USE OF ENZYMES IN PREPARATION OF ENANTIOPURE 1,4-DIHYDROPYRIDINES

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Abstract - Various chemoenzymatic routes to optically pure biologically active dihydropyridines are reviewed. The review iš focused on influence on the bio-transformation of the main *in vivo* enzymatic activity of the hydrolase (lipase, esterase, protease), the microorganism source of the lipase and the substituents of the 1,4-dihydropyridine ring, as well as the physicochemical properties of the solvent and the nature of the nucleophile. The influence of these variables in the desymmetrisation of prochiral diesters or in the resolution of racemic compounds is analyzed.

1. INTRODUCTION

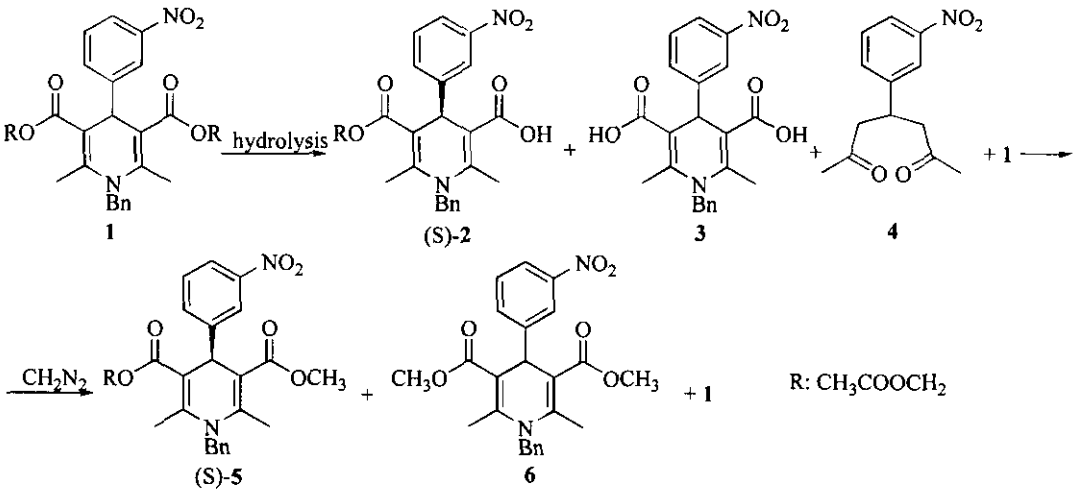
4-Aryl-1,4-dihydropyridine drugs (DHP) calcium channel modulators are important peripheral vasodilators and are widely used in treatment of cerebro-circulatory disorders, hypertension and angina pectoris.[1] Furthermore, certain 4-alkyl-1,4-dihydropyridines can act as potent and specific PAF antagonists.[2] In the case of 1,4-dihydropyridines having different ester groups at C-3 and C-5 or different substituents at C-2 and C-6 possess a stereogenic center at C-4. Both enantiomers have opposite activity as Ca(II) channel blockers or activators, specially when nitro group is on C-3 of the aromatic ring.[3] In other cases, 4*S* enantiomer is generally more active than the antipode in the heart diseases.[4]

Classical Hantzsch synthesis of dihydropyridines produces, however, only racemic mixtures of the target substances. This is the reason why there is an intensive interest for resolution of racemates by means of diastereomers crystallization, preparative chiral chromatography or biocatalytic synthesis.[3] Since a number of dihydropyridines drugs have esters or acids on C-3 and C-5, hydrolases seem to be the most profitable class of enzymes for the production of their pure enantiomers. Hydrolases offer additional advantages coming from easy work-up, usually low cost of enzyme and possibilities to work in organic solvents or start from prochiral molecules. Especially the latter case is economically very interesting because the desymmetrisation of prochiral compound gives nearly theoretical 100% yield in pure enantiomer. This approach was already used for synthesis of wide range of chiral monoesters starting from prochiral alcohols or diesters.[5]

2.1 PROCHIRAL- 2,6-DIMETHYL-1,4-DIHYDROPYRIDINES

The first attempts to desymmetrize symmetric 2,6-dimethyl-1,4-dihydropyridines bearing two ester moieties in positions 3- and 5- of their skeleton were done by groups of Sih and Achiwa.[6-8] These experiments were unsuccessful when dimethyl ester of 1,4-dihydro-2,6-dimethyl-4-phenyl-3,5-pyridine-dicarboxylate was used as the starting material, apparently due to the steric congestion at the ester carbonyl groups. To relieve this problem, less sterically hindered bis(acetoxymethyl) ester of 1,4-dihydropyridine (**1**) was used as starting material for enzymatic hydrolysis or alcoholysis after which the resulting hydroxymethyl group can decompose spontaneously to the desired hemiester.[6,7]

Scheme 1



This philosophy was successful in lipase catalysed hydrolysis of the diesters, low yields, ee and creation of side-products led authors to replace hydrolysis by alcoholysis. n-Butanol - water (10:1) was found as the best reaction medium. The alcoholysis both improved the enantiomeric excesses and suppressed creation of diketone (**4**) as by-product (Scheme 1). Table 1 summarizes results of alcoholysis of **1** carried out by various lipases originating from *Pseudomonas* and *Candida*. It is interesting to note, that unlike the enzymes from *Pseudomonas*, the use of *Candida rugosa* lipase OF-360 lead to creation of the opposite enantiomer.

