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Reactivity of Tyrosyl–Proline toward Benzoylation in Aqueous 1,4-Dioxane

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Abstract—The kinetics of the reaction of L-tyrosyl-L-proline (Tyr–Pro) with di- and trinitrophenyl benzoates in aqueous 1,4-dioxane (40 wt % of water) were studied in the temperature range 298–313 K. The reaction rate constant k_{298} was found to vary in the range from 0.035 to 0.564 $L \cdot mol^{-1} \cdot s^{-1}$, and the activation barrier changed from 39 to 51 kJ/mol. The neutral and anionic forms of the dipeptide were simulated by the DFT/B3LYP/cc-pVTZ method. Natural bond orbital analysis of the electron density distribution in these forms showed that the preferred nucleophilic center in the dipeptide molecule is the nitrogen atom of the primary amino group rather than the oxygen atom of the phenolic hydroxy group.

Keywords: dipeptide, tyrosylproline, benzoylation, kinetics, 1,4-dioxane, 2,4- and 2,6-dinitrophenyl benzoates, picryl benzoate

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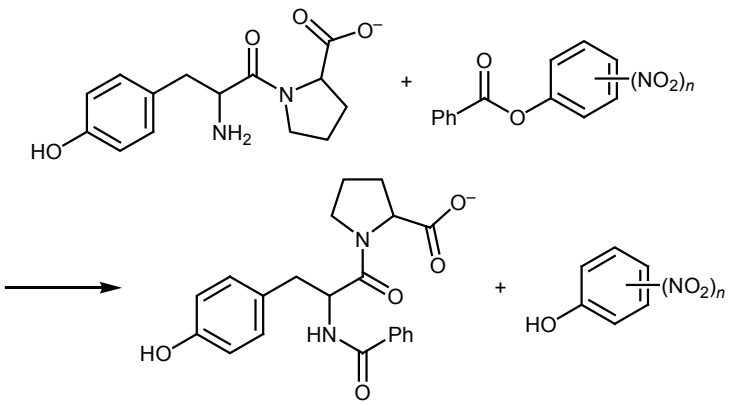
Up-to-date systematic data on the reactivity of dipeptides in acyl transfer reactions are confined to our previous data for the reactions of glycyl–glycine and alanyl–alanine with a number of esters and for the reactions of dipeptides with benzoyl chloride and sulfonyl chlorides in aqueous 1,4-dioxane [1–4]. Meanwhile, in recent publications, much attention has been given to biological activity of both natural oligopeptides and their functional derivatives, as well as to metabolism of these compounds. Guzevatykh et al. [5, 6] analyzed amino acid sequences of 130 opioid peptides belonging to 30 families and revealed a high occurrence rate of the Tyr–Pro dipeptide fragment; the authors also found experimentally that Tyr–Pro is the shortest among known amino acid sequences exhibiting analgesic activity in thermal, mechanical, and chemical pain stimulation in rats. It was found that Tyr–Pro analogs obtained by modification at the proline carboxy group, Tyr–Pro–X (where X = NH_2, OMe, OEt), showed a high activity. However, synthesis of Tyr–Pro derivatives at the tyrosine α-amino group is restrained due to the lack of data on the reactivity of this nucleophilic center.

The goal of the present work was to perform a comprehensive study of the reactivity of two nucleophilic centers in the Tyr–Pro molecule, nitrogen atom of the primary amino group and oxygen atom of the phenolic hydroxy group, by quantum chemical calculations of structural, energetic, and electronic parameters of the neutral and anionic forms of the dipeptide, as well as by measuring the kinetics of its acylation.

The kinetics of the reactions of L-Tyr–L-Pro with 2,4- and 2,6-di- nitrophenyl and 2,4,6-trinitrophenyl benzoates were studied in aqueous 1,4-dioxane in the temperature range 298–313 K (Scheme 1). As we showed previously [2], the only reactive species of α-amino acids and dipeptides in reactions with esters at pH 8.5–9 are the corresponding anions. The N-acylation in aqueous–organic medium can be accompanied by hydrolysis of esters. We found in [2–4] that phenyl benzoates are not hydrolyzed in water; therefore, only alkaline hydrolysis of the ester (Scheme 2) was taken into account in addition to the main reaction (Scheme 1).

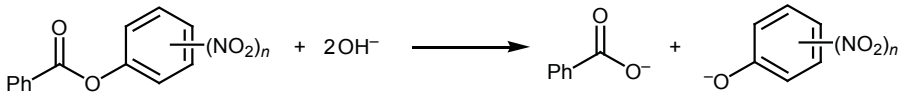
The reaction kinetics were measured by spectrophotometry (λ 400 nm) using a large excess of the dipeptide whose concentration was higher by two orders of magnitude than the concentration of the acylating agent (ester). The reaction rate was monitored by the concentration of the products, di(tri)nitrophenoxide

Scheme 1.



n = 2, 3.

