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ENANTIOSELECTIVE HYDROLYSES BY BAKER'S YEAST - II. ESTERS OF N-ACETYL AMINO ACIDS[1]

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Abstract - D-N-Acetyl amino acid esters were obtained via enantioselective hydrolysis of their racemates by use of fermenting yeast. Evidence is given that proteinases are the enzymes involved.

INTRODUCTION

Enantioselective transformations of organic compounds using baker's yeast (*Saccharomyces cerevisiae*) are well documented[2]. The large majority of these conversions take advantage of the oxidoreductases[3] present in this microorganism. On the contrary much less is known about its hydrolytic properties[4,5] and it was shown by competition experiments among almost 250 species of microorganisms that the oxidoreductase activity of yeasts in general is greater than their hydrolytic action[6]. In the past decade asymmetric hydrolyses using a variety of different microorganisms have become a frequently used method[7] for the resolution of racemic esters bearing their chirality either in their acid component[6] or (more frequently) in the alcohol moiety[8] Only a few reports[4] describe hydrolytic conversions by means of *yeast* following either an enantioselective[4a] or achiral course[4b,5]. Most of the latter cases seem to have occurred as undesired side reactions. Among all microorganisms hitherto employed for asymmetric hydrolysis yeast is preferable for a preparative organic chemist since it is easily available and its use obviates the need of sterile fermentation equipment[5]. The foregoing reasons in mind we started a study on the hydrolytic properties of yeast[1] choosing N-acetyl amino acid esters as substrates for the following reasons:

a) they are easily accessible and
b) show a broad variety of structural features.
c) Enantiomerically pure material is available for comparison from natural sources and
d) both enantiomers play an important role in biological systems.

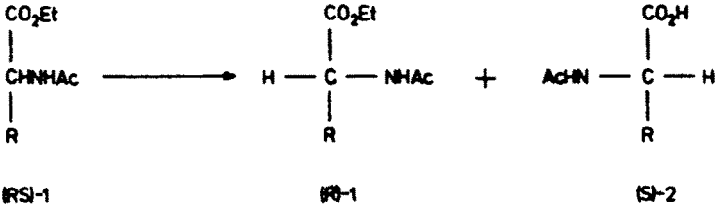
RESULTS AND DISCUSSION

A series of racemic N-acetyl amino acid esters **1**, **3** and **5** was subjected to the action of fermenting yeast (*Saccharomyces cerevisiae Hansen*). We found that in all cases the carboxylic ester was hydrolysed and the amide group remained unchanged. Similar to the action of α-chymotrypsin on some of these compounds[9] only the "natural" L-derivatives were cleaved while the "unnatural" D-enantiomers (R)-**1**, (R)-**3** and (R)-**5** remained unchanged and could be recovered with low to excellent enantiomeric excess. The corresponding (S)-N-acetyl amino acids **2**, **4** and **6** were not isolated.

1) Steric Influence of Substituents

As shown in scheme 1 the majority of substrates bearing unbranched alkyl or arylalkyl substituents (**1a**, **1b**, **1d** and **1e**) is hydrolyzed with excellent enantiomeric excess. Side chains only inhibit the reaction if branching is located close to the asymmetric center in β-position (substrates **1c** and **1k**). In these cases the speed of conversion is reduced significantly as indicated by high recovery of starting ester and therefore only marginal enantiomeric excess is found. A γ-substituent (substrate **1d**) shows no negative effect on the course of the enzymatic transformation.

