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KINETICS OF THE STEPWISE HYDROLYSIS OF MONONITROFLUORESCEIN DIACETATES IN ORGANIZED SOLUTIONS

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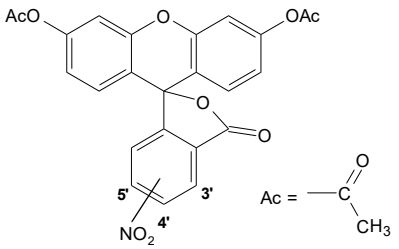
The kinetics of the stepwise alkaline hydrolysis of three isomeric mononitrofluorescein diacetates, containing the nitro group in the residue of the phthalic acid, were investigated in water and organized aqueous solutions of cationic surfactants and a cationic calix[4]arene. The reaction follows a consecutive two-stage mechanism involving the formation of a spectroscopically distinguishable monoacetate intermediate. UV-visible spectrophotometric monitoring allowed for the separate determination of the pseudo-first-order rate constants for both hydrolytic steps. The catalytic efficiency of the organized systems was found to increase in the order: cetyltrimethylammonium bromide (CTAB) < N,N-dimethyl-N-hydroxyethyl-4-dodecyloxybenzylammonium chloride (C_{12}-Bn-Ch) < 5,11,17,23-Tetra(N,N-dimethyl-N-hydroxyethylammonium)methylene-25,26,27,28-tetradodecyloxy calix[4]arene tetrachloride (C_{12}-C4A-Ch), with acceleration factors reaching nearly three orders of magnitude for the 3'-nitro derivative. The positioning of the nitro substituent markedly influenced the relative rates of the two hydrolysis stages, with the k_2'/k_1' ratio being governed by the interplay between intrinsic electronic effects and the nature of the organized environment. These results suggest that supramolecular assemblies based on calixarene derivatives provide a highly efficient microenvironment for ester hydrolysis, likely through a synergy between specific host-guest recognition within the macrocyclic cavity and collective electrostatic effects at the aggregate interface.

Keywords: *mononitrofluorescein, ester hydrolysis, micellar catalysis, cationic calixarenes, organized solutions, host-guest recognition.*

Introduction

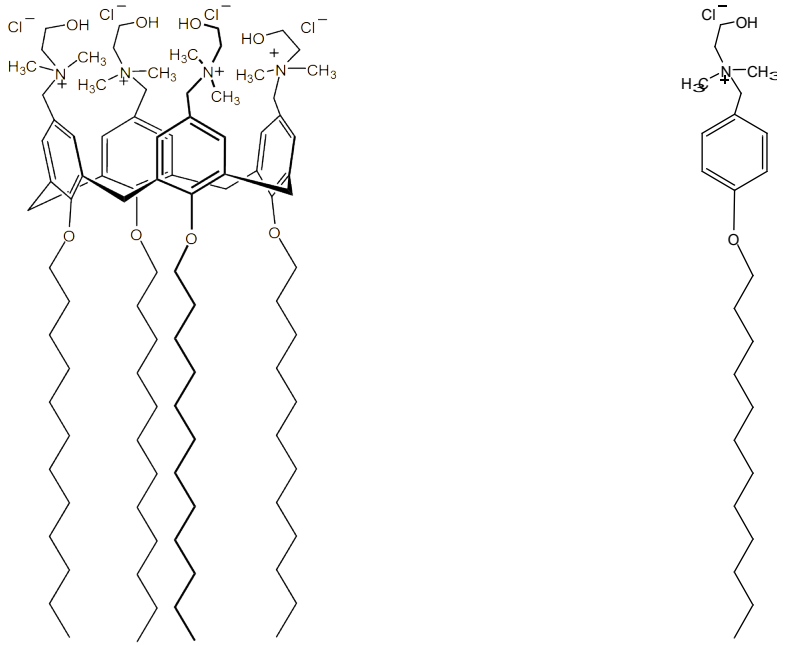
The hydrolysis of fluorogenic substrates belonging to the fluorescein ester series is a popular and well-documented indicative reaction, widely utilized in biochemistry and related fields [1–3]. Typically, this process is monitored via fluorescence spectroscopy, where the two-step transformation is conventionally treated as a single-stage reaction [4–7]. However, such a simplified approach neglects the kinetic complexity of the system. In aqueous alkaline media, the hydrolysis of fluorescein diacetates proceeds through two successive steps via a monoacetate intermediate. Building upon our previous findings regarding the kinetic resolution of these stages for the unsubstituted dye [8], the present work focuses on the impact of electron-withdrawing nitro groups at the 3'-, 4'-, and 5'-positions of the phthalic acid residue. It is well established that such substituents significantly alter the protolytic and tautomeric equilibria of the resulting xanthene dyes, which in turn should influence the reactivity of the ester bonds during the nucleophilic attack of the hydroxide ion.