Table 1: Hydrolysis of **1** with lipases in n-butanol/water (10:1)

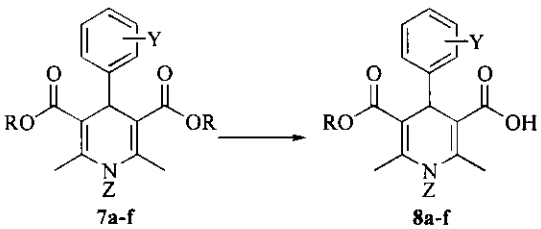
Lipase	Reaction time (h)	Yields (%)			Absolute conf. **5**	ee (%)
		1	**6**	**5**		
P-30	49	5	21	34	*S*	88
K-10	49	32	14	20	*S*	63±5
AK	2.5	28	trace	27	*S*	35±4
AK	5	20	34	30	*S*	65±3
AK	7.5	0	27	29	*S*	77
AK	16.5	0	37	16	*S*	89
AK	22	0	31	8	*S*	97
OF-360	49	46	1	24	*R*	73±3

AK a K-10 - *Pseudomonas* sp., P-30 - *Pseudomonas cepacia*, OF-360 - *Candida rugosa*.

Taking into consideration that hydrolysis of diesters is in fact a two-step process leading at the end to diacids, prolongation of reaction time represents a tool for controlling the optical purity of produced enantiomers (Table 1). Similarly to the previous work, Achiwa *et al.* hydrolysed bis(pivaloyloxymethyl) and bis(propanoyloxymethyl) esters of dihydropyridines (**7a-f**) by *Pseudomonas fragilipase* B. and *Pseudomonas cepacia lipase* P.[8] Hydrolysis of bis(pivaloyloxymethyl) ester (**7a**) by lipase B in buffered solution gave pure hemiester [(*S*)-**8a**] in high yield. Exchange of the buffer by water-saturated diisopropyl ether represents an excellent protocol to obtain pure product in almost equimolar yields with easy separation and sixfold shortened reaction time (Table 2). Similar results were obtained with bis(propanoyloxymethyl) ester (**7e**). However it was necessary to prevent the second hydrolysis step by carrying the reaction at temperature near 0°C.

It is noteworthy that *Pseudomonas fragilipase* B. has opposite stereoselectivity against bis(acyloxymethyl) diesters of 1,4-dihydropyridines, bis(propionyloxymethyl) ester being more suitable for the hydrolysis according to reaction time and higher ee and chemical yields.

Table 2: Lipase-catalysed enantioselective hydrolysis of **7a-f**



7a-f **8a-f**

	R	Z	Y
a	$(CH_3)_3CCO_2CH_2$	CH_3OCH_2	3-NO_2
b	$(CH_3)_3CCO_2CH_2$	CH_3OCH_2	H
c	$(CH_3)_3CCO_2CH_2$	CH_3OCH_2	2-CF_3
d	$(CH_3)_3CCO_2CH_2$	CH_3OCH_2	2,3-diCl
e	$C_2H_5CO_2CH_2$	CH_3OCH_2	3-NO_2
f	$(CH_3)_3CCO_2CH_2$	H	3-NO_2

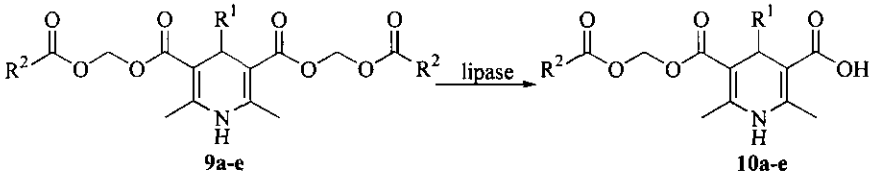
Substrate	Lipase	Solvent	Time (h)	Product[a]	Yield (%)	ee (%)[b]	$[\alpha]_D^{20}$ [c]
7a	B	buffer (pH 8)	24	**8a**	80	>99	+42.6
7a	B	IPE/H_2O	4	**8a**	95	>99	+42.6
7b	B	IPE/H_2O	5	**8b**	76	>99	+43.5
7c	B	IPE/H_2O	7	**8c**	83	>99	+33.8
7d	B	IPE/H_2O	6	**8d**	81	96	+22.0
7a	P	IPE/H_2O	72	**8a**	44	69	-28.7
7e	B	IPE/H_2O	8[d]	**8e**	71	>99	+49.7
7e	P	IPE/H_2O	10	**8e**	78	88	-42.1
7f	B	IPE/H_2O	35	**8f**	83	93	+27.9

IPE-diisopropyl ether, [a] isolated yields, [b]based on HPLC analysis using column packed with Chiracel OD, [c]acetone, c = 1 - 2, [d] 0-5 °C

Summarising previous observations it is evident that similarly to other enzymatic processes, the nature of enzyme, as well as type of media and structure variations of substrate may affect stereoselectivity of hydrolysis and alcoholysis of 1,4-dihydropyridines. Results from another work of Achiwa *et al.* show that

also the type of the substituent in position 4 has strong effect on hydrolysis of activated dihydropyridine diesters by *Pseudomonas fragilipase* B and *Pseudomonas cepacia lipase* P (Table 3).[9] Based on these results authors synthesized chiral building blocks of **PCA 4248** (Scheme 2) and **PCA 4233**.