Scheme 1



Substrate (R)-1	R	Recovery [%][a]	Enantiomeric Excess [%][b]	Fermentation Conditions[c]
1a	Me	47	100[10,d]	an
1b	Et	48	>96[11,d,e]	an
1c	*i*-Pr	75	13[12]	an
1c	*i*-Pr	38	80[12]	ae
1d	$CH_2CH(CH_3)_2$	48	92[13]	an
1e	CH_2Ph	38	>97[14,d]	an
1f	CH_2CO_2Et	49	86[15]	an
1g	$(CH_2)_2CO_2Et$	46	89[16]	an
1h	$(CH_2)_4NHAc$	94	nonsubstrate[17]	an
1i	CH_2OAc	37	43[d,e]	an
1i	CH_2OAc	36	47[d,e]	ae
1k	$CH(OAc)CH_3$[f]	64	3[e]	an

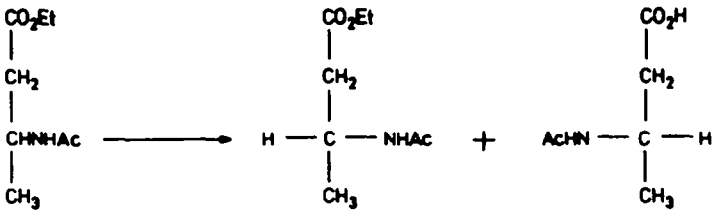
[a] Ester (R)-**1**, isolated from reaction. [b] Determined by measurement of optical rotation, references for data of enantiomerically pure material. [c] an=anaerobic, ae=aerobic fermentation conditions (see experimental). [d] In addition determined by ^{1}H-NMR spectroscopy using the chiral shift reagent Eu(hfc)$_3$. [e] See experimental. [f] N-Acetyl-O-acetyl threonine ethyl ester.

If the distance between the center of chirality and the carboxylic ester group is extended by a CH_2-unit microbially mediated hydrolysis fails completely showing compound **3** to be a nonsubstrate (see scheme 2). Obviously an N-acetylamino function in α-position to the ester moiety is necessary for an effective conversion.

This fact is confirmed by an additional finding: If two chemically different ester groups are present in a substrate (compound **1f** and **1g**) an enzymatic regioselection is observed: While the α-ester is preferentially cleaved the ω-ester moiety remains unchanged yielding the ω-half esters of (S)-N-acetyl aspartic [(S)-**2f**] and (S)-N-acetyl glutamic acid [(S)-**1g**] as determined by comparison of their ^{13}C-NMR spectra and their behaviour on TLC with independently synthesized material[18]. The remaining (R)-diesters **1f** and **1g** both showed almost 90% e.e. These facts are in

accordance with the hydrolytic behaviour of α-chymotrypsin[9,15,16].

Scheme 2

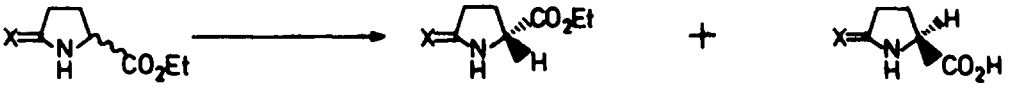


Recovery[a]	Enantiomeric Excess [%][b]	Fermentation Conditions[c]
82	nonsubstrate	an
64	0	ae

[a] Ester (R)-**3**, isolated from reaction. [b] Determined by measurement of optical rotation. [c] an= anaerobic, ae=aerobic fermentation conditions (see experimental).

Low enantioselection is observed in the hydrolysis of cyclic derivatives **5a** and **5b**. Similarly, amino acid esters bearing an additional polar substituent (**1h**, **1i** and **1k**) turn out to be unsuitable as substrates for *Saccharomyces cerevisiae:* even after 48 hours of exposure to the culture medium low conversion - indicated by a general high recovery level - is found and the optical purity of isolated esters is either disappointing (**1i**: e.e.=43%) or exceedingly low (**1k**: e.e.=3%). The most polar derivative among this group turns out to be even a nonsubstrate.

Scheme 3

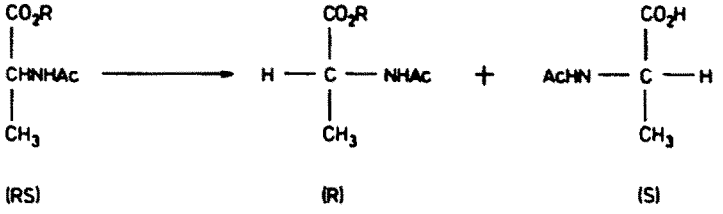


Substrate (R)-5	X	Recovery [%][a]	Enantiomeric Excess [%][b]	Fermentation Conditions[c]
5a	H_2	10	25[19]	an
5b	O	59	0[20]	an

[a] Ester (R)-**5a**, **b**, isolated from reaction. [b] Determined by measurement of optical rotation, references for data of enantiomerically pure material. [c] an=anaerobic, ae=aerobic fermentation conditions, see experimental.