Scheme 2.



n = 2, 3.

ions. The reaction rate with respect to the reagent (c_{ac}) is given by Eq. (1):

$$-\frac{\partial c_{ac}}{\partial \tau} = [k_h + (k\alpha)c_0]c_{ac} = k_{obs}c_{ac}, \quad (1)$$

where α is the concentration ratio of the reactive (anionic) form of Tyr–Pro and its overall concentration in solution c_0; k (L·mol^{-1}·s^{-1}) is the rate constant of acylation of the reactive form; k_h (s^{-1}) is the rate constant of hydrolysis of the acylating agent; and k_{obs} (s^{-1}) is the observed rate constant:

$$k_{obs} = k_h + (k\alpha)c_0. \quad (2)$$

In order to maintain a definite concentration of dipeptide anions, potassium hydroxide was added to the reaction solution in an amount ensuring that a part of the dipeptide remained in the zwitterionic form which is inactive in the acylation. The concentration of Tyr–Pro anions was equal to the concentration of added alkali ($c = c_{KOH}$), and its fraction in solution α and the observed rate constant k_{obs} were defined by Eqs. (3) and (4):

$$\alpha = c_{KOH}/c_0 = c/c_0; \quad (3)$$

$$k_{obs} = k_h + k\,c. \quad (4)$$

According to the results of our previous kinetic studies [2–4], when the concentration ratio of the unreactive zwitterionic ($c_\pm$) and reactive anionic forms (c) of an α-amino acid or dipeptide is higher than 4 ($c_\pm/c > 4$), the rate of alkaline hydrolysis of the ester can be neglected. In this case, the rate constant of the reaction shown in Scheme 1 can be calculated by the equation

$$k = k_{obs}/c. \quad (5)$$

The contribution of hydrolysis of the acylating agent to the overall reaction rate was estimated by special kinetic experiment. For this purpose, the reac-

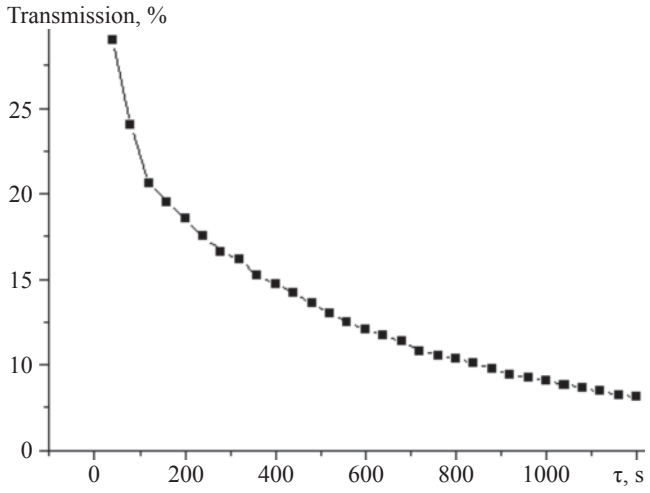


Fig. 1. Transmission of the working solution versus time in the reaction of Tyr–Pro with 2,4-dinitrophenyl benzoate in aqueous 1,4-dioxane (40 wt % of water); temperature 313 K.

Table 1. Rate constats k_{obs} and k for the reaction of L-proline with 4-nitrophenyl benzoate at different concentrations of the amino acid anion c; X_1 is the mole fraction of water in its mixture with 1,4-dioxane; temperature 298 K.

X_1,	c, M	$k_{obs}\times10^2$, s^{-1}	k, $L\cdot mol^{-1}\cdot s^{-1}$
0.765	0.0169	1.87±0.06	1.10±0.04
	0.00846	0.93±0.01	1.10±0.02
	0.00564	0.64±0.01	1.13±0.02
	0.00423	0.493±0.007	1.16±0.02
0.951	0.0196	5.0±0.6	2.6±0.3
	0.00980	2.46±0.07	2.51±0.07
	0.00653	1.67±0.06	2.56±0.09

Table 2. Kinetic parameters of the reaction of Tyr–Pro with di(tri)nitropheyl benzoates in aqueous 1,4-dioxane (40 wt % of water)

