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Investigation of lipase-catalyzed Michael-type carbon–carbon bond formations

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ABSTRACT

Conjugate additions of carbon nucleophiles to appropriate acceptor molecules were investigated with respect to the synthetic potential and stereochemistry of the products. Reactions of short-chain α,β-unsaturated ketones and mono-substituted nitroalkenes with CH-acidic carboxylic ester derivatives were catalyzed by various immobilized lipases. Using methyl nitroacetate complete conversion with methyl vinyl ketone and *trans*-β-nitrostyrene was obtained. The reactions proceeded without enantioselectivity. Evidence for enzyme catalysis is provided by the observation that after denaturation of *Candida antarctica* lipase B or inhibition no reaction took place. Docking studies with the chiral addition product methyl 2-methyl-2-nitro-5-oxohexanoate did not reveal any specific binding mode for this reaction product, which would have been the requirement for stereoselective additions. These results support the experimental findings that the conjugate addition takes place without enantiopreference.

1. Introduction

Conjugate additions, such as the Michael addition, represent fundamental reactions in synthetic organic chemistry to produce carbon–carbon and carbon–heteroatom bonds. With this type of reaction up to three chiral centres can be generated in a single event with perfect atom-economy. Classical Michael additions are performed under acid/base catalysis.[1] Moreover, several asymmetric procedures using organometallic catalysts or organocatalysts have emerged in the literature.[2]

Contrary to the importance for synthetic chemistry in nature enzyme-catalyzed Michael-type reactions have not been as well studied yet. Examples are transformations by enoate reductases,[3] some ligases[4] and a few other enzymes.[5] Non-natural enzymatic reactions[6] for conjugate additions were reported with *N*-ethyl-maleimide[7] and hetero-nucleophiles adding to α-trifluoromethyl acrylic acid[8,9] or cinnamic esters,[10] catalyzed by serine hydrolases and bakers yeast. In the latter cases the authors could demonstrate that these additions proceeded with moderate to good enantioselectivity.[9,10] More recently, several reports on conjugate additions of hetero-nucleophiles to various acceptors catalyzed by proteases and lipases were published.[11,12] The few successful examples of enzyme-catalyzed carbon–carbon bond formations via Michael additions were mainly restricted to acetylacetone, acetoacetates and malonates in combination with α,β-unsaturated carbonyl compounds.[13,14] From the data from literature it was apparent that only highly activated donor molecules like acetylacetone and ethyl acetoacetate were able to react. Most of the products reported did not possess a chirality centre and therefore no conclusions on the stereochemical course of the reactions could be drawn. To elucidate a potential enzyme-catalyzed Michael addition we decided to investigate this biocatalytic transformation.

2. Results and discussion

2.1. Michael additions of methyl nitroacetate (2a) and of methyl 2-nitropropionate (2b) to 3-buten-2-one (1)

At the beginning of our investigations it was necessary to ascertain conditions under which enzyme-catalyzed Michael additions were least affected by non-enzymatic acid/base catalysis. It quickly turned out that buffered aqueous solutions were not applicable due to a fairly strong base-induced activation of reactants thus leading to dominant background reactions. Moving from aqueous conditions to organic solvents required the careful selection of immobilization techniques in order to avoid mass transfer limitations associated with suspended powders or the use of large amounts of enzymes. We therefore investigated several solid supports, which had already been used for the immobilization of enzymes by deposition or adsorption. Deposition onto Celite[15] was quickly ruled out because of the strong activity to catalyze our model reaction, the Michael addition of methyl nitroacetate (**2a**) to 3-buten-2-one (**1**). Lewatit VP OC 1600[16] and Amberlite XAD-7,[17] which are well known supports