Scheme 1. The molecular structures of esters of nitrofluoresceins

Nowadays, organized solutions, such as micellar solutions of surfactants and related liquid-phase systems, are among the most widespread environments for governing the rates of chemical processes. Cationic micelles, for instance, cetyltrimethylammonium bromide (CTAB), are known to accelerate ester hydrolysis by several orders of magnitude [8–10]. Within the framework of the pseudophase ion-exchange model, this effect is attributed to the electrostatic concentration of OH^- ions in the Stern layer of the aggregates. More recently, amphiphilic calix[4]arenes have emerged as a sophisticated class of "nanoreactors". These macrocyclic assemblies provide a more complex catalytic environment, where multivalent electrostatic effects are coupled with specific host-guest recognition [11,12].

In this paper, we report the kinetic regularities of the alkaline hydrolysis of three isomeric mononitro-derivatives of fluorescein diacetate in the presence of CTAB, a benzyl-choline-derived surfactant (C_{12}-Bn-Ch), and a cationic calix[4]arene (C_{12}-C4A-Ch) (Scheme 2). The focus is placed on the separate determination of the pseudo-first-order rate constants, k_1' and k_2', for both hydrolytic steps. By analyzing the acceleration factors and their dependence on the substituent position, we aim to elucidate how the confined environment of calixarene-based aggregates can either reinforce or override the intrinsic electronic effects of the substrate.



C_{12}-C4A-Ch C_{12}-Bn-Ch

Scheme 2. Structural formulas of the calix[4]arene bearing choline groups (C_{12}-C4A-Ch) and its monomeric "quarter" unit (C_{12}-Bn-Ch)

Experimental section

Three isomeric mononitrofluoresceins, containing the nitro group in the residue of the phthalic acid (3'-, 4'-, and 5'-nitro derivatives), were synthesized as described in our previous reports [13,14].

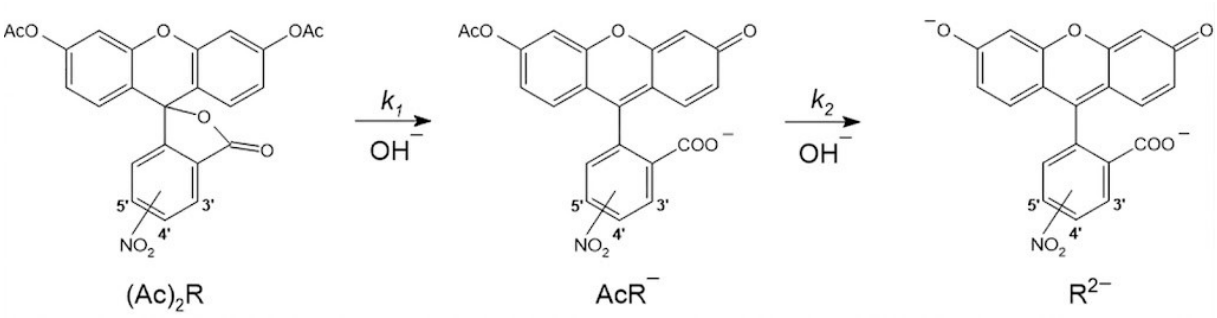
The purity of the dyes was confirmed by elemental analysis, NMR spectroscopy, and by the constancy of their molar absorptivities. Cetyltrimethylammonium bromide (CTAB, Sigma-Aldrich) was used without further purification. The tetracationic calix[4]arene 5,11,17,23-tetra(N,N-dimethyl-N-hydroxyethylammonium)methylene-25,26,27,28-tetradodecyloxy calix[4]arene tetrachloride (C_{12}-C4A-Ch), as well as its monomeric analog, N,N-dimethyl-N-hydroxyethyl-4-dodecyloxybenzylammonium chloride (C_{12}-Bn-Ch), were synthesized at the Institute of Organic Chemistry, National Academy of Sciences of Ukraine, following the procedures reported elsewhere [8,15]. All other reagents, including buffer components and sodium chloride, were of analytical grade. Carbonate-free NaOH solutions were prepared using deionized water.