Temperature, K	c, M	$k_{obs}\times10^4$, s^{-1}	$k\times10^2$, $L\cdot mol^{-1}\cdot s^{-1}$	$\Delta H^{\neq}_{298}$, kJ/mol	$-\Delta S^{\neq}_{298}$, $J\cdot mol^{-1}\cdot K^{-1}$
2,4-Dinitrophenyl benzoate					
298	0.00876	4.19±0.17	4.79±0.19	37±2	145±7
303		5.21±0.16	5.94±0.18		
308		6.49±0.43	7.40±0.49		
313		9.43±0.57	10.8±0.65		
2,6-Dinitrophenyl benzoate					
298	0.00876	3.02±0.22	3.45±0.25	–	–
2,4,6-Trinitrophenyl benzoate					
298	0.00876	25.3±0.3	28.9±0.3	49±2	91±6
303		37.3±0.5	42.6±0.6		
308		54.8±0.5	56.4±0.1		

tion of L-proline with 4-nitrophenyl benzoate was run several times in a solvent with the same composition at the same temperature with variation of the initial amino acid concentration. The fact that the k values remained constant within the examined temperature range (Table 1) indicated insignificant contribution of hydrolysis of the ester to the overall reaction rate.

Figure 1 shows one of the experimental kinetic curves for the benzoylation of Tyr–Pro. The shape of the kinetic curve suggests the absence of catalytic or autocatalytic processes in the system. It also implies that the attack of the acylating agent is directed at only one nucleophilic center of the dipeptide molecule, nitrogen atom of the primary amino group. As will be shown below, just that group exhibits strongly pronounced electron-donor properties in comparison to the alternative nucleophilic center, oxygen atom of the phenolic hydroxy group.

The experimental kinetic parameters of the acylation process are presented in Table 2. It is seen that the rate constants for the reactions of Tyr–Pro with di(tri)nitrophenyl benzoates increase in the series 2,6-DNPB < 2,4-DNPB < 2,4,6-TNPB. The same ester reactivity order was observed previously in the reactions with Gly–Gly and Ala–Ala [2, 4]; obviously, it is related to increase of the electrophilicity of these esters in the same series. In addition, similarity of the activation parameters for the reactions of 2,4-DNPB with Tyr–Pro (Table 2) and glycine should be noted ($\Delta H^{\neq}_{298}$ = 31 $kJ\cdot mol^{-1}$, $\Delta S^{\neq}_{298}$ = –134 $J\cdot mol^{-1}\cdot K^{-1}$) [4].

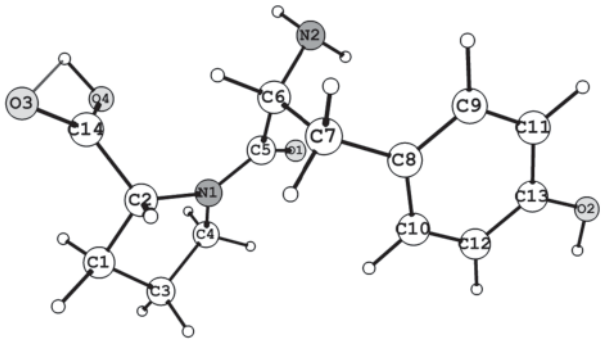
Comparison of the kinetic parameters for the benzoylation of α-amino acids and dipeptides in aqueous 1,4-dioxane (Table 3) indicates the determining effect of the basicity of amino groups subjected to acylation on the reaction rate. In going from Gly to Gly–Gly, the pK value decreases by 1.55 log unit, and the acylation

Table 3. Rate constants of N-acylation of α-amino acids and dipeptides in aqueous 1,4-dioxane (40 wt % of water), temperature 298 K

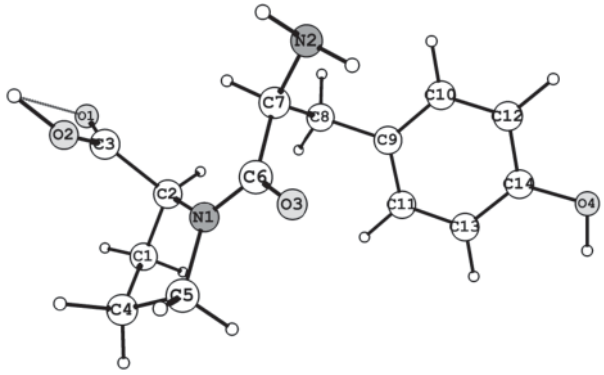
Substrate	pK[a]	Ester	k,[b] L·mol^{-1}·s^{-1}	Substrate	pK[a]	Ester	k,[b] L·mol^{-1}·s^{-1}
Gly	9.78	2,4-DNPB	2.12	L-Pro	10.64	2,4-DNPB	11.1
		2,5-DNPB	0.42			2,5-DNPB	7.9
		2,6-DNPB	0.30			2,6-DNPB	3.0
		2,4,6-TNPB	11.2			2,4,6-TNPB	36.5
DL-Val	9.72	2,4-DNPB	0.54	L-Asn	8.80	2,4,6-TNPB	0.75
		2,5-DNPB	0.09	DL-Ser	9.15	2,4,6-TNPB	1.67
		2,6-DNPB	0.06	Gly–Gly	8.23	2,4,6-TNPB	3.1
		2,4,6-TNPB	5.2	L-Ala–L-Ala	8.14	2,4-DNPB	0.35[c]

