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Enantioselective hydrolysis of diethyl acetamidomalonate catalyzed by α-chymotrypsin †

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Abstract: Hydrolysis of diethyl acetamidomalonate **1** catalyzed by α-chymotrypsin proceeded enantioselectively affording the dextrorotatory monoester, most likely having the (R)-configuration, which racemized in the course of the reaction with the rate being slower than that of the hydrolysis. © 1997 Published by Elsevier Science Ltd. All rights reserved.

Aminomalonic acid (**Ama**) is the first representative of the homologous α-amino dicarboxylic acid series, including important proteinogenic acids such as glutamic and aspartic. **Ama** has never been considered a proteinogenic amino acid until recently when it was detected in the alkaline hydrolyzates of *Echerichia coli* proteins (*ca* 0.3 units per 1000 amino acids units).[1] It seems to be the first case of establishing **Ama** moiety as the component of a natural peptide. The participation of **Ama** as a peptide building block may not be so rare as it appeared because the old routine methods of analytical decomposition of peptides involved drastic conditions under which any **Ama** initially present should have decarboxylated directly to glycine[2] and obviously avoided detection. Recently peptide derivatives of **Ama** have invoked significant interest because of their possible physiological activities as enzyme inhibitors, *i.e.* renin[3,4] and HIV-1 protease.[5] The **Ama** molecule has a prochiral center and all the derivatives, having different substituents at the carboxyl groups, should be chiral. Obviously, the chiral moiety of the **Ama** derivatives might become an important stereocontrolling element if the **Ama** residue was incorporated in the peptide chain. There are some reports on the separation of diastereoisomerically and enantiomerically pure H-Ama-PheOR derivatives of **Ama**.[6] However, to date no enantiomerically enriched **Ama** derivatives in which the only stereogenic centre belongs to the **Ama** moiety have been obtained and their properties investigated. A promising approach to the simplest compounds in the series seemed to be a stereodifferentiating enzymatic hydrolysis of the symmetric diesters of N-protected Ama. Nevertheless a search of the literature indicated that the earlier attempts at enzymatic hydrolysis of diethyl acetamidomalonate **1** with carboxylesterase NP[7] or α-chymotrypsin (**CT**),[8] as catalysts, afforded racemic AcNHCH(COOEt)COOH **2**.

In the present work we have reinvestigated **CT** catalyzed hydrolysis of **1** and been able to demonstrate that the reaction proceeds enantioselectively with the formation of dextrorotatory monoester **2** that slowly racemizes in the reaction mixture.

The experiments were carried out at ambient temperature, pH 6.5 or 7.5 in 0.01 M aqueous phosphate buffer solutions (15–20 ml) and the amount of **1** initially was 1.07 g. The spontaneous hydrolysis of **1** was not detected under the experimental conditions. The weight ratio of **1/CT** was equal to 82.3 and **CT** activity corresponded to 40–60 units/mg of protein. The pH was monitored with a routine pH-meter equipped with a glass and a reference calomel electrodes. The pH of the mixtures decreased in the course of the reaction and had to be maintained in the range of ±0.2 units by the addition of

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Table 1. Change of optical rotation of water solution during hydrolysis of **1** catalyzed by **CT**[a]

pH	Time (min)	Conversion [b] (%)	$(\alpha_{578})_{obs}$ [c]	$[\alpha]_{578}$ [d]	$\Delta[\alpha]_{578}/\Delta t$ [e]
7.5	132	76	+1.008	+54.4	
	225	94	+1.093	+49.9	0.048
	270	96	+1.078	+48.4	0.033
	315	98	+1.054	+46.6	0.040
6.5	65	20	+0.314	+57.7	
	140	40	+0.560	+52.7	0.067
	220	60	+0.720	+47.4	0.063
	320	80	+0.763	+39.7	0.077
	410	90	+0.708	+33.7	0.067

[a] Enzymatic reaction (1.07 g of **1** and 13 mg of **CT**) and optical rotation determination were carried out at room temperature. [b] Calculated from the volume of consumed 1 N NaOH solution (v). [c] Determined in a cell $l = 0.5$ dm. [d] Calculated with the equation $(\alpha_{obs}-\alpha_{enz})/l(10^{-3}\ vM/V+v)$, where α_{obs} - observed rotation angle of solution, α_{enz} - rotation angle of **CT** (-0.021 at pH 7.5 and -0.027 at pH 6.5; dilution was not taken into account), M - molecular weight of **2** (189.2), V - initial volume of reaction mixture (15.2 ml at pH 7.5 and 15.0 ml at pH 6.5). [e] Absolute values are given.

1 M NaOH aqeous solution. Initial heterogeneous reaction mixtures became homogeneous after *ca* 70% conversion of the substrate **1** and consumption of NaOH stopped after 1 equivalent of the latter had been added. This indicated that only one of the two ester groups of **1** was hydrolyzed to afford monoester **2** which was recovered as a crystalline solid from the reaction mixture after completion of the reaction (vacuum evaporation to *ca* 1/4 of final volume at 40°C and careful acidification with conc. HCl at 5°C) and had satisfactory combustion analysis and ^{1}H NMR spectra. As the results summarized in Table 1 indicate, the reaction proceeded faster at pH 7.5 than at pH 6.5 in accordance with the pH-activity profile of **CT**.[9]

The hydrolysis was accompanied initially by the increase of the positive optical rotation (α_{obs}) of the solution at 578 nm. Taking into account that the contribution of **CT** to the observed rotation is negligible (*see* footnotes to Table 1), the optical activity should only be associated with the formation of (+)-**2** which is the only chiral product that can be formed from **1** in sufficient amounts. The specific rotation of **2** at different stages of the reaction could be easily calculated from the data and the extent of the substrate conversion. The results summarized at Table 1 clearly indicated that the hydrolysis of **1** catalyzed with **CT** was enantioselective.