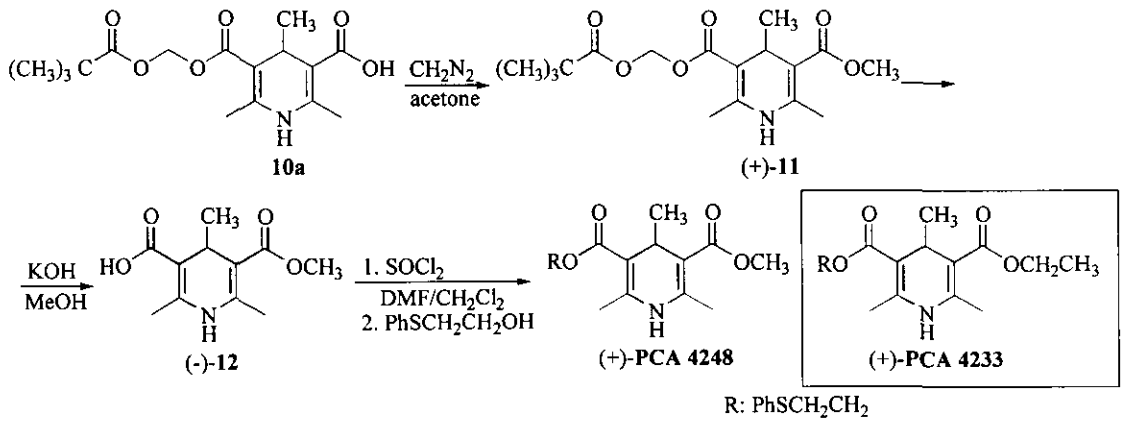
Table 3: Lipase-catalysed synthesis of enantiopure 4-substituted 1,4-dihydropyridines (**9a-e**)



Substrate			Lipase	Time	Product			
No.	R^1	R^2		(h)	No.	Yield (%)[a]	ee (%)[b]	$[a]_D^{20}$ [c]
9a	CH_3	$(CH_3)_3C$	B	8	**10a**	76	91	+25.8
9b	$C_6H_5CH_2$	$(CH_3)_3C$	B	151	**10b**	67	~0	+2.6
9c	$C_6H_5CH_2$	C_2H_5	B	4	**10c**	56	48	-27.2
9c	$C_6H_5CH_2$	C_2H_5	P	48	**10c**	71	91	-42.9
9d	C_6H_{11}	$(CH_3)_3C$	B	96	**10d**	62	56	+11.5
9e	C_6H_{11}	C_2H_5	B	89	**10e**	16	20	-13.4
9e	C_6H_{11}	C_2H_5	P	118	**10e**	29	26	-16.3

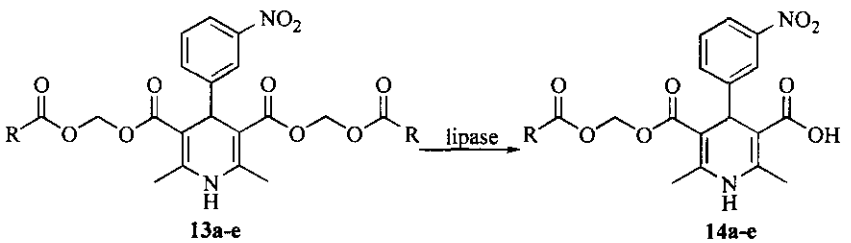
Lipase B-*Pseudomonas fragilipase*, [a]isolated yields, [b]based on HPLC analysis using column packed with Chiracel OJ, [c]acetone, c = 0.5-1

Scheme 2



The effect of the acyl structure and type of solvent is well documented on the hydrolysis[10] of bis(acyloxymethyl) esters of 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylic acid (**13a-e**) by *Pseudomonas fluorescens* lipase AH and *Pseudomonas cepacia lipase* PS (Table 4).