[a] The values refer to protonation of the primary amino group in water [7].
[b] The error in the determination of rate constants did not exceed 5%.
[c] In aqueous 1,4-dioxane containing 60 wt % of water.

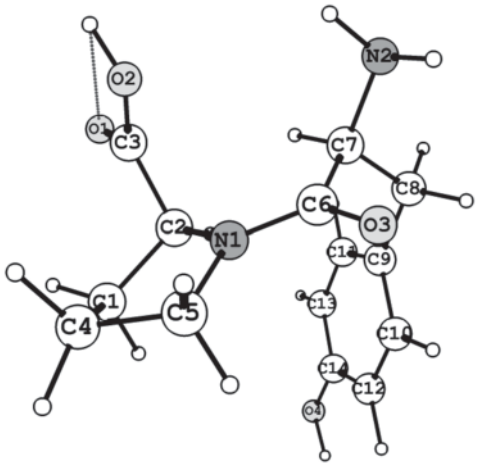
rate constant decreases by a factor of 3.6. According to the data of [7], Tyr–Pro is characterized by a low basicity in aqueous solution [pK(Tyr–Pro) 7.81, cf. pK(Tyr) 9.11], which is likely to be responsible for the lower (by an order of magnitude) rate constant k of benzoylation of Tyr–Pro (Table 2) than of Gly–Gly. Elongation of the peptide chain up to seven amino acid residues leads to an insignificant decrease of pK



ΔE = 0 kcal/mol (conformer **1**)



ΔE = 0.64 kcal/mol (conformer **2**)



ΔE = 2.96 kcal/mol (conformer **3**)

Fig. 2. Calculated strutures of the most stable conformers of the neutral form of Tyr–Pro and their relative energies. There is intramolecular hydrogen bond in the carboxy group of each conformer.

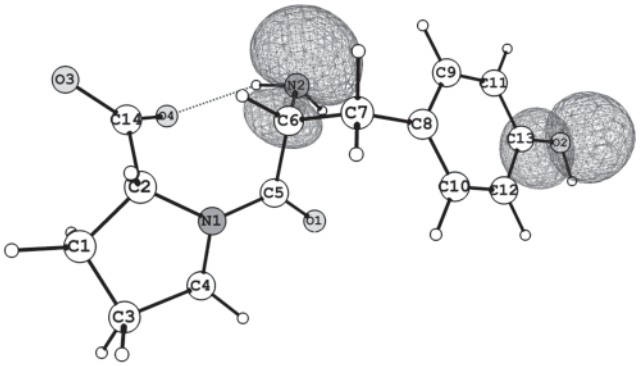


Fig. 3. Calculated structure of the anionic form of Tyr–Pro; localization of $LP(N^2)$ and $LP_2(O^2)$ on the nucleophilic centers is shown.

(p*K* 7.31 and 7.34 for Tyr–Pro–Phe–Pro–Gly–Pro–Ile and Tyr–Pro–Phe–Val–Glu–Pro–Ile, respectively) [7]. The linear correlation between log*k* for the reactions of amino acids and dipeptides with 2,4,6-TNPB (Table 3) and p*K* values of their amino groups was used to predict the rate constants for the reactions of heptapeptides with 2,4,6-TNPB. Extrapolation of this dependence gave *k* values at a level of 0.080–0.084 $L \cdot mol^{-1} \cdot s^{-1}$.

Taking into account that the Tyr–Pro molecule possesses two nucleophilic centers capable of undergoing electrophilic attack by benzoyl group, namely the primary amino group and phenolic hydroxy group of the tyrosine moiety, it seemed reasonable to compare electron-donor properties of these groups on the basis of quantum chemical calculations. For this purpose, the neutral and anionic forms of Tyr–Pro were simulated by DFT calculations using B3LYP functional and cc-pVTZ basis set (Gaussian 03 software package [8]). Geometric parameters of both forms were completely optimized, and the energy minima on the potential energy surface were identified by the absence of imaginary frequencies in the corresponding Hessian matrices. The anionic form of the dipeptide was generated by deprotonation of the proline carboxy group, and its charge was assumed to be equal to –1, and multiplicity, to 1.