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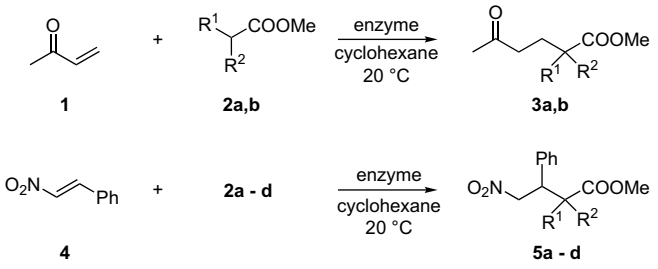
for the immobilization of lipases by adsorption, catalyzed the model reaction remarkably strong and were therefore also excluded from the studies. At the end only Accurel MP1000 remained as suitable support for the investigation of enzyme-catalyzed carbon–carbon bond formations via Michael addition. Furthermore, using Accurel MP1000[15] it was also possible to deposit the lyophilized enzymes on the carrier. The results obtained with immobilized lipases via deposition and adsorption after 24 h did not differ significantly in the case of CALB. Initial screenings of α,β-unsaturated ketones and aldehydes in the presence of immobilized CALB and CH-acidic compounds comprising malonates, β-ketoesters, acetylacetone and activated acetates revealed only a few successful reactions. Moreover, only the donor molecule methyl nitroacetate (**2a**) reacted noticeably with α,β-unsaturated carbonyl compounds during the time span of a few hours. Regarding the acceptor molecules it was not surprising that acrolein turned out as the most active substrate followed by 3-buten-2-one (**1**) and 1-penten-3-one. β-Substituted analogues like *trans*-4-phenyl-3-buten-2-one did not react at all. Since in reactions with methyl nitroacetate (**2a**) acrolein gave a product mixture we used 1-buten-3-one (**1**) as model compound, which gave a clean Michael addition to a single product. All immobilized lipases were screened for their efficiency to catalyze the conjugate addition (see Scheme 1) using 0.2 M solutions of reagents in cyclohexane at room temperature.



2, 3, 5	R^1	R^2
a	H	NO_2
b	CH_3	NO_2
c	H	COMe
d	$-(CH_2)_3CO-$	

Scheme 1. Enzyme-catalyzed Michael additions of nitroesters (**2a**) and (**2b**) to 3-buten-2-one (**1**), respectively, nitroesters (**2a**) and (**2b**) and β-ketoesters (**2c**) and (**2d**) to *trans*-β-nitrostyrene (**4**).

The experiments clearly identified *Candida antarctica* lipases A and B along with the lipases from *Mucor miehei* and porcine pancreas as efficient catalysts for the Michael-addition reaction. Besides the addition reaction we always observed a loss of methyl nitroacetate (**2a**) during the reaction which most likely resulted from lipase-catalyzed ester hydrolysis caused by traces of water in the system. This latter fact might be the reason why some enzyme-containing reactions gave lower conversions than Accurel alone. In accordance with the literature examples concerning the formation of carbon–carbon bonds we also noticed a better conversion with the Ser105Ala mutant[13] compared to wild-type CALB. An explanation for this result might be that the partial deletion of the catalytic triad by the replacement of Ser105 against non-coordinating Ala105 makes the enzyme more prone to non-natural reactions. Moreover, the possibly enhanced activation of the acceptor molecule and easier deprotonation of methyl nitroacetate (**2a**) by a more basic His224 might also be the reason for improved Michael-addition activity.[12,13,18] Using a higher loading of the lipase on the carrier and an excess of methyl nitroacetate (**2a**) it was possible to convert 3-buten-2-one (**1**) almost completely into the corresponding product (entry 20, Table 1).

Table 1
Michael-addition reactions between 3-buten-2-one **1** (0.2 M) and methyl nitroacetate **2a** (0.2 M) in cyclohexane at 20 °C, catalyzed by various immobilized lipases

No.	Manufacturer's code[a]	Catalyst[b]	pH[c]	Conv. [%][d]
1	ICR-101	*Aspergillus* sp. lip.	—	8
2	ICR-102	*Rhizopus* sp. lip.	—	11
3	ICR-103	*Rhizopus oryzae* lip.	—	6
4	ICR-104	*Penicillium* sp. I lip.	—	9
5	ICR-105	*Penicillium* sp. II lip.	—	16
6	ICR-106	*Candida rugosa* lip.	—	5
7	ICR-107	*Pseudomonas cepacia* lip.	—	7
8	ICR-109	*Pseudomonas fluorescens* lip.	—	9
9	ICR-110	CALB	—	46
10	ICR-111	*Candida* sp. lip.	—	7
11	ICR-112	CALA	—	63
12	ICR-113	*Pseudomonas* sp. lip.	—	7
13	ICR-114	Porcine pancreas lip.	—	27
14	ICR-115	*T. lanuginosas* lip.	—	10
15	ICR-116	*M. miehei* lip.	—	40
16	ICR-117	*Alcaligenes* sp. lip.	—	14
17	ICR-128	Lipase modified by directed evolution for large substrates	—	15
18		CALB WT	7.65	10
19		CALB Ser105Ala	7.65	26
20		CALB (2.5% w/w)	7.65	99[e,f]
21		CALB (2.5% w/w) denatured	7.65	1[e]
22		CALB (2.5% w/w) inhibited	7.65	1[e]
23		Accurel MP1000		11[e]
24		BSA (1.26% w/w) on Accurel	7.00	42
25		Blank		1[e]

[a] BioCatalytics Inc.
[b] Immobilized on Accurel MP1000 (1.6% w/w).
[c] pH not adjusted or pH of the sodium phosphate immobilization buffer.
[d] Conversion after 16.5 h at 20 °C.
[e] Conversion after 20 h at 20 °C.
[f] Methyl nitroacetate (0.4 M) **2a**.

