $J=7.2$ Hz, CH_2CH_3), 2.34 (3H, s, CH_3), 2.36 (3H, s, CH_3), 4.16 (2H, q, $J=7.1$ Hz, CH_2CH_3), 4.54 (2H, ABq, $COOCH_2COO$), 4.60 (2H, s, $COOCH_2COO$), 5.48 (1H, s, CH), 6.28 (1H, s, NH), 7.00–7.23 (3H, m, C_6H_4), 7.39 (1H, dd, 2-Cl-C_6H_4); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 14.04 (CH_3), 19.20 (CH_3), 19.31 (CH_3), 37.05 (CH), 59.76 (CH_2), 60.12 (CH_2), 61.40 (CH_2), 102.60, 126.98 (CH), 127.41 (CH), 129.20 (CH), 131.60 (CH), 132.40, 145.66, 146.18, 146.32, 166.74, 166.78, 168.95, 172.97.

4.5. (+)-1,4-Dihydro-2,6-dimethyl-5-ethoxycarbonylmethoxycarbonyl-4-(3′-pyridyl)-3-pyridinecarboxylic acid ***4f***

A solution of 223 mg (0.5 mmol) of **3f** in 20 ml of acetonitrile was added to 380 ml of 20 mM K_2HPO_4/KH_2PO_4 buffer solution and heated until 45°C after which 600 mg of Novozym 435 was added. The resulting mixture was shaken at 350 rpm for 17 h at 45°C. After removal of enzyme by filtration, the filtrate was extracted with chloroform (2×100 ml). The filtrate was acidified to pH 5.0 by adding 1 M HCl and concentrated in vacuum. The residue was flash chromatographed on silica gel using chloroform/isopropyl alcohol/acetic acid (100:20:0.1) to (50:50:0.1) to give **4f**. Crystallization from ethyl acetate gave 122 mg (58%) of **4f** as a powder, mp 187–192°C (dec.); $[\alpha]_D^{20}$ +23.7 (*c* 1.0, MeOH); ^{1}H NMR (DMSO-d_6): δ 1.13 (3H, t, $J=7.0$ Hz, CH_2CH_3), 2.30 (6H, s, 2×CH_3), 4.06 (2H, q, $J=7.1$ Hz, CH_2CH_3), 4.21 (2H, ABq, $COOCH_2COO$), 4.59 (2H, s, $COOCH_2COO$), 4.95 (1H, s, CH), 7.23 (1H, dd, Py), 7.57 (1H, dt, Py), 8.29 (1H, d, Py), 8.39 (1H, br s, Py), 9.13 (1H, s, NH).

4.6. (+)-4-(2′-Difluoromethoxyphenyl)-1,4-dihydro-2,6-dimethyl-5-ethoxycarbonylmethoxycarbonyl-3-pyridinecarboxylic acid ***4b*** *by hydrolysis in wet isopropyl ether*

To a solution of 204 mg (0.40 mmol) of **3b** in 50 ml of water-saturated isopropyl ether, 600 mg of Novozym 435 was added and the resulting mixture was shaken at 350 rpm for 12 days (282 h) at 45°C. The enzyme was removed by filtration and washed additionally with methanol and the filtrate was concentrated under reduced pressure. The residue was flash chromatographed on silica gel with chloroform/isopropyl alcohol/acetic acid (100:20:0.1) to give 86 mg (45%) of **4b** as a solid and 95 mg (47%) of unreacted **3b**. Data for **4b**: $[\alpha]_D^{20}$ +72.6 (*c* 1.0, MeOH); ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz) are identical to those for **4b** described in Section 4.4.2.

4.7. General procedure for the preparation of **6a–f**

All derivatives were prepared in the same manner as described below for compound **6c**.

4.7.1. Ethoxycarbonylmethyl methoxycarbonylmethyl 1,4-dihydro-2,6-dimethyl-4-(3′-nitrophenyl)-pyridine-3,5-dicarboxylate **6c**

To a solution of 65 mg (0.14 mmol) of **4c** in 1 ml DMF 29 mg (0.21 mmol) of K_2CO_3 was added at rt and the resulting mixture was stirred for 2 h after which 0.017 ml (0.28 mmol) of MeI was added. The reaction mixture was stirred for additional 2 h. The mixture was poured into water and extracted with $CHCl_3$. The extract was washed successively with water and brine, then dried over $MgSO_4$. After removal of solvent in vacuum the residue was flash chromatographed on silica gel with petroleum ether (bp 40–60°C)/chloroform/isopropyl alcohol (8:2:2) to give 46 mg (69%) of **6c**, mp 85–88°C (triturated with hexane–ether); ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.24 (3H, t, $J=7.2$ Hz, CH_2CH_3), 2.42 (6H, s, $2\times CH_3$), 3.72 (3H, s, CH_3), 4.19 (2H, q, $J=7.2$ Hz, CH_2CH_3), 4.60 (2H, s, $COOCH_2COO$), 4.61 (2H, s, $COOCH_2COO$), 5.24 (1H, s, CH), 6.03 (1H, s, NH), 7.39 (1H, t, C_6H_4), 7.73 (1H, dd, C_6H_4), 8.02 (1H, dt, C_6H_4), 8.14 (1H, t, C_6H_4); MS m/z (rel. abund.): 476 (M^+, 4), 387 (3), 373 (4), 356 (3), 355 (16), 354 (100), 345 (3), 326 (3), 296 (10), 268 (6); HRMS calcd for $C_{22}H_{24}N_2O_{10}$: 476.1431; found: 476.1428.