Table 4: Lipase-catalysed enantioselective hydrolysis of **13a-e**



13a-e **14a-e**

	a	**b**	**c**	**d**	**e**
R	$(CH_3)_3C$	$(CH_3)_2CH$	n-C_3H_7	C_2H_5	CH_3

Substrate	Lipase	Water-saturated IPE				Water-saturated cyclohexane			
		Time (h)	Yield	ee (%)	Abs.conf.	Time (h)	Yield	ee (%)	Abs.conf.
13a	AH	48	87	>99.0	*S*	48	88	88.8	*R*
13a	PS	200	8	73.5	*R*	200	23	97.0	*R*
13b	AH	24	83	89.0	*S*	48	71	91.0	*R*
13b	PS	20	34	86.0	*R*	48	32	99.0	*R*
13c	AH	24	71	88.9	*S*	48	62	91.1	*R*
13c	PS	20	91	96.0	*R*	48	29	99.0	*R*
13d	AH	1	83	68.1	*S*	17	57	91.4	*R*
13d	PS	24	86	99.0	*R*	72	31	92.0	*R*
13e	AH	5	77	42.3	*S*	17	32	88.2	*R*
13e	PS	24	87	>99.0	*R*	72	28	>99.0	*R*

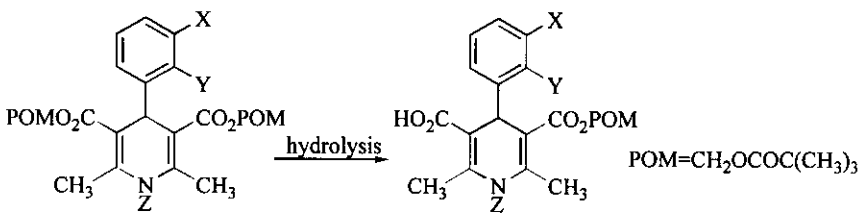
Lipase: AH-*Pseudomonas fluorescens lipase AH*, PS-*Pseudomonas cepacia lipase PS*, IPE-diisopropyl ether.

According to Table 4 it is evident that in water-saturated diisopropyl ether the bulkiness of the acyloxymethyl moiety affected the optical purity of enantiomers produced by above mentioned lipases. This effect was not so clear when the hydrolysis was carried out in cyclohexane saturated with water. It is

however important to note, that hydrolysis in water-saturated cyclohexane catalysed by *Pseudomonas fluorescens* lipase AH gave opposite enantiomer than the reaction in diisopropyl ether. The influence of the solvent in the enantioselectivity has been explained as a consequence of the different solvatation of both enantiomers by the solvent molecules (related to polarity and geometry of the solvent).[11] Nevertheless the structure of the lipase is very important because lipase PS does not alter the enantiopreference by the solvent.

Other structural variations of dihydropyridine diesters can affect the lipase-catalysed hydrolysis/alcoholysis, specially *N*-protection of the nitrogen in dihydropyridine ring. Moreover, synergic effect of both the substituent in position 4- and *N*-protecting group can occur. Thus hydrolysis of bis(pivaloyloxymethyl) ester of *N*-methoxymethyl-4-(3-nitrophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylic acid (**15a**) by lipase AH gave the corresponding hemiester (**16a**) in higher ee compared to the non-protected diester (**15b**). On the contrary, when 4-(2,3-dichlorophenyl) derivatives (**15c,d**) were used, the non-protected form (**15d**) gave better results.

Table 5: Enzyme and solvent effects on lipase-catalysed hydrolysis of 1,4-dihydropyridines (**15a-d**)



15a-d (*R*)- or (*S*)-**16a-d**

Substrate				Lipase	Solvent	Time	Product (**16**)		
No.	X	Y	Z			(h)	Yield (%)[a]	ee (%)[b]	Abs. conf.
15a	NO_2	H	CH_2OCH_3	AH	IPE	4	95	>99	*S*
15a	NO_2	H	CH_2OCH_3	PS	IPE	72	44	69	*R*
15b	NO_2	H	H	AH	IPE	35	83	93	*S*
15b	NO_2	H	H	AH	CH	48	88	89	*R*
15c	Cl	Cl	CH_2OCH_3	AH	IPE	6	81	96	*S*
15c	Cl	Cl	CH_2OCH_3	PS	IPE	300	19	81	*S*
15c	Cl	Cl	CH_2OCH_3	AH	CH	80	62	93	*S*
15d	Cl	Cl	H	AH	IPE	360	78	>99	*S*
15d	Cl	Cl	H	AH	CH	312	88	47	*R*

[a] Isolated yields, [b] ee determined by HPLC analyses by using chiral columns, IPE=diisopropyl ether, CH=cyclohexane

Moreover, the reversal in enantioselectivity in cyclohexane was suppressed in case of the protected 4-(2,3-dichlorophenyl) derivative (**15c**) (Table 5).[12] Based on these results, (*S*)-(-)-**NB 818** and (*R*)-(+)-nilvadipine were synthesised using *Pseudomonas fluorescens* lipase AH in diisopropyl ether or cyclohexane saturated with water.[13,14]

Among other *Pseudomonas candida* lipases used for methanolysis or hydrolysis of *N*-protected and unprotected 1,4-dihydropyridine diesters, lipase AK (*Pseudomonas* sp.) changed its stereopreference from pro-*R* to pro-*S* when *N*-methoxymethyl group was used for protection of nitrogen in the dihydropyridine ring.[15] The use of benzyl as protective group generally produces hemiesters possessing low or moderate ee.[15]