Little influence on the microbial hydrolysis is effected by variation of the alcohol moiety of N-acetyl alanine esters (see scheme 4). While the methyl ester only was converted slightly slower, all other esters of primary and secondary alcohols were hydrolyzed at the same rate yielding products with an enantiomeric excess greater than 96%. The *t*-butyl ester was not converted at all.

Scheme 4



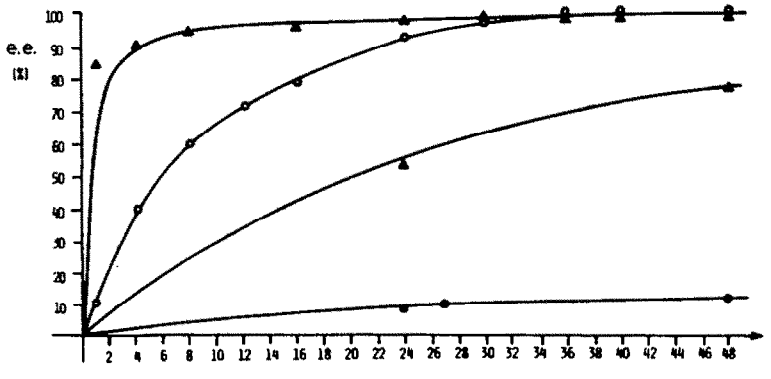
R = methyl, ethyl, *n*-propyl, *i*-propyl, *n*-butyl, *n*-octyl, benzyl, cyclohexyl, *t*-butyl.

2) Influence of Fermentation Conditions

In general *anaerobic* conditions[21] were used for the fermentation (see schemes 1-3) providing a sufficient rate of hydrolysis to accomplish a conversion greater than 50% within 48 hours necessary for an optimum of enantiomeric excess. Beyond this time gradual cell decomposition begins making workup more troublesome.

As can be seen in figure 1 various substrates are hydrolysed at different rates, **1e** reaching maximum optical purity already within 12 hours and **1a** within 36 hours. On the contrary compound **1c** shows a low speed of conversion. Aiming to increase the rate of hydrolysis by accelerating the metabolism of the microorganism we performed the hydrolysis of three selected substrates [**1c**, **1i** and **3** (see scheme 1 and 2)] - which did *not* give satisfying results using *anaerobic* conditions - with aeration of the culture medium (see experimental).

Figure 1



△ N-Acetyl phenylalanine ethyl ester (**1e**), *anaerobic* conditions.

○ N-Acetyl alanine ethyl ester (**1a**), *anaerobic* conditions.

● N-Acetyl valine ethyl ester (**1c**), *anaerobic* conditions.

▲ **1c**, *aerobic* conditions.

In case of **1c** where probably steric reasons caused a low speed of conversion under *anaerobic* conditions this method improved the result drastically: aeration raised the enantiomeric excess of recovered ester **1c** from 13% (*anaerobic*) to 80% (*aerobic* conditions). Unfortunately, this behaviour may not be taken as a rule: although rapid hydrolysis was found with substrate **3** enantioselection was entirely lost. No influence of fermenting conditions on the hydrolysis was ascertainable with substrate **1i** (see scheme 1).

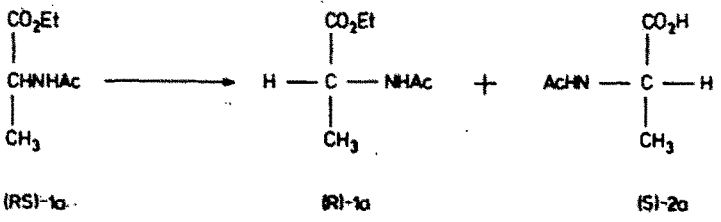
3) Enzyme System Involved

Various types of hydrolytic enzymes are active in fermenting yeast[22]. To elucidate the nature of those involved in the hydrolysis of N-acetyl amino acid esters experiments were performed with compound **1a** as a model substrate.