The time dependence of α_{obs} was not linear and a maximum at 80% of conversion, followed by decrease of α_{obs}, was observed (Figure 1). At the same time the specific rotation (*see* $[\alpha]_{578}$, Table 1) was continuously decreasing. In the case of a simple accumulation of an optically active product the permanent increase of α_{obs} with time and a plateau at 100% conversion should have been expected (Figure 1), and $[\alpha]_{578}$ of the product within the reaction time span should have been constant. The racemization of (+)-**2** yielding (±)-**2** seems to be the only reasonable explanation of the observation. Racemization was faster (*see* $\Delta[\alpha]_{578}/\Delta t$, Table 1) at pH 6.5 than 7.5. Assuming carbanionic mechanism of the racemization, this phenomenon can be rationalized in terms of ionization of **2** to $AcNHCH(COOEt)COO^-$ **2′**. The electron-withdrawing effect of COOH is greater than that of COO^- (compare σ_p 0.45 and 0.0 respectively[10]), which causes the increase of CH-acidity of unionized **2** in comparison with **2′**, providing greater rates of racemization of **2**. The ratio **2**/**2′** must be higher at pH 6.5 than at pH 7.5 and this was reflected in greater rates of racemization at low pH.

Owing to the racemization reaction, we were unable to determine the enantioselectivity of **CT** catalyzed hydrolysis of **1** and the absolute configuration of (+)-**2**. Nevertheless, the latter can be

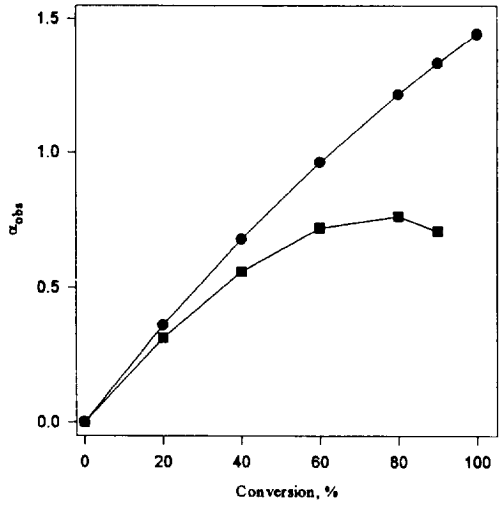

Figure 1. Experimental (■) and theoretical (●) curves of α_{obs} (λ=578 nm) variation *vs* conversion. (Theoretical curve was calculated in accordance with equation $\alpha_{obs}=\{[\alpha]_{578}\}\cdot l\cdot(10^{-3}$ v·M/V+v) (*see* footnotes to Table 1) using $[\alpha]_{578}$=+61 for the enantiomerically pure final product.)

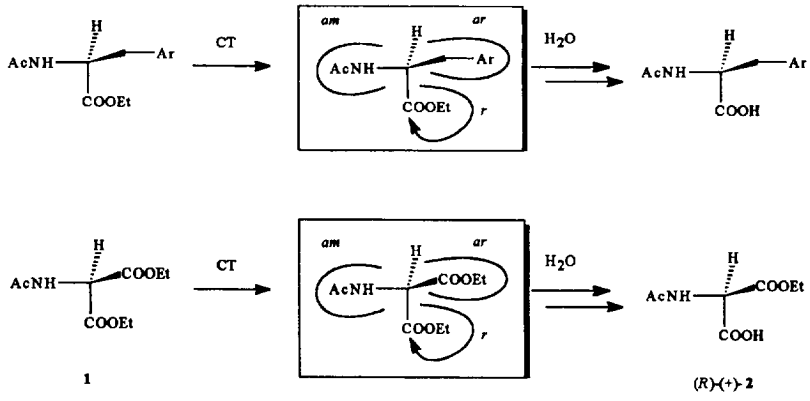

Figure 2. Schematic representation of stereochemical course of **CT** catalyzed hydrolysis of a) ethyl N-acetyl-L-phenylalaninate and b) diethyl acetamidomalonate (**1**); where *am*=amide domain, *ar*='hydrophobic pocket' and *r*=reactive site of **CT**.

deduced, assuming that on the active site of the enzyme the stereochemical orientation of AcNH and H moieties adjusted to the α-C of **1**, was the same as for the **CT** specific substrates, aromatic N-acetyl-L-amino acid esters.[11] In this case *pro-S* COOEt group would be placed in the 'hydrophobic pocket' of the enzyme active site and the *pro-R* COOEt group would be subjected to the attack of the nucleophilic enzyme OH-group thus affording product with (*R*)-configuration (Figure 2).

The extrapolation of the dependence of the calculated values of the specific rotation ($[\alpha]_{578}$, *see* Table 1) *vs* time to the beginning of the hydrolysis gave +61±2 as the value for the specific rotation of the initially formed unracemized sample of (*R*)-**2**.

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