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BIOCATALYTICAL TRANSFORMATIONS II.* ENANTIOSELECTIVE HYDROLYSIS OF N-ACETYL-FLUORO-PHENYLALANINE-ETHYLESTERS BY LYOPHILISED YEAST

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SUMMARY

The three nuclear substituted monofluoro D-N-acetylphenylalanine-ethylesters were obtained in excellent yield via enantioselective hydrolysis of their respective racemates by use of lyophilised yeast (*Saccharomyces cerevisiae* Hansen).

INTRODUCTION

The replacement of hydrogen by fluorine has become a useful tool for pharmacological and structural analysis of organic compounds [1-3]. Fluorine containing amino acids, in particular, the fluorinated phenylalanines, are of special interest, since they have been shown to be poisonous analogues of phenylalanine. They are useful in the isolation of temperature-conditional phenylalanine transport HeLa-mutants [4], they have been used for inhibition studies on 3-deoxy-D-*arabino*heptulosonate-7-phosphate synthetase from *E. coli*

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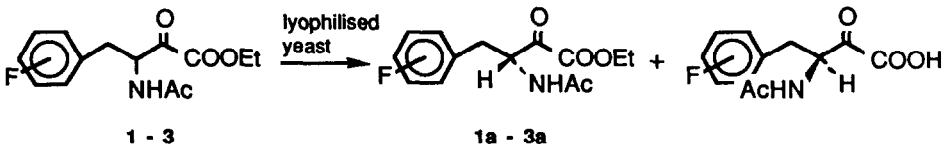
[5] and yeasts [6,7], and were used for studies of transport of aromatic amino acids in bacteria [8]. In addition, fluorophenylalanine-containing peptides [9], angiotensin II analogues [10] oxytocin-derivatives [11], and gramicidin A analogues [12] have been prepared and investigated.

D- and L-forms of these compounds exhibit different transport and enzyme properties [13,14] and therefore, high yield and enantiospecific syntheses of these targets are of continuing interest.

RESULTS AND DISCUSSION

Usual synthetic routes proceed to the racemic amino acids and resolution is often achieved by enzymatic reactions [15-17]. These reactions proceed either by deacylation of the DL-N-acylamino acids (or esters) making use of acylases [18] or carboxypeptidases [19] or via papain-mediated enantioselective formation of their respective phenylhydrazides [16,20,21]. Enantioselective hydrolysis has previously been achieved with α-chymotrypsin [12,22,23] and subtilisin [17], the latter approach being limited, however, to N-benzyloxycarbonyl-protected compounds. Instability of the enzymes [18], small-scale preparations [18] and rather low rates of hydrolysis [19] are drawbacks of some of these approaches.

Since organic preparations performed by microorganisms are often limited to "easy-to-get-and-easy-to-handle" microorganisms, we started a study on the hydrolytic properties of baker's yeast (*Saccharomyces cerevisiae* Hansen). It was shown that this microorganism is very easy to handle and a highly efficient "reagent" for enantioselective hydrolyses [24-26]. Fermenting yeast has been proven a valuable biocatalyst and therefore we decided to investigate hydrolysis of the racemic fluoro-phenylalanine ethylesters **1-3** (**1** *ortho*-, **2** *meta*- and **3** *para*- fluoro-substituted). Difficulties are encountered [26], however, on monitoring the rate of hydrolysis: Acids arising from metabolic processes are liberated during the reaction, thus inhibiting the monitoring of the rate of hydrolysis which is normally carried out using an autoburette [27]. From the close analogy of the hydrolytic behaviour of fermenting *Saccharomyces cerevisiae* Hansen with α-chymotrypsin [25] it can be assumed that the active enzyme is an unspecific proteinase. In order to obtain the optimal reaction conditions the pH-optimum was determined and found to be at 7.5 as exemplified with (**3**) (cf. fig. 2).