Figure 2 shows three most stable structures of the neutral form of Tyr–Pro, which were selected among the possible conformers, and the most stable structure of the deprotonated dipeptide is presented in Fig. 3. It is seen that the geometric parameters of the neutral form (conformer 1) significantly change in going to the anionic form. In particular, the torsion angle $N^1C^5C^6C^7$ and the distance between the cyclic fragments of the molecule increase, whereas the torsion angle $N^1C^5C^6N^2$ decreases due to formation of intramolecular hydrogen bond between the oxygen atom of the carboxylate group and hydrogen atom of the primary amino group. As a result, the N–H bond becomes longer (from 1.015 to 1.021 Å), which facilitates its dissociation in the course of acylation. Furthermore, the dipole moment of the molecule changes both its direction and magnitude (μ = 4.38 and 11.23 D for the neutral and anionic forms, respectively).

The electronic parameters of the two possible nucleophilic centers, nitrogen atom of the primary amino group (N^2) and oxygen atom of the phenolic hydroxy group (O^2) were compared on the basis of NBO analysis [9, 10] of electron density distribution in the neutral and anionic forms. The results showed that the oxygen atom has two unshared electron pairs, $LP_1(sp^{1,2})$ with a lower energy and $LP_2(p_\pi)$ with

Table 4. Electronic characteristics of the two potential nucleophilic centers in the neutral and anionic forms of the dipeptide Tyr–Pro

Characteristic	Neutral form			Anionic form		
	N^2	O^2		N^2	O^2	
Charge, a.u.	–0.826	–0.629		–0.849	–0.782	
Energy of lone electron pair, eV	–8.51	–16.55 (LP_1) –8.68 (LP_2)		–5.15	–14.54 (LP_1) –6.52 (LP_2)	
Population of LP orbital	1.95	1.98 (LP_1) 1.88 (LP_2)		1.94	1.98 (LP_1) 1.89 (LP_2)	
Interacting orbitals (donor → acceptor)	LP → $\sigma^*(C^5–C^6)$	LP_1→ $\sigma^*(C^{12}–C^{13})$	LP_2→ $\pi^*(C^{12}–C^{13})$	LP → $\sigma^*(C^5–C^6)$	LP_1→ $\sigma^*(C^{12}–C^{13})$	LP_2→ $\pi^*(C^{12}–C^{13})$
Energy of donor–acceptor interaction, kcal/mol	8.45	6.70	25.23	9.44	7.13	27.88

a higher energy (Fig. 3; Table 4). However, the LP_2 orbital is involved in strong conjugation with the aromatic π-system $LP_2 \rightarrow \pi^*(C^{12}–C^{13})$, so that is electron-donor power is reduced. The N^2 atom possesses only one lone electron pair LP($sp^{3,6}$) whose energy is higher than the energy of LP_2 on the oxygen atom. The energy of LP(N^2) increases in going from the neutral form of the dipeptide to the anion, which enhances its electron-donor power.

Thus, the NBO analysis of electron density distribution in the anionic form of the dipeptide showed that the nitrogen atom of the primary amino group is a more probable center for electrophilic attack than the oxygen atom of the phenolic hydroxy group. The results of quantum chemical calculations and experimental kinetic data suggest the possibility of modification of the dipeptide Tyr–Pro at the α-amino group of the tyrosine residue.

EXPERIMENTAL

The dipeptide L-Tyr–L-Pro as hydrochloride with a purity of 99.95% was synthesized at the Institute of Chemical Reagents and Highly Pure Chemical Substances ("Kurchatov Institute" National Research Center). 2,4-, and 2,6-Dinitro- and 2,4,6-trinitrophenyl benzoates were prepared by acylation of the corresponding nitrophenols with benzoyl chloride. All reagents and solvents were purified until their physical properties (melting or boiling point and refractive index) completely coincided with reference data. 1,4-Dioxane of chemically pure grade was kept over potassium hydroxide over 7 days and was then distilled under atmospheric pressure over metallic sodium to remove organic peroxide impurities. The binary solvent was prepared from deionized water which was obtained using a DV-1 deionizer. Working solutions of the dipeptide and KOH in aqueous 1,4-dioxane and of di(tri)-nitrophenyl benzoate in 1,4-dioxane were prepared from accurately weighted amounts of the reagents and were kept at a constant temperature over a period of 30 min before a kinetic run. The initial reactant concentrations were 10^{-2} and 10^{-4} M, respectively. The optical densities of solutions were measured with an SF-56 spectrophotometer equipped with a temperature-controlled cell compartment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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