In contrast 1-penten-3-one gave a conversion of 45% under the same conditions indicating strong substrate dependence. Most noticeably immobilized bovine serum albumin catalyzed the conjugate addition fairly strong, which points out that some proteins without enzyme function can also catalyze conjugate additions.[19] Therefore one has to prove that the catalytic activities of the lipases for Michael-addition reactions did not arise from unspecific protein-derived activation of the reagents by, e.g., the surface of the enzyme. It was experimentally verified that inhibition and denaturation of CALB caused a complete disruption of the catalytic activity of the enzyme leading to a similar conversion as found for the blank reaction. As a consequence one must conclude that the enzyme-catalyzed Michael addition occurs in the active site of the enzyme or in close proximity. On the other hand one can assume that the protein surface of CALB is predominately catalytically inactive in the process. Having discovered active lipases for the catalysis of conjugate additions of methyl nitroacetate (**2a**) to 3-buten-2-one (**1**) we became encouraged to investigate the stereochemical course of the transformations. Because the hitherto used reactions (Scheme 1) furnished products, which tend to racemize in the α-positions, thereby loosing all chiral information from potential stereoselective additions, we decided to replace the donor molecule **2a** by methyl 2-nitropropanoate (**2b**). This starting material has a maximum of structural similarity to methyl nitroacetate (**2a**) but leads to a racemization-free quaternary centre in the product. Though the substituents are not very different in size in this case it seems feasible to obtain enantio-enriched products in enzyme-catalyzed Michael additions if there was any preferred stereochemical course of the reaction (see Scheme 1).

As depicted in Table 2 the Michael-addition reactions of 2-nitropropanoate (**2b**) to 3-buten-2-one (**1**) were catalyzed by various immobilized lipases but the conversions were significantly lower than with methyl nitroacetate (**2a**) although the reaction

Table 2
Michael-addition reactions between 3-buten-2-one (**1f**) (0.6 M) and methyl 2-nitropropanoate (**2b**) (0.2 M) in cyclohexane at 20 °C, catalyzed by various immobilized lipases, pH as in Table 1

No.	Manufacturer's code[a]	Catalyst[b]	Conv. [%][c]	e.e. [%]
1	ICR-101	*Aspergillus* sp. lip.	4	0
2	ICR-102	*Rhizopus* sp. lip.	6	0
3	ICR-103	*R. oryzae* lip.	2	0
4	ICR-104	*Penicillium* sp. I lip.	9	0
5	ICR-105	*Penicillium* sp. II lip.	2	0
6	ICR-106	*C. rugosa* lip.	2	0
7	ICR-107	*P. cepacia* lip.	1	0
8	ICR-109	*P. fluorescens* lip.	1	0
9	ICR-110	CALB	9	0
10	ICR-111	*Candida* sp. lip.	1	0
11	ICR-112	CALA	16	0
12	ICR-113	*Pseudomonas* sp. lip.	2	0
13	ICR-114	Porcine pancreas lip.	10	0
14	ICR-115	*T. lanuginosas* lip.	5	0
15	ICR-116	*M. miehei* lip.	10	0
16	ICR-117	*Alcaligenes* sp. lip.	8	0
17	ICR-128	Lipase modified by directed evolution for large substrates	10	0
18		Accurel MP1000	2	0

[a] BioCatalytics Inc.
[b] Immobilized on Accurel MP1000 (1.6% w/w).
[c] Conversion after 40 h at 20 °C using 0.2 M methyl 2-nitropropanoate (**2b**) and 0.6 M 3-buten-2-one (**1**) in cyclohexane.

time and the amount of unsaturated ketone were increased (0.2 M **2b** and 0.6 M **1** as compared to 0.2 M **2a** and 0.2 M **1**). The most active enzymes in the reaction with methyl 2-nitropropanoate (**2b**) match exactly those four most efficient ones mentioned in Table 1. However, in product formation no enantioselectivity was found.