Kinetic measurements were performed using a Hitachi U-2000 spectrophotometer equipped with a thermostated cell holder at 25.0 ± 0.1°C. The pH values of the working solutions were maintained using borate or Veronal buffer systems. The total ionic strength was adjusted to 0.05 M with NaCl.

The pH was monitored using a glass electrode (calibrated with standard buffers at 1.68, 4.01, 6.86, and 9.18) in a cell with a liquid junction.

The stock solutions of the nitrofluorescein diacetates were prepared in acetonitrile. To initiate the reaction, an aliquot of the stock solution was introduced into the thermostated buffer solution containing the surfactant or calixarene. The final substrate concentration in the working solutions was (5-10) × 10^{-6} M, while the acetonitrile content did not exceed 1–2 vol%. In all experiments involving surfactants, their concentrations were kept above the critical micelle concentration (CMC).

The alkaline hydrolysis of nitrofluorescein diacetates (Scheme 3) proceeds as a sequence of two consecutive steps. Since the reaction was carried out in buffer solutions, the concentration of hydroxide ions remained constant, and the process was described using pseudo-first-order rate constants, k_1' and k_2' (s^{-1}) where $k'=k\cdot c(OH^-)$:



Scheme 3. Alkaline hydrolysis of the diacetyl derivative of nitrofluoresceins

The progress of the reaction was monitored by recording the absorbance (A) over time. The spectral characteristics of the reaction's final product were determined spectrophotometrically in the corresponding systems. The spectrum of the monoester was modeled by the spectrum of methylnitrofluorescein, which has an analogous structure of the chromophoric xanthene moiety of the dye. The reaction rate was considered sufficiently low compared to the spectrum measurement time (approximately 10 s). The values of k_1' and k_2' were determined separately based on the following analytical dependencies of the mole fractions (α) on time (t):

$$\alpha_{R_2Fl} = e^{-k'_1 t} \quad (1)$$

$$\alpha_{RFl^-} = \frac{k'_1}{(k'_2 - k'_1)\left(e^{-k'_1 t} - e^{-k'_2 t}\right)} \quad (2)$$

$$\alpha_{Fl^{2-}} = 1 - \frac{k'_2}{k'_2 - k'_1} e^{k'_1 t} + \frac{k'_1}{k'_2 - k'_1} e^{k'_2 t} \quad (3)$$

Results and Discussion

Consistent with our previous observations in homogeneous aqueous and aqueous-ethanol media [16], the alkaline hydrolysis of nitro-substituted fluorescein diacetates in organized solutions follows a stepwise mechanism involving a spectroscopically distinguishable intermediate. In all studied organized systems, CTAB micelles, C_{12}-Bn-Ch aggregates, and C_{12}-C4A-Ch calixarene assemblies a pronounced catalytic effect was observed, leading to a significant acceleration of the reaction rate compared to pure water.