4.7.2. Ethoxycarbonylmethyl methoxycarbonylmethyl 1,4-dihydro-2,6-dimethyl-4-phenyl-pyridine-3,5-dicarboxylate **6a**

Yield: 65% as a powder triturated with hexane, mp 112–114°C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.25 (3H, t, $J=7.1$ Hz, CH_2CH_3), 2.38 (6H, s, $2\times CH_3$), 3.72 (3H, s, CH_3), 4.19 (2H, q, $J=7.2$ Hz, CH_2CH_3), 4.59 (2H, ABq, $COOCH_2COO$), 4.61 (2H, s, $COOCH_2COO$), 5.14 (1H, s, CH), 5.89 (1H, s, NH), 7.10–7.36 (5H, m, C_6H_5); MS m/z (rel. abund.): 431 (M^+, 4), 355 (11), 354 (100), 238 (3); HRMS calcd for $C_{22}H_{25}NO_8$: 431.1580; found: 431.1578.

4.7.3. Ethoxycarbonylmethyl methoxycarbonylmethyl 4-(2′-difluoromethoxyphenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate **6b**

Yield: 80% as a powder triturated with hexane, mp 83–85°C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.22 (3H, t, $J=7.1$ Hz, CH_2CH_3), 2.35 (6H, s, $2\times CH_3$), 3.67 (3H, s, CH_3), 4.15 (2H, q, $J=7.1$ Hz, CH_2CH_3), 4.55 (2H, ABq, $COOCH_2COO$), 4.57 (2H, s, $COOCH_2COO$), 5.40 (1H, s, CH), 5.96 (1H, s, NH), 6.49 (1H, t, $J=75.3$ Hz, $OCHF_2$), 6.97–7.42 (4H, m, C_6H_4); MS m/z (rel. abund.): 497 (M^+, 6), 495 (4), 408 (3), 394 (4), 391 (4), 368 (9), 366 (5), 355 (16), 354 (100), 296 (5); HRMS calcd for $C_{23}H_{25}F_2NO_9$: 497.1497; found: 497.1501.

4.7.4. Ethoxycarbonylmethyl methoxycarbonylmethyl 4-(4′-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate **6d**

Yield: 71% as a precipitate from hexane–ether, mp 131–132°C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.25 (3H, t, $J=7.2$ Hz, CH_2CH_3), 2.39 (6H, s, $2\times CH_3$), 3.72 (3H, s, CH_3), 4.20 (2H, q, $J=7.2$ Hz, CH_2CH_3), 4.60 (2H, s, $COOCH_2COO$), 4.61 (2H, s, $COOCH_2COO$), 5.11 (1H, s, CH), 5.83 (1H, s, NH), 7.19 and 7.28 (4H, two d, $J=9$ Hz, C_6H_4); MS m/z (rel. abund.): 465 (M^+, 4), 378 (3), 376 (3), 362 (3), 356 (3), 355 (17), 354 (100), 348 (3), 334 (4), 268 (3); HRMS calcd for $C_{22}H_{24}ClNO_8$: 465.1190; found: 465.1185.

4.7.5. Ethoxycarbonylmethyl methoxycarbonylmethyl 4-(2′-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate ***6e***

Yield: 40% as a precipitate from hexane–ether, mp 91–93°C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.21 (3H, t, $J=7.1$ Hz, CH_2CH_3), 2.35 (3H, s, CH_3), 2.36 (3H, s, CH_3), 3.64 (3H, s, CH_3), 4.15 (2H, q, $J=7.2$ Hz, CH_2CH_3), 4.57 (2H, ABq, $COOCH_2COO$), 4.58 (2H, s, $COOCH_2COO$), 5.50 (1H, s, CH), 6.23 (1H, s, NH), 7.00–7.24 (3H, m, C_6H_4), 7.40 (1H, dd, C_6H_4); MS m/z (rel. abund.): 465 (M^+, 3), 429 (3), 428 (13), 370 (4), 362 (3), 355 (14), 354 (100), 334 (3), 296 (5), 268 (4); HRMS calcd for $C_{22}H_{24}ClNO_8$: 465.1190; found: 465.1186.

4.7.6. Ethoxycarbonylmethyl methoxycarbonylmethyl 1,4-dihydro-2,6-dimethyl-4-(3′-pyridyl)-pyridine-3,5-dicarboxylate ***6f***

Yield: 60% as a powder triturated with hexane, mp 124–125°C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.24 (6H, t, $J=7.2$ Hz, CH_2CH_3), 2.39 (6H, s, 2×CH_3), 3.71 (3H, s, CH_3), 4.19 (4H, q, $J=7.2$ Hz, CH_2CH_3), 4.59 (2H, s, $COOCH_2COO$), 4.61 (2H, s, $COOCH_2COO$), 5.14 (1H, s, CH), 6.28 (1H, br s, NH), 7.18 (1H, dd, Py), 7.68 (1H, dt, Py), 8.40 (1H, br d, Py), 8.50 (1H, br s, Py); MS m/z (rel. abund.): 432 (M^+, 5), 355 (18), 354 (100), 343 (5), 329 (6), 301 (5), 297 (3), 296 (23), 268 (8), 211 (3); HRMS calcd for $C_{21}H_{24}N_2O_8$: 432.1533; found: 432.1532.

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