Both *Pseudomonas* lipases AH and PS belong to the same class of serine hydrolases and possess the same number of amino acids in their active center with similar sequences, the differences being in sixteen amino acids.[16] This difference causes however significant changes in the behaviour of the above mentioned lipases towards 1,4-dihydropyridine diesters. To elucidate the role of particular amino acids of the active center in enantioselectivity of lipases, Achiwa *et al.* prepared twenty lipases by site mutagenesis of lipase PS.[16] Among them, only the mutant possessing three amino acids replaced (Val266 - Leu, Leu 287 - Ile, Phe221 - Leu) changed in its enantioselectivity, substrate requirements and behaviour in organic solvents. All these new properties were closely similar to the properties of *Pseudomonas fluorescens* lipase AH.

Table 6: Enantioselective hydrolysis of **17**

Enzyme	Origin	Yield [%][a]	ee [%][b]
Seaprose S	*Aspergillus melleus*	99	>99
Protease P6	*Aspergillus melleus*	99	>99
Acylase 30,000	*Aspergillus sp.*	90	>99
Protease Type VIII	*Bacillus licheniformis*	41	>99
Deamyzime®	*Aspergillus sp.*	24	>99
Pronase E	*Streptomyces griseus*	10	>99
Protease Type XIX	*Aspergillus sojae*	7	>99

[a] Yields determined by HPLC analysis, [b] ee determined by HPLC using chiral column

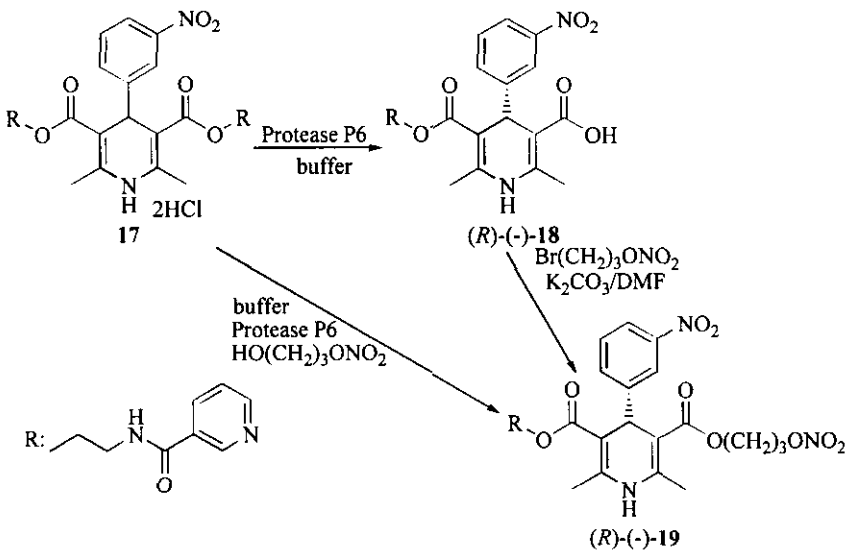
Esterases and proteases differ from lipases in their requirement for water-soluble substrates in ester hydrolyses. As an example of the solubilization of dihydropyridine can serve the synthesis[17] of bis[2-(nicotinoylamino)ethyl] ester (**17**). 84 Enzymes were tested for hydrolysis of this compound in buffer solutions, protease P6 and seaprose S (*Aspergillus melleus*) being the best ones (Table 6). High chemical yields were obtained due to only one step hydrolysis of the diester, the second undesired step occurred only to a negligible extent.

Use of protease P6 and Seaprose S (both from *Aspergillus melleus*) for desymmetrisation of such diesters differs from lipase catalysed transformation in two important points:

1. Protease attacks directly the carboxyl group of DHP ester skeleton instead of primary hydrolysis of the amide bond
2. In the presence of alcohols, these proteases catalyse transesterification of the ester even in excess of water or buffer.

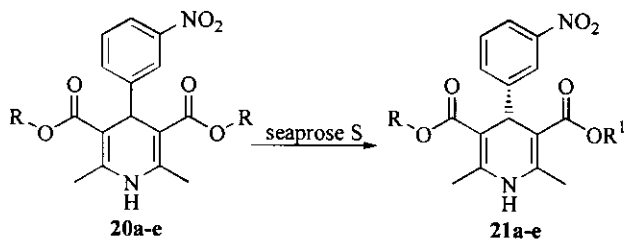
Attacking of ester moiety has been proven by presence of 2-(nicotinoylamino)ethanol in the reaction mixture as well as by direct alcoholysis. Using 1,3-propanediol mononitrate as nucleophile, a new calcium(II) channel modulator ((*R*)-**19**) as more active enantiomer was prepared directly (Scheme 3).[17]

Scheme 3



Achiwa *et al.* tested hydrolysis and acidolysis of activated dihydropyridine esters (**20a-e**) with Seaprose S as catalyst (Tables 7 and 8).[18,19] Again, direct alcoholysis by aqueous methanol was observed as well.[19]