Figure 1 displays the representative normalized absorption spectra recorded during the hydrolysis of 3'-, 4'-, and 5'-nitrofluorescein diacetates in the presence of the tetracationic calixarene C_{12}-C4A-Ch. The spectral evolution clearly demonstrates the formation of two distinct colored species: the monoacetate intermediate (AcR^-) and the final dianion (R^{2-}). These forms exhibit overlapping but distinguishable absorption bands in the 400–500 nm range, allowing for the kinetic resolution of the individual stages.

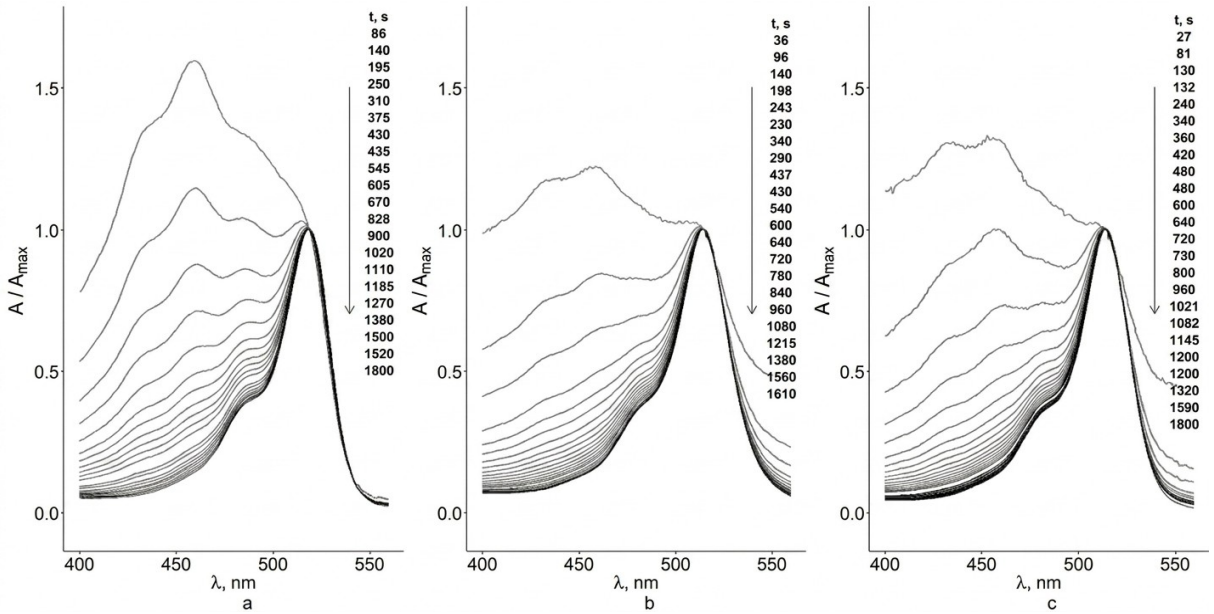


Figure 1. Normalized absorption spectra during the hydrolysis of 3′- (a), 4′- (b) and 5′- (c) nitrofluorescein diacetates in aqueous solution of calixarene C_{12}-C4A-Ch, 25 °C, I = 0.05 M (NaCl + buffer mixture).

Figure 2 shows the temporal evolution of molar fractions of the three forms during hydrolysis of 3'-, 4'-, and 5'-nitrofluorescein diacetates in aqueous solution of C_{12}-Bn-Ch. The characteristic profiles expected for consecutive first-order reactions are clearly observed: the diester $(Ac)_2R$ decreases monotonically, the monoester AcR^- exhibits a rise-and-fall profile with a maximum at intermediate times, and the dianion R^{2-} accumulates progressively as the final product.