1 - 3 1a - 3a

Fig. 1. Enantioselective hydrolysis of racemic N-acetyl fluoro-phenylalanine ethyl esters **1 - 3**

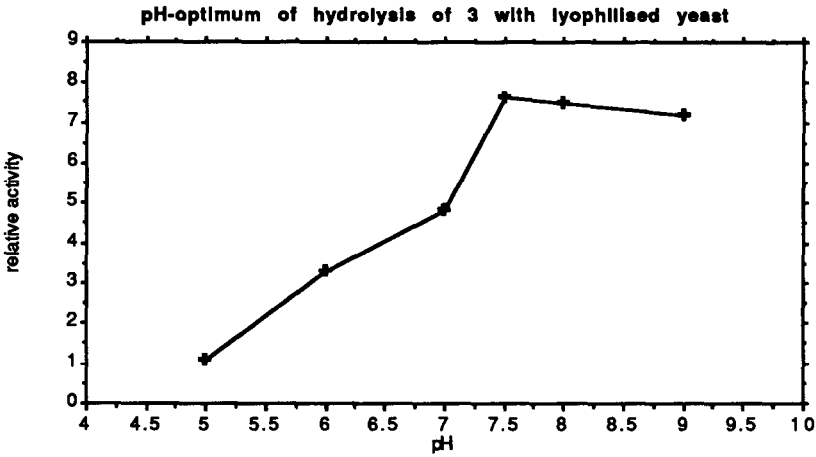


Fig. 2. The pH-optimum for the hydrolysis of **3** was determined at room temperature using 0.2 g (0.8 mmol) of **3** and 0.3 g of lyophilised yeast. **3** and the yeast were incubated at the respective pH-values (which were kept constant during this time with the autoburette) for 30 minutes. The relative activity [in µmol/min g (dry weight)] of the yeast corresponds to the degree of conversion which was calculated from the amount of liberated acid (titrated with 0.02 M NaOH)

Fermenting *Saccharomyces cerevisae* Hansen, however, showed a pH-optimum for these reactions between pH 3 and 4, whereas its lyophilised form showed no reaction at these low pH values. Hence the reactions were performed at pH 7.5- keeping the pH constant throughout the reaction by addition of 1 M NaOH from the autoburette. The reactions stopped after 6-8 hours at 52-53 % conversion. This shows the high enantioselectivity of the hydrolysis. No differences were observed in the rate of hydrolysis for substrates **1-3.** Both the enantiomeric excess and the rates (conversion vs time) are almost identical. It can

be reasoned therefore that the position of the fluorine has no effect on the hydrolytic activity of the enzyme(s) involved. This assumption can be supported by determination of the rate of hydrolysis and of the enantiomeric excess obtained with unsubstituted DL-N-acetyl-phenylalanine ethylester, **4** (cf. Fig.3). Contrary to other enzymes [13,14] the enzyme(s) actually involved in these transformations is (are) not affected by fluorine containing phenylalanines at all.

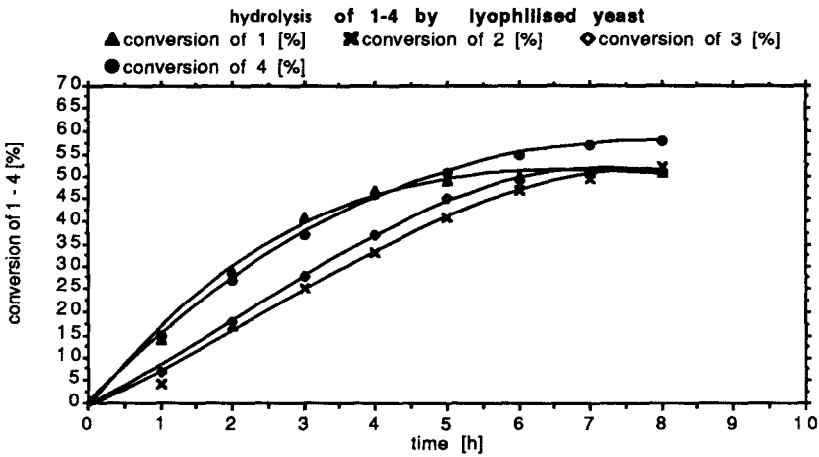


Fig. 3. Hydrolysis of **1-4** (yielding products **1a-4a**; for conditions cf. EXPERIMENTAL.