Table 7: Seaprose S-catalysed enantioselective hydrolysis of **20a-e**



20a-e **21a-e**

	a	**b**	**c**	**d**	**e**
R	$(CH_2)_2CN$	$(CH_2)_2SO_2CH_3$	CH_2CONH_2	$CH_2CO_2C_2H_5$	CH_2OCH_3

Substrate	Time [h]	R^1	Yield [%]	ee [%][a]	$[a]_D^{20b}$
20a	72	H	87	>99	-44.0
20b	72	H	50	>99	-60.9
20c	20	H	83	>99	-61.9
20d	72	H	60	>99	-55.8
20e	72	H	54	98	-35.0

[a]ee Determined by HPLC on a Chiracel OJ column, [b]for **21a,b,d,e** acetone c = 0.4-0.8; for **21c** ethanol, c = 0.473

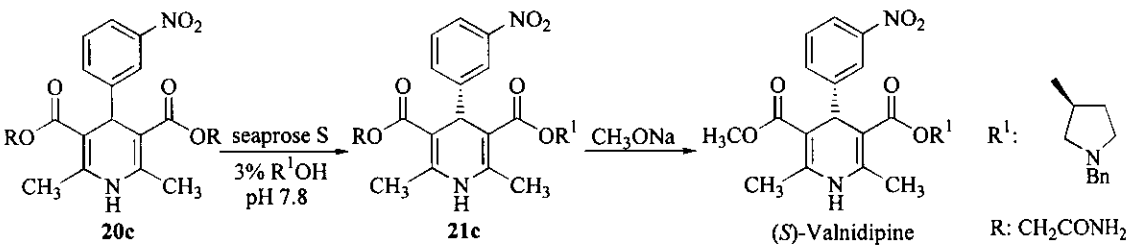
Table 8: Seaprose S-catalysed enantioselective transesterification of **20a-e**

Substrate	Time [h]	R^1	Yield [%]	ee [%][a]	$[a]_D^{20b}$
20a	120	CH_3	35	99	-32.6
20b	120	CH_3	48	99	-64.0
20c	20	CH_3	83	99	-16.0
20d	48	CH_3	52	99	-34.8
20e	120	CH_3	26	99	-15.5

[a] ee determined by HPLC on a Chiracel OJ or AS column; [b] acetone, c = 0.4-0.8

(*S*)-Valnidipine was prepared by transesterification of **20c** by (*S*)-*N*-benzyl-3-pyrrolidinol followed by chemical transesterification with sodium methoxide (Scheme 4), ee of the final product being more than 99%.[19]

Scheme 4



20c **21c** (*S*)-Valnidipine

Hydrolases were used also for kinetic resolution of non-symmetric 1,4-dihydropyridine diesters. This non-symmetry may be caused both by different substituents in position 2 and 6 and/or by different alcohol part of ester groups in positions 3 and 5. According to the results of de Castro *et al.*,[20] enantioselectivity of *Pseudomonas* lipases AK and PS in hydrolysis (Scheme 5) of racemic acetoxymetyl, ethyl 2,6-dimethyl-1,4-dihydro-4-phenyl-3,5-pyridinedicarboxylate (**22a,b**) remained the same as with prochiral bis(acetoxymethyl) analogues (**15a-d**) (compare Tables 5 and 9).

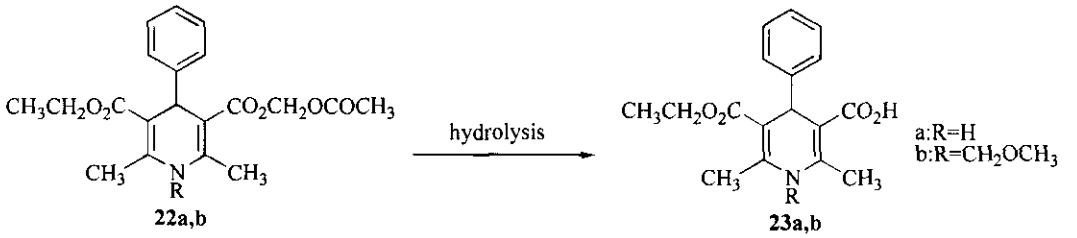
Table 9:

Effect of substituent in nitrogen on lipase-catalysed hydrolysis of prochiral 1,4-dihydropyridines (**22a,b**)

Substrate	Lipase	Time [h]	Yield of **22** [%][a]	Yield of **23** [%][a]	ee of **22** [%][b]	ee of **23** [%][b]
22a	AK	26.5	50	30	15 (*R*)	20 (*R*)
22a	PS	24.0	51	39	70 (*R*)	70 (*R*)
22b	AK	7.0	42	51	95 (*S*)	63 (*S*)
22b	PS	11.0	39	39	18 (*R*)	24 (*R*)

[a] Isolated yields, [b] ee determined by ^{1}H NMR ($Eu(hfc)_3$)