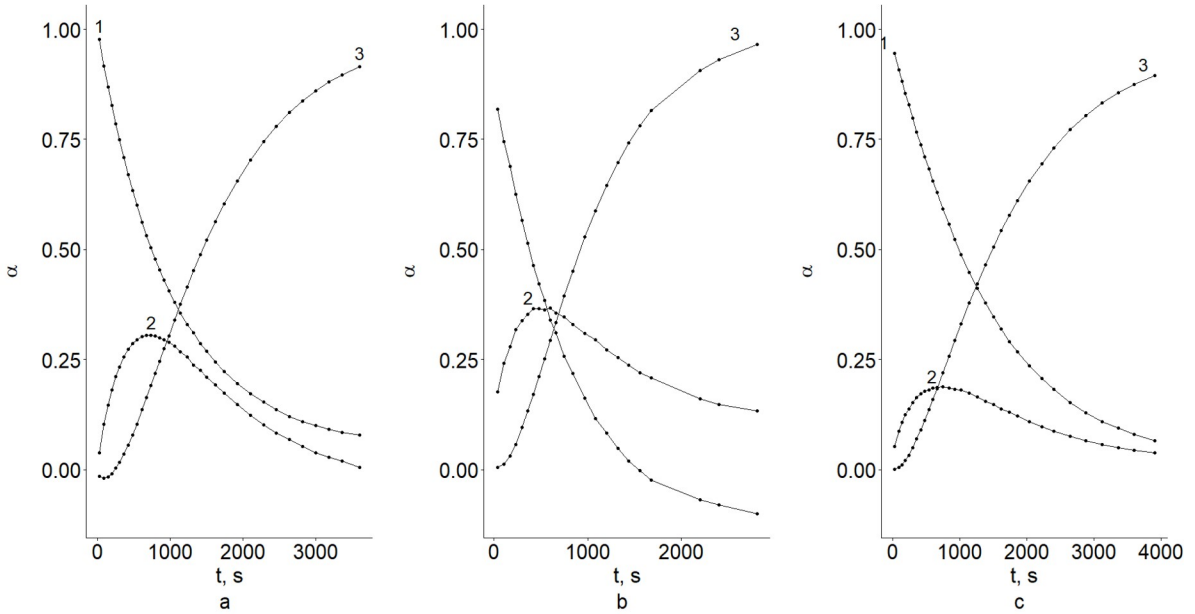


Figure 2 Dependences of the molar fractions of $(Ac)_2R$ (1), AcR^- (2), and R^{2-} (3) of 3′-nitrofluorescein (a), 4′-nitrofluorescein (b) and 5′-nitrofluorescein(c) in in aqueous solution of C_{12}- Bn -Ch, 25 °C, I = 0.05 M (NaCl + borate buffer).

Table 1 presents the experimentally determined values of k_1' and k_2' for diacetyl fluorescein and its 3'-, 4'-, and 5'-nitro derivatives in the three types of organized media. The rate constants were measured at the indicated pH values, which varied slightly depending on the medium and buffer composition.

Table 1. The rate constants of hydrolysis of nitrofluoresceins in solution, 25 °C, I = 0.05 M

Compound	pH	λ_{max}	k_1', s^{-1}	k_2', s^{-1}	k_2/k_1
CTAB (0.003 M)					
Diacetyl fluorescein [8]	9.16	501	3.49±0.08	10.5±0.3	3.0
Diacetyl 3′-nitrofluorescein	9.22	514	3.21±0.06	19.2±0.4	5.97
Diacetyl 4′-nitrofluorescein	9.14	509	3.4±0.3	10.7±0.9	3.18
Diacetyl 5′-nitrofluorescein	9.17	509	1.80±0.19	9.3±0.9	5.17
C_{12}-Bn-Ch (0.001 M)					
Diacetyl fluorescein [8]	8.64	502	7.9±0.2	9.6±0.2	1.2
Diacetyl 3′-nitrofluorescein	7.87	514	7.6±0.2	10.9±0.3	1.4
Diacetyl 4′-nitrofluorescein	7.81	510	19.5±1.5	19.6±1.5	1
Diacetyl 5′-nitrofluorescein	7.85	511	7.0±0.1	21.8±0.1	3.1
C_{12}-C4A-Ch ($1.8\cdot10^{-4}$ M)					
Diacetyl fluorescein [8]	8.44	505	36.2±0.08	182.1±0.4	5.0
Diacetyl 3′-nitrofluorescein	7.87	519	21.33±0.9	29.0±1.2	1.4
Diacetyl 4′-nitrofluorescein	7.83	514	32.52±1.8	50±3	1.5
Diacetyl 5′-nitrofluorescein	7.85	514	10.4±0.2	39.9±0.6	3.8