Taking these results one must conclude that the Michael additions investigated are completely unselective in terms of enantiomer formation although a certain type of interaction in the active site of enzymes must have occurred. This assumption is again supported by the observation that inhibited CALB (CALB treated with methyl *p*-nitrophenyl *n*-hexylphosphonate) was not catalytically active at all (conversion <1%). The reaction takes place via the attack of methyl 2-nitropropanoate (**2b**) at the β-position of 3-buten-2-one (**1**), forming a single chiral centre at the quasi γ-position. The chiral centre itself stems exclusively from the donor molecule, which is initially deprotonated, thereby loosing the original chiral information, and then covalently bound to the acceptor molecule. Apparently the lack of enantioselectivity must be attributed to the unselective addition of methyl 2-nitropropanoate (**2b**) to the α,β-unsaturated ketone (**1**), where within the active site no attack from a preferred direction occurs. The donor molecule's substituents might not be sufficiently diverse to ensure a discrimination of reaction pathways during the conjugate addition. All further attempts to gain additional information on the selectivity at the β-position failed because the alternative substrates benzalacetone and cinnamic aldehyde did not react under these conditions. It is worth mentioning that the literature on CALB and CALB S105A catalyzed reactions of thiol heteronucleophiles with similar acceptor substrates also reports the lack of any enantioselectivity.[12]

2.2. Michael additions to *trans*-β-nitrostyrene (4)

Since in conjugate addition reactions α,β-unsaturated ketones turned out to be rather unreactive substrates we were seeking some more reactive Michael acceptors. During our initial screenings of activated olefins with various CH-acidic compounds in the presence of Novozyme 435 we had discovered high reactivities with aliphatic and aromatic nitroalkenes. Due to the higher reactivity of these substrates it became feasible to study the stereochemistry of conjugate addition to give products with more than one stereocentre.

Table 3
Michael-addition reactions between *trans*-β-nitrostyrene (**4**) (0.1 M) and methyl nitroacetate (**2a**) (0.1 M), methyl 2-nitropropanoate (**2b**) (0.1 M), methyl 2-oxocyclopentanecarboxylate (**2d**) (0.2 M) or methyl 3-oxobutanoate (**2c**) (0.2 M) in cyclohexane at 20 °C, catalyzed by various immobilized lipases

No.	Manufacturer's code[a]	Catalyst[b]	**5a**		**5b**		**5d**		**5c**	
			Conv. [%][c]	d.r.[d]	Conv. [%][c]	d.r.[d]	Conv. [%][c]	d.r.[d]	Conv. [%][c]	d.r.[d]
1	ICR-101	Aspergillus sp. lip.	26	1.3	1	2.8	3	6.7		
2	ICR-102	*Rhizopus* sp. lip.	7	1.1						
3	ICR-103	*R. oryzae* lip.	4	1.3						
4	ICR-104	*Penicillium* sp. I lip.	4	1.2						
5	ICR-105	*Penicillium* sp. II lip.	10	1.2						
6	ICR-106	*C. rugosa* lip.	3	1.4						
7	ICR-107	*P. cepacia* lip.	6	1.4						
8	ICR-109	*P. fluorescens* lip.	16	1.2						
9	ICR-110	CALB	40	1.0	17	1.2	8	4.9		
10	ICR-111	*Candida* sp. lip.	7	1.1						
11	ICR-112	CALA	67	1.1	34	1.2	35	4.0	65	0.9
12	ICR-113	*Pseudomonas* sp. lip.	29	1.1	1	2.2	3	5.8		
13	ICR-114	Porcine pancreas lip.	28	1.1	3	2.0	6	4.4		
14	ICR-115	*T.s lanuginosas* lip.	70	1.1	3	1.3	17	4.9		
15	ICR-116	*M. miehei* lip.	89	1.2	8	1.2	3	4.9		
16	ICR-117	*Alcaligenes* sp. lip.	37	1.1	2	2.5	5	4.7		
17	ICR-128	Lipase modified by directed evolution for large substrates	37	1.1	2	2.6	5	3.9		
18		CALB WT	59	1.1	8	1.8	5	4.3	3	1.0
19		CALB Ser105Ala	52	1.1	19	1.8	15	5.0		1.1
20		CALB (2.5% w/w)	91	1.1					7	
21		CALB (2.5% w/w) denatured	1							
22		CALB (2.5% w/w) inhibited	2							
23		Accurel MP1000	8	1.1	0	—	1	—	1	—
24		BSA (1.26% w/w) on Accurel	13	1.1	13	1.2	17	5.8	2	1.0
25		Blank	0		0	—	1	—	0	—
		Reference substance		1.1		1.2		4.1		1.4

[a] BioCatalytics Inc.
[b] Immobilized on Accurel MP1000 (1.6% w/w).
[c] Conversion after 20 h determined by chiral HPLC.
[d] Diastereomeric ratio determined by chiral HPLC.

