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Useful hydrolytic enzymes: Proteases, lipases and nitrilases

Brigitte Berger, Anna de Raadt, Herfried Griengl, Walter Hayden, Petra Hechtberger, Norbert Klempier and Kurt Faber*

Institute of Organic Chemistry, Graz University of Technology, Stremayrgasse 16, A-8010 Graz, Austria.

Abstract - The most recent developments in the use of hydrolytic enzymes - proteases, lipases and nitrilases - are summarized: Sterically demanding α,α-disubstituted carboxylic esters can be enzymatically resolved using a commercially available protease derived from *Aspergillus oryzae*. A significant enhancement of selectivity and activity of *Candida cylindracea* lipase in irreversible acyl transfer reactions may be achieved by removal of by-products or *via* covalent modification of the enzyme. Enzymatic hydrolysis of nitriles proceeds under mild conditions but enzyme-catalysed side-reactions such as ester- or epoxide-hydrolysis may occur.

INTRODUCTION

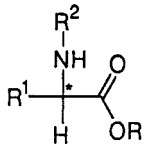
About two thirds of the research reported on the biotransformation of non-natural organic compounds makes use of enzymes from the group of the hydrolases. Their main advantages, which makes them the favourite class of enzymes for organic chemists, is their lack of cofactors and their ready availability.

PROTEASES–RESOLUTION OF α,α-DISUBSTITUTED CARBOXYLATES

Numerous proteases, the most prominent of which are α-chymotrypsin, subtilisin and papain, have extensively been used to resolve not only α-amino acid derivatives of type **1**, but also many α-monosubstituted carboxylic esters (type **2**, ref. 1). Although the majority of the structural features (R^1, R^2) of the general substrate types can be varied considerably, one restriction remains for both types of substrates: The remaining hydrogen atom on the α-carbon atom must not be replaced, since the sterically more demanding α,α-disubstituted counterparts of **1** and **2** are generally not accepted as substrates (ref. 2). A protease from *Aspergillus oryzae* (AOP, ref. 3) seems to be an exception to this rule by being able to accept also bulky substrate esters.

From the two ester moieties of diethyl 4,5-dihydroisoxazole-3,5-dicarboxylate (**3**), only the 5-ester moiety was hydrolysed in a regioselective manner by AOP to give monoacid **4** (60% yield). Similarly, a *cis/trans*-mixture of epoxy esters **5** and **6** could be separated *via* a regioselective hydrolysis using this protease. The reaction ceased, when the *trans*-isomer **6** was converted to **7**, leaving the *cis*-counterpart **5** untouched. The potential of AOP to resolve sterically demanding esters was recently demonstrated by the enantioselective hydrolysis of α-substituted mandelic esters such as atrolactic (**8**) or a precursor of Mosher's acid (**9**, ref. 4). This concept was extended to the 5-methyl-4,5-dihydroisoxazole-5-carboxylates **10-12**, which are precursors of α-hydroxy γ-amino acids (ref. 5). Depending on the size of the substituent in position 3, low to good enantioselectivities (E) were achieved. In analogy to the regioselective transformation of diester **3**, the enantioselective hydrolysis of diester **12** only occurred on the 5-carboxylate by leaving the ester in position 3 untouched. Chemical transformation of the recovered unhydrolysed (*R*)-**12** (e.e. >97%) led to optically pure (*R*)-citramalic acid (ref. 6). An interesting reversal of the stereochemical preference of AOP was observed with substrate **13** (ref. 7). This behaviour is not uncommon among hydrolytic enzymes and may be explained by an alternative fit of the sterically more and less hindered substrate esters (**11** and **13**, resp.) within the active site of the enzyme (ref. 8). The highly substituted *trans*-configurated heterocyclic ester **14**, which represents a precursor for a rare amino acid in the antibiotic Nikkomycin (ref. 9), was resolved with excellent selectivity. The general applicability of this concept is demonstrated by replacement of the dihydroisoxazole core with a dioxolane moiety, leading to substrate **15** (ref. 10).

Scheme 1 Regio-, diastereo- and enantioselective hydrolyses using *Aspergillus oryzae* protease