For quantitative comparison across different systems, all rate constant values were normalized to a single pH value of 8.64.

Table 2. The rate constants of hydrolysis of nitrofluoresceins in solution, 25 °C, I = 0.05 M recalculated to pH=8.64

Compound	k_1', c^{-1}	k_2', c^{-1}	k_2/k_1
CTAB (0.003 M)			
Diacetyl fluorescein [8]	1.1	3.2	3.0
Diacetyl 3′-nitrofluorescein	0.8	5.0	5.97
Diacetyl 4′-nitrofluorescein	1.1	3.4	3.18
Diacetyl 5′-nitrofluorescein	0.5	2.7	5.17
C_{12}-Bn-Ch (0.001 M)			
Diacetyl fluorescein [8]	7.9	9.6	1.2
Diacetyl 3′-nitrofluorescein	45.0	64.2	1.4
Diacetyl 4′-nitrofluorescein	131.6	132.6	1
Diacetyl 5′-nitrofluorescein	43.1	134.2	3.1
C_{12}-C4A-Ch (1.8 Ч 10^{-4} M)			
Diacetyl fluorescein [8]	57.4	288.6	5.0
Diacetyl 3′-nitrofluorescein	125.6	170.9	1.4
Diacetyl 4′-nitrofluorescein	210.0	323.9	1.5
Diacetyl 5′-nitrofluorescein	64.0	245.8	3.8
Water			
Diacetyl fluorescein [8]	0.29	0.37	1.29
Diacetyl 3′-nitrofluorescein[16]	0.13	0.63	4.83
Diacetyl 4′-nitrofluorescein[16]	0.34	0.97	2.85
Diacetyl 5′-nitrofluorescein[16]	0.19	0.76	3.92

Organized aqueous solutions of cationic surfactants and calixarene aggregates exhibit a profound catalytic effect on both hydrolytic stages, while also modulating kinetic selectivity.

The introduction of the nitro group at various positions of the phthalic acid residue exerts a differentiated influence on both hydrolytic stages, and this impact is markedly governed by the nature of the organized environment (Figure 2, Table 3).

As previously reported [16], the introduction of nitro substituents in the phthalic acid residue significantly modulates the kinetic profile in aqueous media. For the first step (k_1'), the 3'- and 5'-isomers exhibit reduced reactivity (0.45–0.66× relative to DAFl), likely due to localized solvation effects near the reaction center. Conversely, k_2' is enhanced for all derivatives (1.7–2.6×) owing to the stabilization of the anionic transition state. The positional trend (3' < 5' < 4') reflects the efficiency of

electronic communication through the sp^3-hybridized C_9 bridge, where the inductive influence of the nitro group is modulated by the specific geometry of the isomer [16].

In CTAB micellar solutions, the impact of nitro-substitution on k_1' follows a qualitative trend similar to that observed in the homogeneous aqueous phase: a reduction in reactivity is observed for the 3'- and 5'-isomers (0.73× and 0.45×, respectively), while the 4'-derivative remains practically unaffected. However, the second hydrolytic stage (k_2') exhibits a more complex dependence on the substituent position: the 3'-isomer shows a 1.56-fold enhancement in reactivity, whereas the 5'-derivative demonstrates a modest decrease (0.84×).