This special interest originates from a report of Kitazume et al. on substituted 3,3,3-trifluoropropanoic acids, whose chiral α-positions were created via Michael additions of hetero-nucleophiles to 2-trifluoromethyl acrylate in the presence of hydrolases.[9]

The Michael-addition experiments were run in cyclohexane at 20 °C using 0.1 M *trans*-β-nitrostyrene (**4f**) and 0.1 M nitroester **2a** or **2b**, respectively, 0.2 M β-ketoester **2c** and **2d**, in the presence of 20 mg/mL of immobilized enzyme. This was half the concentration used in the reactions with α,β-unsaturated ketones. Successful conjugate additions of this type (see Scheme 1) lead to products with two chiral centres in the β- and γ-position with respect to the acceptor molecule, thus providing more information on the steric course of the reaction. The transformation of *trans*-β-nitrostyrene (**4**) and methyl nitroacetate (**2a**) turned out to be the most reactive combination with conversions up to 90% after 20 h (Table 3). Conversions greater than 60% were found with immobilized lipases from *M. miehei*, *Thermomyces lanuginosas* and *C. antarctica* (type Aand B). The lipases from *Aspergillus* sp., *Pseudomonas* sp., porcine pancreas, *Alcaligenes* sp. and the lipase ICR-128 gave conversions around 30–40%. *C. antarctica* lipase B and the S105A mutant, both expressed in *Pichia pastoris*, displayed roughly the same activity (Table 3, entries 19 and 20). All these conversions are significantly higher than those obtained with pure Accurel or immobilized albumin. Denatured and inhibited CALB were almost completely inactive. Apart from the conversions it is even more interesting to look at the stereochemical outcome of the reactions, which did not proceed with any preferred stereoselectivity. All products had a diastereomeric ratio of around 1.1 and no clear indications of any enantioselectivity. The product composition was virtually the same as obtained by non-stereoselective base catalyzed Michael addition and was obviously hardly affected by side reaction caused by the lipases' ability to catalyze partial hydrolysis with traces of water in the system. In the cases of other donor molecules we observe similar results as for methyl nitroacetate (**2a**) (Table 3).

Concerning the stereochemistry one can see that the diastereomeric ratio of products from experiments with decent conversions match those of the chemically synthesized reference substances. Minor deviations of the diastereomeric ratios were only found in cases of reactions with low yields. Disappointingly, we could not detect any enantioselectivity in any of the experiments performed. The S105A mutant of CALB was generally more active than the corresponding wild-type enzyme from the same expression host (Table 1, entries 19 and 20). Moreover it was also apparent that Accurel-supported bovine serum albumin was often among the best catalysts tested but could not introduce any chiral selection into the reaction products, either. Since *trans*-β-nitrostyrene (**4**) was, at least in combination with methyl nitroacetate (**2a**), a highly reactive acceptor molecule we also tested some other aromatic and an aliphatic analogues. More crowded *trans*-β-methyl-β-nitrostyrene and *trans*-4-fluoro-β-nitrostyrene did not react with methyl nitroacetate (**2a**) in the presence of CALA and higher-loaded CALB. *trans*-4-Dimethylamino-β-nitrostyrene, although only little soluble, gave a conversion of less than 10% in toluene under similar conditions. CALB catalysis for the reaction of the aliphatic nitroalkene *trans*-1-nitrohept-1-ene and methyl nitroacetate (**2a**) at a concentration of 0.2 M in cyclohexane gave a conversion of 35% after 20 h. Again in none of these further successful examples we could observe a stereoselective course of the reaction.

All attempts to convert 2-trifluoromethyl acrylate and the corresponding methyl ester with methyl nitroacetate (**2a**) in the presence of immobilized CALB and cyclohexane or toluene as solvents failed. Surprisingly, although acetylacetone is a reactive *C*-nucleophile, it did not react with *trans*-β-nitrostyrene (**4**) under our conditions.