After completion, the reactions were worked-up and the corresponding D-N-acetyl phenylalanine ethyl esters **1a-3a** were obtained in excellent chemical yield. To determine the enantiomeric purity some ^{1}H-N.M.R. spectroscopic studies were performed at 200 and 400 MHz using the chiral shift reagent Eu(hfc)$_3$. In order to obtain the highest $\Delta\Delta\delta$ values, increasing amounts of shift reagent were added to chloroform solutions of the racemates **1-3**. The best results were achieved in the presence of 0.25-0.3 molar equivalents of the shift reagent, which is in good agreement with results recently published for methylesters of amino acids [28]. The products **1a-3a**, however, showed no line splitting under these optimized conditions, thus indicating an enantiomeric excess > 96 %. In addition, the optical rotations and melting points of **1a-3a** did not change on repeated recrystallizations from ethyl acetate/hexane, hence giving further indication of their enantiomeric purity. We detected no line

splitting with the racemates **1-3** by means of addition of $Eu(hfc)_3$ in the ^{19}F-NMR spectra. However, addition of $Yb(hfc)_3$ gave rise to line splittings of ca 1.5-2.4 Hz in the ^{19}F-NMR spectra but there was no base-line separation and the signals were very broad and allowed no exact determination of ratios of mixtures of enantiomers.

The hydrolysis by means of lyophilised *Saccharomyces cerevisiae* Hansen has been shown to be very enantioselective for these substrates, thus allowing a very convenient, high yield access to the D-configurated fluorophenylalanine ethylesters. Since both lyophilised yeast is easily prepared and the reactions proceed on preparative scale in an enantioselective manner, this approach seems to be superior to previous enzyme or microorganism mediated syntheses of these targets.

EXPERIMENTAL

General

^1H- ^{13}C-, and ^{19}F-NMR spectra were recorded on a Bruker WH90, AM400 or a Varian XL200 instrument. Optical rotations were measured on a Perkin-Elmer 141 polarimeter; the melting points are uncorrected (Tottoli). The amino acid serving as starting materials were obtained from SIGMA (F.R.G.) and used as received. They were esterified according to *Brenner* [29] and subsequently acetylated with acetic anhydride/pyridine in dry dichloromethane under usual conditions.

Lyophilised yeast

A suspension of fresh yeast (*Saccharomyces cerevisiae* Hansen, 1000 ml, about 160 g of dry weight) from the late exponential growth phase is incubated with saccharose (100g). After all the saccharose has been consumed (34°C, ca. 2 h), the cells are isolated by centrifugation (3oooxg), suspended in 800 ml of distilled water and recentrifuged. The resulting pellet is lyophilised at -20 °C to give approx. 130 g of a powder (stored at 5 °C).

General procedure for the hydrolysis by lyophilised yeast

1.5 g lyophilised yeast is suspended in 70 ml of phosphate buffer (0.1M, pH =7.5) and equilibrated for 20 min at room temperature. Then **1a-3a** (1.0 g, 3.98 mmol) are each added at once. The mixture is stirred vigorously and the pH is kept at 7.5 by addition of 1M NaOH from an autoburette. The hydrolysis terminates at 52-53 % conversion, the reaction mixture is centrifuged and the products **1a-3a** are extracted from the supernatant liquid with dichloromethane. The extracts are dried (Na_2SO_4), the solvent evaporated and the residue subjected to column chromatography (cyclohexane / ethyl acetate = 1/1).