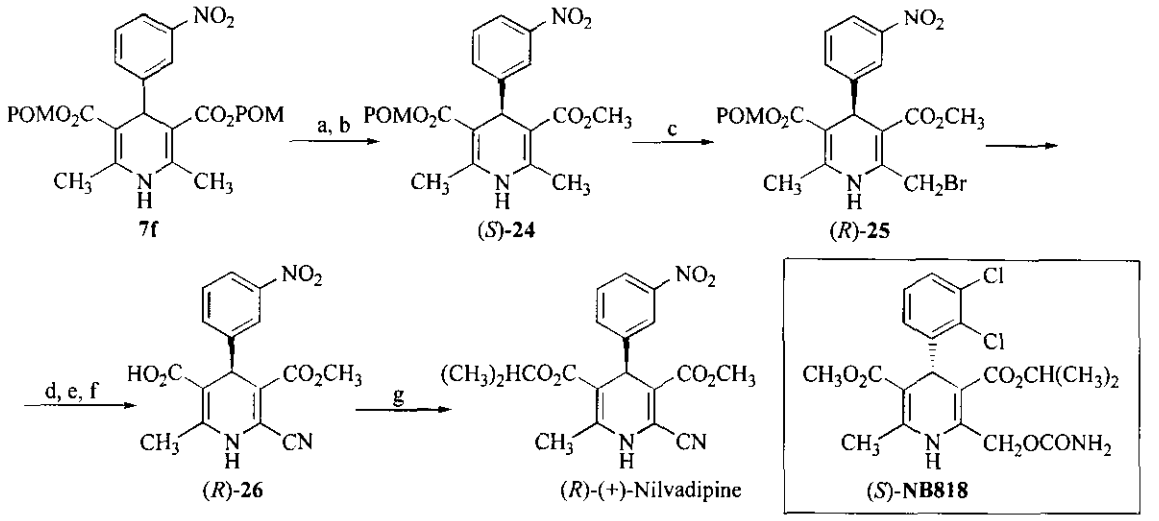
Scheme 5



2.2 Kinetic resolution of racemic substrates

Preparation of chiral 1,4-dihydropyridines with different substituents in positions 2 and 6 is possible by enzymatic hydrolysis or alcoholysis of prochiral diesters (**7a-f**) (Table 2) followed by regioselective bromination of methyl group on dihydropyridine ring and transformation of bromomethyl derivative to the desired compound.[13,14] Although chiral intermediate (**24**) could be obtained in good yield and ee (83% resp. 93%), other steps, particularly oxidation of 2-bromomethyl derivative (**25**) to an aldehyde and esterification of **26** (Scheme 6), significantly decrease overall yield of (*R*)-(+)-nilvadipine (only about 1%). (*S*)-**NB 818** was prepared in similar manner.[14]

Scheme 6



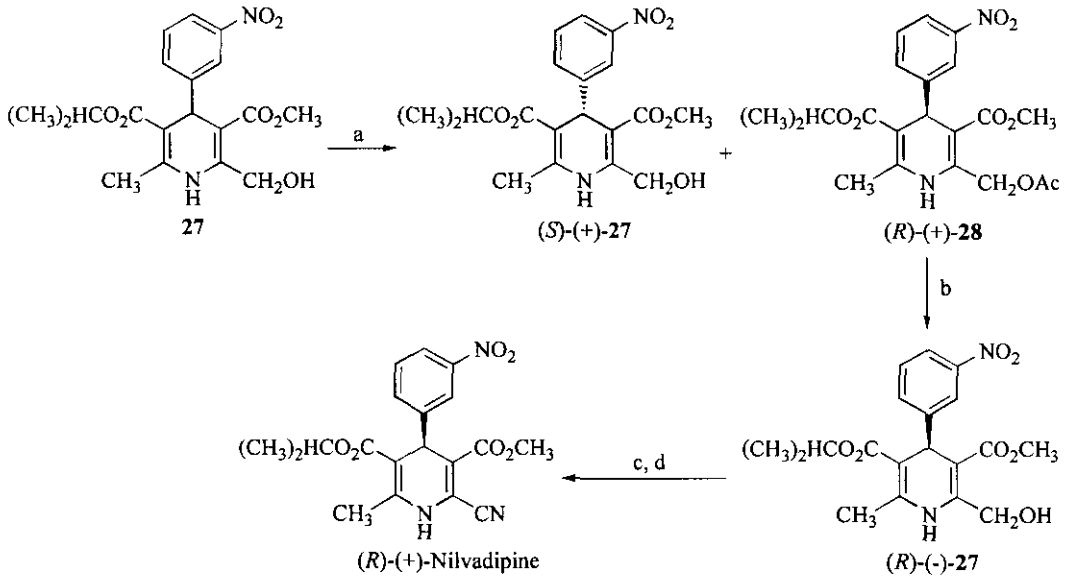
POM: $(CH_3)_3CCO_2CH_2$

Reagents: a: lipase AH/IPE/H_2O, b: CH_2N_2/acetone, c: DMAP.HBr_3/pyridine/CH_2Cl_2, d: $NaHCO_3$ /DMSO, e: $NH_2OH.HCl$/AcONa/AcOH/Ac_2O, f: KOH/MeOH, g: $SOCl_2$/2-propanol

Second approach in the synthesis of chiral 2-substituted-1,4-dihydropyridines consists in kinetic resolution of racemic substrates. However, the theoretical yields for one enantiomer are 50% at their best.