Considering other successful examples for enzyme-catalyzed Michael additions in the literature one must take into account that the amounts of enzyme or concentrations of reagents used in these investigations were much higher.[13,14] The published data are therefore not fully comparable with our results. On the other hand using higher amounts of enzymes, higher concentrations of reagents or modified reaction conditions one can probably find even more, hitherto undiscovered examples of enzyme-catalyzed conjugate additions. However, stereoselective enzyme-catalyzed Michael additions with carbon-nucleophiles remain unknown so far.

3. Molecular modelling

A modelling of the interaction of the substrates with the amino acids of the active site was performed for an interpretation of the experimental result that the Michael additions investigated were catalyzed by the enzymes applied but no enantioselectivity was encountered.

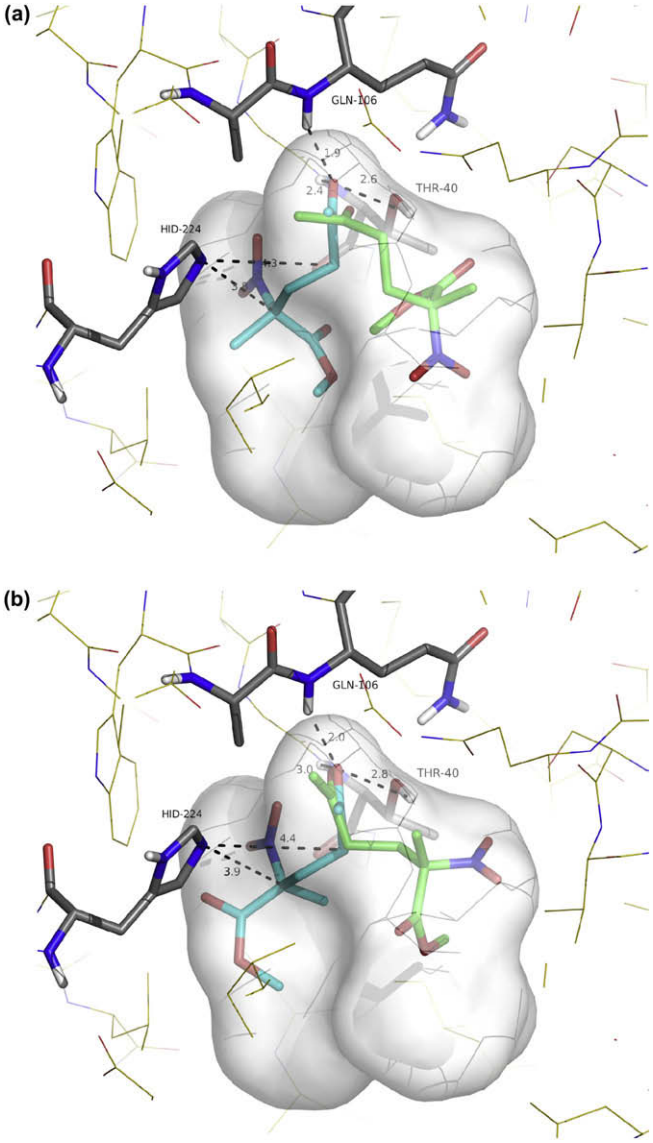


Figure 1. Docking results of methyl 2-methyl-2-nitro-5-oxohexanoate (**3b**) in CALB S105A. (a) Active site pocket with the (*R*)-enantiomer. The most frequent binding mode is coloured in green and the docked product located near the putative active site residues is coloured in cyan. (b) Active site pocket with the (*S*)-enantiomer. The most frequent binding mode is again coloured in green and the docked structure located near the active site residues is represented in cyan. The presentation of the docking results was created by using the PyMOL visualization program.

The docking of both enantiomers of methyl 2-methyl-2-nitro-5-oxohexanoate (**3b**) into the active site of the S105A mutant of the lipase from *C. antarctica* was carried out using the program AutoDock v. 3.05.[20] With the settings applied, the docked structures showed only insignificant energetic differences and many different binding modes (18 binding modes for the (*R*)-form and 14 binding modes for the (*S*)-form of **3b**). In each of the figures are depicted two examples for the (*R*)-enantiomer (Fig. 1a) and the (*S*)-enantiomer (Fig. 1b).