TABLE I

Yields and physical data of **1a-4a**

Substitution		Yield (g)	Yield (%)	$[\alpha]_D^{25}$ [a]	MP [°C]	Elemental analysis[b]
1a	*ortho*	0.40	80	-78.8 (c 0.8)	69-70	C:61.72, H:6.40; N:5.60
2a	*meta*	0.38	76	-83.7 (c 2.1)	97-98	C:61.75; H:6.48; N:5.65
3a	*para*	0.43	86	-82.4 (c 0.9)	66-67	C:61.56; H:6.45; N:5.59
4a		0.42	84	-13.6 (c 2.0)[c,d]	89-90	

[a] in $CHCl_3$ solution; [b] calculated for $C_{13}H_{16}FNO_3$: C: 61.65, H:6.37, N:5.53 %; [c] for the L-enantiomer an optical rotation of 13.8 (ethanol) is reported [30]; [d] in ethanol

^{1}H-NMR (in δ from internal tetramethylsilane, couplings in Hz):
1a: 1.25 (t, 3H, J= 7.2, CH_3-Ester), 1.98 (s, 3H, CH_3-NAc), 3.21 and 3.14 (dxAB, 2H, J= 6.1, J_{AB}= 13.9, CH_2), 4.16 and 4.20 (qxAB, J= 7.2, J_{AB}= 10.7, CH_2-ester), 4.85 (ddd, 1H, J= 6.1, 6.1, and 7.9, CH), 6.03 (bd, 1H, J= 7.9, NH), 6.44-7.26 (m, 4H, aromat)
2a: 1.25 (t, 3H, J= 7.1, CH_3-Ester), 2.00 (s, 3H, CH_3-NAc), 3.09 and 3.15 (dxAB, 2H, J= 5.9, J_{AB} = 13.9, CH_2), 4.18 (q, 2H, J= 7.1, CH_2-ester), 4.86 (ddd, 1H, J= 5.9, 5.9, and 7.3, CH), 6.01 (bd, 1H, J= 7.3, NH), 6.8-6.96 (m, 3H, aromat), 7.22-7.27 (m, 1H, aromat).
3a: 1.25 (t, 3H, J= 7.1, CH_3-Ester), 2.0 (s, 3H, CH_3-NAc), 3.07 and 3.12 (dxAB, 2H, J= 5.9, J_{AB}= 13.9, CH_2), 4.16 and 4.19 (qxAB, J= 7.1, J_{AB}= 10.8, CH_2-ester), 4.84 (ddd, 1H, J= 5.9, 5.9, and 7.4, CH), 5.92 (bd, 1H, J= 7.4, NH), 6.95-7.09 (m, 4H, aromat).

TABLE II

^{13}C-NMR and ^{19}F-NMR-Data for **1a-3a**[c]

Carbon /fluorine	**1 a**	**2 a**	**3 a**
CH_3-Ester	13.9[d]	14.1	14.0
CH_3-N-Ac	23.0	23.1	23.0
CH_2	31.4	37.6[a]	37.1
CH	52.5	53.0	53.2
CH_2-Ester	61.6	61.6	61.5
C-1	123.0 (J= 16.1)	138.5 (J= 7.2)	131.6 (J= 3.4)
C-2	161.3 (J= 245.2)	113.9 (J= 20.8)[b]	130.7 (J= 8.1)
C-3	115.3 (J= 23.0)	162.7 (J= 246.1)	115.2 (J= 21.2)
C-4	128.9 (J= 8.4)	116.2 (J= 21.1)[b]	161.9 (J= 245.2)
C-5	124.1 (J= 3.6)	129.9 (J= 8.4)	115.2 (J= 21.2)
C-6	131.6 (J= 4.6)	124.9 (J= 2.9)	130.7 (J= 8.1)
COO[b]	169.7	169.6	169.6
NHCO[b]	171.5	171.4	171.5
^{19}F[e]	-115.9	-111.9	-116.0

[a] $J(^{13}C-^{19}F)$= 1.6 Hz, [b] assignments may have to be reversed; [c] in $CHCl_3$ solution [d] in δ from internal tetramethylsilane; [e] in δ from internal $CFCl_3$; [f] $J(^{13}C-^{19}F)$ in Hz.

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