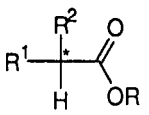


1

R^1 = alkyl, aryl

R^2 = acyl

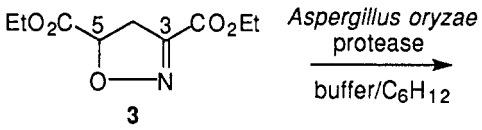
R = Me, Et



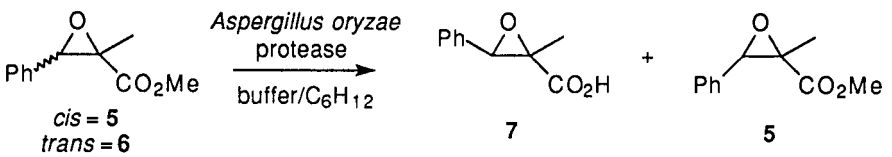
2

R^1, R^2 = alkyl, aryl, halogen

R = Me, Et, 2-haloethyl

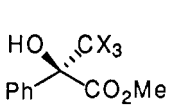


3 **4**

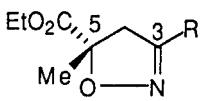


cis = **5**
trans = **6**

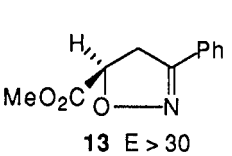
7 **5**



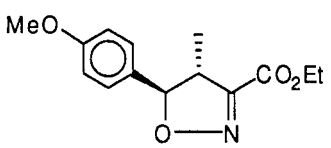
Compound	X	E
8	H	14
9	F	26



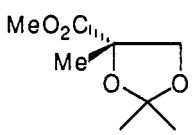
Compound	R	E
10	Me	3
11	Ph	10
12	CO_2Et	>20



13 E > 30



14 E > 30



15 E = 14

For racemic substrates only the hydrolysed enantiomer is shown

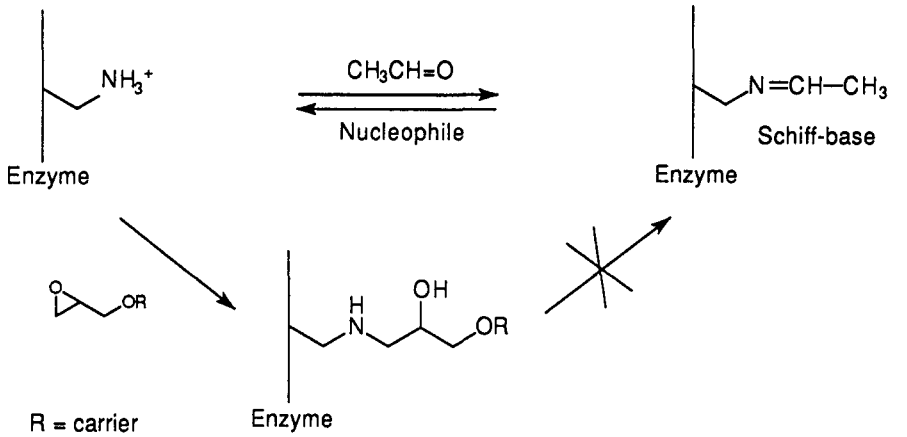
LIPASES – SELECTIVITY ENHANCEMENT BY MEDIUM-ENGINEERING AND COVALENT ENZYME MODIFICATION

Lipases are the biocatalysts of choice for the resolution of esters of secondary alcohols (ref. 11). Especially for lipophilic solid substrates they have frequently been used to catalyse an enantioselective acyl transfer reaction in organic solvents with low water content. Acyl donors which provide a highly desirable irreversible type of reaction (ref. 12), such as acid anhydrides (ref. 13) and enol esters (ref. 14), can be recommended as long as certain precautions are met:

Employing acid anhydrides as acyl donors, the liberated co-product acid acidifies the micro-pH of the enzyme, thus causing a depletion in selectivity of *Candida cylindracea* lipase (CCL). The addition of a weak base to the reaction medium, which functions as an acid scavenger, was an effective counter-measure to increase the selectivity of CCL in the order of one magnitude (ref. 15).

When vinyl acetate is used as acyl donor, acetaldehyde is liberated as by-product during the course of the reaction. The latter can lead to a severe loss of activity and selectivity *via* the formation of Schiff's-bases particularly with the ε-amino groups of lysine residues.

Scheme 2 Avoiding the formation of Schiff's bases by enzyme immobilisation



Chemical derivatisation of CCL by blocking the reactive amino residues *via* covalent immobilisation (ref. 16) is an effective method to stabilize the lipase's activity and selectivity (ref. 17). When the same lipase sample was repeatedly used for the resolution of substrate (±)-**16**, a severe loss of activity and selectivity - being already low from the first run (E = 8) - was observed when untreated native enzyme was used. The covalently modified lipase, however, showed a five-fold increase in selectivity (E = 42), which was entirely stable towards deactivation during repeated use.

Scheme 3 Selectivity enhancement of *Candida cylindracea* lipase by covalent enzyme modification

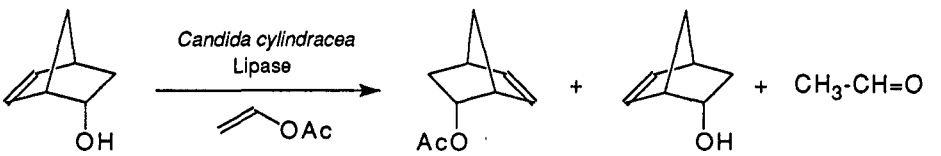


TABLE 1. Optical purity of products.