The most frequent binding mode is clustered at the opposite side of the putative active site residues Ala105, His224, Gln106 and Thr40 and is shown in green within the active site pocket in Figure 1. The binding mode closest to the putative active site residue (His224) with the lowest energy is coloured in cyan in Figure 1a for the (*R*)- and in Figure 1b for the (*S*)-enantiomer. The oxyanion hole is formed by the main chain polar hydrogens of the amino acids Gln106 and Thr40 as well as by the Thr40 side chain polar hydrogen.

A common feature of the docking results is that the carboxyl group binds in the oxyanion hole, which may indicate the activation of the acceptor via polarization. His224 may play a role as acid/base catalyst. Both enantiomers in fact have similar docked energies and similar preferred binding modes. The active site is very voluminous and our calculations do not indicate differential binding of (*R*)- and (*S*)-enantiomers of the docked product methyl 2-methyl-2-nitro-5-oxohexanoate (**3b**). Therefore we conclude that a stereoselective course of the enzyme-catalyzed Michael addition is very unlikely from a theoretical point of view. All these facts support the experimental findings that all reactions presented herein proceeded without detectable stereoselective guidance by the enzymes used.

4. Conclusion

Several novel lipase-catalyzed Michael-type carbon–carbon bond formations for the addition of CH-acidic esters to small size α,β-unsaturated ketones and nitroalkenes are reported. Methyl nitroacetate (**2a**) turned out as the most active donor molecule, which led to almost complete conversion in reactions with methyl vinyl ketone (**1**) and *trans*-β-nitrostyrene (**4**) within 20 h. More sterically demanding donor and acceptor substrates were less reactive or even completely unreactive. Only a limited number of enzymes tested, such as CALA, CALB and the lipases from *M. miehei* and *T. lanuginosas*, catalyze all or most of the investigated conjugate additions appropriately. The reactions required strong electron withdrawing substituents at the alkene substrate and highly nucleophilic CH-acidic compounds to proceed. The activity of the ester derivatives corresponded to the inverse order of pK_a values, descending from methyl nitroacetate (**2a**) to methyl acetoacetate (**2c**). Enzyme induced stereoselectivity was not found, which indicates that the catalytic mechanism is not as highly specific as the natural ester hydrolysis reaction. Taking into account that denatured or inhibited CALB is catalytically inactive one must conclude that the enzyme-catalyzed conjugate addition occurs in or in close proximity of the enzyme's active site. One must assume that the unique arrangement of amino acids in the protein cavity attributes to the activation of the substrates in a certain way to facilitate the C–C-bond formation. The absence of a reaction of *trans*-β-nitrostyrene (**4**) with acetylacetone, which is a fairly reactive *C*-nucleophile, may also point out that any unspecific catalysis by, e.g., the protein surface may not be sufficient. These facts let us believe that a certain type of substrate recognition must be involved in the process. Docking simulations indicate that the enantiomers of methyl 2-methyl-2-nitro-5-oxohexanoate (**3b**), the conjugate addition product of methyl 2-nitropropanoate (**2b**) and 3-buten-2-one (**1**), have not clearly distinguished binding modes in the voluminous active site of the CALB S105A mutant. These data allow the conclusion that a stereoselective enzyme-catalyzed Michael addition is unfavoured. Due to the fact that only esters were accepted, it is not out of the question that an interim binding to the serine of the catalytic triad via transesterification is involved, although the CALB S105A mutant was still found catalytically active. As conclusion one must point out that the biocatalysis of Michael additions, originating from promiscuous activities of enzymes, is basically feasible. Enzymes that are capable of performing the reaction stereoselectively still remain to be discovered from natural sources or by creation with molecular biology techniques.

5. Experimental section

5.1. Methods and materials

Chemicals were purchased from Aldrich or Fluka. The hydrolytic enzymes kit was obtained from BioCatalytics Inc. (Pasadena, California, USA) and CALB from *Aspergillus oryzae* was from Fluka. Accurel MP1000 was kindly donated by Membrana GmbH (Obernburg, Germany). Details on the synthesis of reference materials and spectroscopic data are provided in Supplementary data. Thin-layer chromatography was performed on Merck silica gel 60 F_{254} plates. Flash chromatography was performed by using Merck silica gel 60H. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra (referenced by using the solvent peak) were recorded on a Varian Inova-500 instrument. Chiral HPLC analyses were performed on an Agilent 1100 and the GC analysis on a Hewlett Packard 6890 instrument.