Direct kinetic resolution of nilvadipine precursors showed the same enantiopreference in both hydrolysis of acetoxymethyl and esterification of free hydroxymethyl group catalysed by cholesterol esterase and lipase PS (Tables 10 and 11).[21]

Scheme 7



Reagents: a: lipase/vinyl acetate; b: NH_3/MeOH; c: 1) $(COCl)_2$/DMSO/CH_2Cl_2, -78 °C, 2) Et_3N; d: 1) $NH_2OH.HCl$/AcOH/AcONa, 2) Ac_2O

Table 10: Enzyme-catalysed esterification of racemic dihydropyridines (**27**)

Enzyme	Solvent	Time	(*S*)-(+)-**27**		(*R*)-(+)-**28**	
		(h)	Yield (%)	ee (%)[a]	Yield (%)	ee (%)[a]
lipase AH	Acetone	33	49	68	46	90
CHE	Acetone	19	49	29	41	35
lipase PS	Acetone	41	46	78	40	89
lipase PS	DMF	136	52	40	26	46
lipase PS	THF	71	46	78	44	89
lipase PS	Acetone[c]	136	40	76	39	89
lipase PS	Acetone[c]	64	40	68	38	82
lipase PS	Acetone[d]	44	42	97	55	72

[a] ee Determined by HPLC analysis using a column packed with Chiracel AS, [c] at 0°C, [d] at 40°C

Table 11: Enzyme-catalysed hydrolysis of racemic dihydropyridines **(28)**

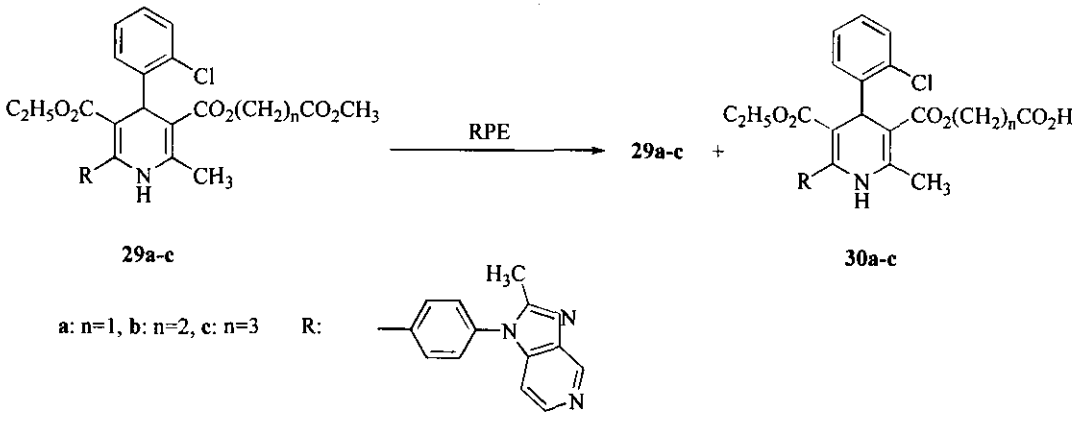
Enzyme	Solvent	Time (h)	(*S*)-(-)-**28**		(*R*)-(-)-**27**	
			Yield (%)[a]	ee (%)[b]	Yield (%)[a]	ee (%)[b]
lipase PS	IPE	96	41	74	36	81
CHE	IPE	24	32	39	50	25

[a] Isolated yields, [b] ee determined by HPLC analysis using a column packed with Chiracel AS

The best results were obtained in esterification of **27** with vinyl acetate catalysed by lipase PS. Derivative [(*R*)-(+)-**28**] was converted to the desired (*R*)-(+)-nilvadipine by standard means (Scheme 7).[21] Also both enantiomers of **NB-818** could be prepared using this method.[22]

As an example of enantioselective transformations of dihydropyridines possessing chirality due to different substituents both in positions 3-, 5- and 2-, 6- may serve also the esterase catalysed hydrolysis of precursors (**29a-c**) of UK-74,505 (Scheme 8), a chiral antagonist of platelet activating factor (PAF) **30.**[23] The precursors with an ester group separated from dihydropyridine skeleton by a long chain undergo easy enzymatic hydrolysis. Unfortunately, the distance from the chiral center decreases the enantioselectivity of the reaction. The best results were observed for rabbit liver esterase, although high degree of conversion (90%) is however necessary to recover the enantiopure starting ester (**29a**).

Scheme 8



3. CONCLUSION

Chemoenzymatic methods represent a promising alternative to classical strategies to obtain pure enantiomers of dihydropyridines possessing important biological activities. Although these enzymatic processes need further optimisation, the results on this field indicate the potential of such syntheses.

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