Enzyme	Run No.	Relative Activity (%)	Selectivity (E)
Native Lipase	1	39	8
	2	0.6	4
	3	not detectable	-
Immobilized Lipase	1	100	42
	2	50	42
	3	23	42

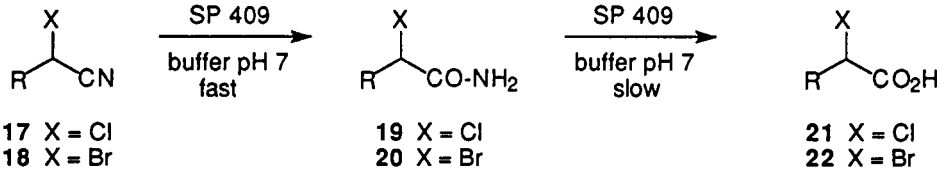
NITRILASES – MILD HYDROLYSIS OF NITRILES

Nitrilase-hydrolysing enzymes may be helpful to broaden the use of cyanide - a 'water-stable carbanion' - as a d_1 synthon by providing a method for the mild and selective hydrolytic transformation of organic nitriles into amides and/or carboxylic acids (ref. 18). Due to the sensitivity of nitrile-converting enzymes most of the transformations have been reported using whole-cell systems, which cannot easily be handled in a standard organic laboratory. The only available ready-to-use nitrilase preparation (ref. 19), which is provided in immobilized form, can accept a large variety of nitriles (ref. 20) but its use is not without limitations: Water-miscible or -immiscible organic solvents, such as methanol or hexane, lead to rapid enzyme deactivation. Similarly, cyanide is also an inhibitor. This hampers the hydrolysis of cyanohydrins as these compounds usually liberate some CN^- at a pH value greater than 4. Other unexpected enzymatic side-activities are also observed:

Methyl carboxylates and acetates of primary and secondary alcohols are rapidly cleaved by esterases present in the crude preparation (ref 21). On the other hand, ethyl carboxylates or 2,2-dimethyl propionates (pivalates) are more resistant. Quite surprisingly, epoxides can be hydrolysed as well to form 1,2-diols (ref. 22). The assumption that this latter reaction was caused by the action of an enzyme present in the crude preparation has been verified by stability experiments in the absence of biocatalyst.

Despite the above mentioned disadvantages, Novo SP 409 is a valuable enzyme preparation for the selective hydrolysis of nitriles at around neutral pH. The α-halonitriles **17** and **18** were hydrolysed to give the corresponding amides **19** and **20**, resp. Further enzymatic hydrolysis of the latter to produce acids **21** and **22** proceeded more slowly.

Scheme 4 Enzymatic hydrolysis of α-halonitriles



17 X = Cl
18 X = Br

19 X = Cl
20 X = Br

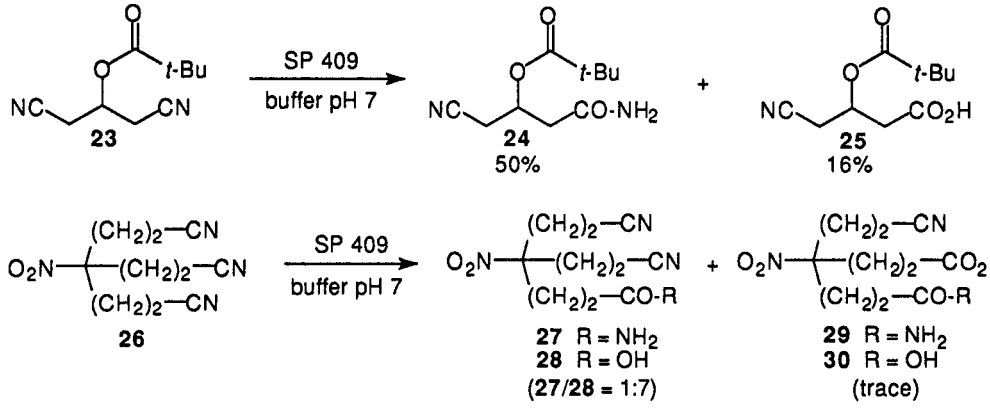
21 X = Cl
22 X = Br

TABLE 2. Yields of α-haloamides and -acids.

R	Time (h)	Amide (%)	Acid (%)
Me	24	40-42	<10
Ph	18	65-70	<10

Regioselective hydrolysis of the 3-acyloxyglutarodinitrile **23** preferentially gave the monoamide **24** with a small amount of monoacid **25**. As may be expected, the intrinsically unstable corresponding 3-hydroxyglutarodinitrile led to decomposition. The trinitrile **26** gave a mixture of monoamide **27** and monoacid **28** in a ratio of about 1:7, whereas the more polar products **29** and **30** were detected only in trace amounts.

Scheme 5 Regioselective enzymatic hydrolysis of di- and trinitriles



23

24 50%

25 16%

26

27 R = NH_2
28 R = OH
(**27**/**28** = 1:7)

29 R = NH_2
30 R = OH
(trace)

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