To clarify whether the enantioselection of the hydrolysis was caused either by the hydrolytic enzyme itself or (less probably) is accomplished via the amino acid transport system of *Saccharomyces cerevisiae*[23] we hydrolyzed compound **1a** using the cytosol and the membrane fraction of mechanically disrupted cells[24] as source of enzyme: The hydrolytic activity was found in equal amounts both in the cytosol and in the membrane fraction yielding ester (R)-**1a** with almost identical optical purity (68% and 64%, respectively, after 24 hours). We regard this as evidence that the enantioselection observed is caused predominantly by the hydrolytic enzyme system itself.

From the close analogy of hydrolytic behaviour of *Saccharomyces cerevisiae Hansen* with α-chymotrypsin[9] we suspected that the enzyme responsible for hydrolysis might be a proteinase[25] rather than a lipase, esterase or phospholipase. To answer this question we performed the hydrolysis of model substrate **1a** with yeast mutant ABYS1 - deficient in unspecific proteinases (yscA, yscB) and unspecific carboxypeptidases (yscY, yscS)[26] - together with the corresponding wild type X 2180-1A as control experiment.

Scheme 5



Time of Fermentation [h]	*Saccharomyces*[a] e.e.[d] [%]	yield [%]	Wild Type[b] e.e.[d] [%]	yield [%]	Mutant[c] e.e.[d] [%]	yield [%]
24	92	n.d.	89	52	1	95
48	100	47	100	43	2	81

[a] *Saccharomyces cerevisiae Hansen.* [b] Wild type X 2180-1A. [c] Strain ABYS1, deficient in unspecific proteinases (yscA, yscB) and unspecific carboxypeptidases (yscY, yscS). [d] E.e. of (R)-**1a**.

While *Saccharomyces cerevisiae Hansen* and the wild type both showed almost identical results indicating their close relationship the proteinase and carboxypeptidase deficient mutant completely failed to hydrolyze substrate **1a** giving a recovery of >80%. from these results we assume the active enzyme system to be a proteinase.

4) Final Remarks

On the basis of these findings we think that the hydrolytic properties of yeast have been underestimated so far. Preliminary results show that yeast mediated hydrolysis is not limited to N-acetyl amino acid esters but can be extended to various types of substrates, being subject of current investigations in this laboratory.

EXPERIMENTAL SECTION

Optical rotations $[\alpha]_D^{20}$ were measured on a Perkin Elmer 141 polarimeter. ^{1}H-NMR spectra were recorded in $CDCl_3$ on a Bruker WH 90 spectrometer. Chemical shifts are reported in δ from TMS as internal standard. TLC was performed on silica gel (Merck 60 F_{254}) using cyclohexane/ethyl acetate 1:1 (v/v) as eluent. Esters were visualized by exposure to Cl_2, subsequent drying at 150° for 1 min and spraying with modified Ehrlich reagent[27]. Elemental analysis data (C,H,N) of novel compounds were within ±0.3% of calculated values.

N-acetyl amino acid esters.

DL- and L-substrates were prepared from amino acids using one of the following esterification methods and subsequent acetylation with acetic anhydride in pyridine:

1) Esterification according to Brenner[28]: **1a**[10], **1b**[11], **1c**[12], **1d**[13], **1e**[14], **1f**[15], **1g**[16], **1h**[17], **5a**[19] and L-N-acetyl alanine methyl ester[29]. L-N-Acetyl alanine propyl ester: yield 87%; bp 96-8°/0.06 mbar (Kugelrohr); $[\alpha]_D^{20}$ -56.9° (c 3.0, EtOH). L-N-Acetyl alanine *i*-propyl ester: yield 80%; bp 98-102°/0.05 mbar (Kugelrohr); mp 49-50°; $[\alpha]_D^{20}$ -54.2° (c 4.8, EtOH). L-N-Acetyl alanine butyl ester: yield 86%; bp 100-5°/0.08 mbar (Kugelrohr); $[\alpha]_D^{20}$ -53.6° (c 5.0, EtOH). DL-3-Acetylamino butanoic acid ethyl ester (**3**): yield 81%; bp 101-3°/0.2 mbar (Kugelrohr); lit.:[33] bp 109-12°/0.02 mbar. D-2-Acetylamino butanoic acid ethyl ester (**1b**)[11] obtained by yeast hydrolysis: $[\alpha]_D^{20}$ +46.0° (c 3.7, EtOH); ^{1}H-NMR spectroscopy using Eu(hfc)$_3$ revealed an optical purity of >96%.

2) Azeotropic esterification using p-toluenesulfonic acid in benzene[30]. L-N-Acetyl-O-acetyl serine ethyl ester (**1i**): yield 45%; mp 63-5°; $[\alpha]_D^{20}$ +3.85° (c 6.0, EtOH). L-N-Acetyl-O-acetyl threonine ethyl ester (**1k**): yield 48%; oil; $[\alpha]_D^{20}$ +38.4° (c 3.7, EtOH). L-N-Acetyl alanine octyl ester: yield 84%; bp 110-5°/0.13 mbar (Kugelrohr); mp 28-9°; $[\alpha]_D^{20}$ -42.9° (c 6.4, EtOH). L-N-Acetyl alanine benzyl ester: yield 81%; bp 125-30°/0.05 mbar (Kugelrohr); mp 48-9°; $[\alpha]_D^{20}$ -50.7° (c 3.9, EtOH). L-N-Acetyl alanine cyclohexyl ester: yield 73%; bp 90-5°/0.13 mbar (Kugelrohr); $[\alpha]_D^{20}$ -49.0° (c 2.1, EtOH).

3) Transesterification using *t*-butyl acetate/$HClO_4$[31]. DL-N-Acetyl alanine *t*-butyl ester: yield 38%; bp 101-6°/0.1 mbar (Kugelrohr).

4) **5b**[20] was synthesized by esterification using DCC[32].

^{13}C-NMR data of half esters of N-acetyl glutamic acid. DL-N-Acetyl glutamic acid γ-ethyl ester[18b]: C-1 173.3, C-2 52.0, C-3 26.9, C-4 30.4, C-5 171.7, γ-ester-CH_3 14.0, γ-ester-CH_2 60.6, amide-CH_3 22.5, amide-CO 174.3 ppm. DL-N-Acetyl glutamic acid α-ethyl ester[18a]: C-1 171.9, C-2 52.0, C-3 27.0, C-4 30.3, C-5 172.3, α-ester-CH_3 14.0, α-ester-CH_2 61.8, amide-CH_3 22.6, amide-CO 176.0 ppm.

General procedure for the asymmetric hydrolysis by yeast:

A suspension of fresh yeast (250ml, about 40g of dry weight, *Saccharomyces cerevisiae Hansen*, Reininghaus Co. Ltd./Graz) is diluted with 750 ml of distilled water and the fermentation is started by addition of saccharose (100g). Few drops of polypropylene glycol P 2000 serve as antifoaming agent. A solution of substrate [3g in 10ml of ethanol/water 1:1 (v/v)] is added dropwise and the mixture is thoroughly stirred at 35° for 48h. Aeration was accomplished by passing compressed air (about 1L/min) through a frit into the culture medium. Then the cells are removed by centrifugation (30 min, 5°, 2000 rpm), again suspended in ethanol/water [30ml, 1:2 (v/v)] and recentrifuged. The combined liquid phase is extracted 4-5 times with CH_2Cl_2 or $CHCl_3$. After drying with Na_2SO_4 the organic layer is evaporated. The residue is subjected to Kugelrohr distillation and subsequent column chromatography on silica gel using hexane/ethyl acetate as eluent. For measurement of optical rotation the material is redistilled.

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