5.2. Immobilization by deposition

The enzymes ICR-101 to ICR-128 (each of 4 mg and 20 μL for ICR-128) were dissolved in distilled water (1.5 mL) and after adding the polypropylene support Accurel MP1000 (250 mg) allowed to stand for 3 h until the mixture was lyophilized. The immobilized enzymes (1.6% w/w loading) were stored at 4 °C until use.

5.3. Immobilization by adsorption

Solutions of wtCALB and CALB S105A were prepared by dissolving the appropriate amounts of lyophilized enzyme (contains phosphate; protein content determined by absorbance assay) in 3 ml of distilled water to obtain a loading of 2.5% (w/w) and subsequent adjustment of the pH to 7.65. Commercially available CALB from *A. oryzae* (12.4 mg) was dissolved in potassium phosphate buffer (3 ml, pH 7.65, 50 mM). After adding Accurel MP1000 (500 mg) the mixtures were slightly stirred during 24 h at 20 °C. Finally the solid supports were filtered off, washed (buffer and distilled water) and dried (20 mbar, $CaCl_2$ followed by P_2O_5). The photometric lipase activity assays based on the cleavage of *p*-nitrophenyl-butyrate revealed less than 2% of the initial activity remaining in solution. Due to a lack of an appropriate assay the immobilization behaviour of CALB S105A was assumed as the same as for wtCALB.

5.4. Denaturation

Immobilized *C. antarctica* lipase B (50 mg) was treated with urea (8 M) in potassium phosphate buffer (1 mL, pH 7.18, 50 mM) over a period of 24 h at 4 °C. After filtering the solid support was washed (potassium phosphate buffer pH 7.65 and distilled water) and dried (20 mbar, $CaCl_2$ followed by P_2O_5). The *p*-nitrophenyl-butyrate assay confirmed less than 1% of remaining activity compared with untreated immobilized enzyme.

5.5. Covalent inhibition

Immobilized *C. antarctica* lipase B was treated with a solution of methyl *p*-nitrophenyl *n*-hexylphosphonate (80 mM)[21] in cyclohexane/

toluene 10:1 (1 mL for 120 mg immobilized lipase) during a period of 18 h at 20 °C. After filtering the solid support was washed (cyclohexane) and dried under vacuum. The remaining activity was less than 1% as determined by a *p*-nitrophenyl-butyrate assay.

5.6. Michael-addition reactions

Typical experiments with α,β-unsaturated ketones were conducted by adding immobilized enzymes (40 mg/mL) to a solution of reagents (0.2 M) in cyclohexane. In the case of nitro-olefins the reactions were conducted at concentrations of 0.1 M. All reactions were run under weak stirring at 20 °C. Samples were taken directly from the solution or after adding a co-solvent (acetone) to the reaction to ensure complete dissolution of all reagents and products. The measurements were generally conducted after evaporation of cyclohexane and re-dissolving in *n*-heptane/2-propanol (HPLC) or dichloromethane (GC). Analysis was done by HPLC (Chiralpak AD-H and Chiralcel OD-H using *n*-heptane/2-propanol as solvent) or GC (Chiraldex G-TA) using chemically prepared products as reference material. All products were confirmed by NMR spectroscopy. The isolated yields of products usually matched those determined by chromatography.

5.7. Computational methods

Docking calculations were carried out using the Autodock 3.05 package.[20] For the docking the lipase B from *C. antarctica* was chosen (PDB Code 1TCA).[22] The protein structure was checked and prepared for the use in the docking experiments. We utilized the NQ-Flipper server[23] to recognize unfavourable rotamers of Asn and Gln residues in the protein structure. The results were checked and the protonation states of the histidines were assigned by visual inspection. The mutation S105A was introduced by replacing the Ser105 with an alanine using PyMOL.[24] Additionally we used the program protonate provided by the amber package[25] to add polar hydrogens to the amino acids. All water molecules were discarded from the structure before docking. SYBYL v.7.3[26] was used to construct the substrate (methyl 2-methyl 2-nitro-6-oxohexanoate) for docking in both enantiomeric forms. Gasteiger–Hückel partial charges were assigned to the atoms and the structures were energy-minimized using the Tripos force field.

Autodock 3.05 was used to dock the substrates to the receptor protein applying a genetic algorithm augmented by a local search (Solis & Wets). The Genetic Algorithm (GA) parameters were set as follows: The number of individuals in the population was set to 50 and the maximum number of energy evaluations was set to 500,000 leading to a typical number of generations of 250. The results of 50 independent runs were clustered.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2009.05.042.

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