This differentiation indicates that the micellar environment significantly influences the manifestation of substituent effects. The enhanced kinetic selectivity (k_2'/k_1') for the 3'-nitro isomer is likely due to its optimal orientation within the Stern layer of the cationic micelles, which facilitates the second hydrolysis step. In contrast, the slight retardation observed for the 5'-derivative during the second stage may be attributed to its less favorable solubilization site or deeper localization within the micellar palisade layer.

In C_{12}-Bn-Ch solutions, a notable inversion of the nitro-substitution effects appears to occur relative to bulk water and CTAB micelles. While in the aforementioned media the 3'- and 5'-substituents were found to retard the first hydrolytic step, in the presence of C_{12}-Bn-Ch aggregates all nitro-derivatives show an acceleration of k_1' relative to the unsubstituted DAFl: 3' (5.70×), 4' (16.66×), and 5' (5.46×). The second step (k_2') is enhanced in a similar manner, with acceleration factors ranging from 6.69 to 13.98.

This behavior could potentially be interpreted in terms of a molecular recognition mechanism. The benzyl moiety in the surfactant headgroup might engage in π-π stacking with the electron-deficient phthalic acid residue of the substrate. Such interactions may preorganize the molecule, perhaps orienting the ester groups more favorably for the nucleophilic attack of the hydroxide ion. The 4'-isomer exhibits the most pronounced effect (a total 387× acceleration relative to DAFl in water), possibly because its symmetric para-position maximizes the electronic polarization of the aromatic system, thus enhancing its complementarity with the benzyl groups at the micellar interface.

Interestingly, the k_2'/k_1' ratios in C_{12}-Bn-Ch tend to decrease toward unity: from 4.83 to 1.40 for the 3'-isomer and from 2.85 to 1.00 for the 4'-isomer. This observation might indicate a preferential acceleration of the first hydrolysis step, which could be attributed to a high degree of structural complementarity of the diacetate substrate within the organized assemblies. The 5'-derivative remains an outlier, maintaining a higher ratio (3.10). This may suggest that its remote position preserves a binding geometry favorable for the monoacetate intermediate, whereas substitutions at the 3'- and 4'-positions might cause subtle geometric rearrangements that slightly alter the stacking efficiency after the first ester bond is cleaved.

The calix[4]arene-based system C12-C4A-Ch displays a specific kinetic profile for nitro-substituted substrates, which could potentially be attributed to a substrate-selective host-guest binding mechanism. In this medium, all nitro-derivatives exhibit enhanced first-step rate constants, k_1', relative to the unsubstituted DAFl, following the order: 4' (3.66×) > 3' (2.19×) > 5' (1.11×). However, the second hydrolysis step, k_2', reveals a notable reversal: while the 4'-isomer shows a modest acceleration (1.12×), the 3'- and 5'-isomers appear to be retarded (0.59× and 0.85×, respectively).

This behavior stands in contrast to the results obtained in C_{12}-Bn-Ch solutions, which represent the monomeric analog of the calixarene. In the monomeric system, all nitro-derivatives exhibit substantial acceleration for both steps. The transition to the cyclic calix[4]arene architecture introduces a rigid macrocyclic cavity that likely imposes certain geometric constraints. While this cavity might provide an environment suitable for the neutral diacetate (facilitating k_1'), the structural rearrangement and charge development in the monoacetate intermediate could potentially lead to a steric or electronic incompatibility within the restricted space, perhaps hindering the second step for the 3'- and 5'-isomers.

The mechanistic difference between the two steps is further supported by our previous studies of the parent DAFl in calix[4]arene analogs with varying alkyl chain lengths from C3 to C12 [8]. These studies suggested that while k_1' is relatively less sensitive to the hydrophobicity of the substituent, k_2' is markedly enhanced by increasing the alkyl chain length. This allows for the suggestion of a mechanistic duality: the first hydrolysis step might be governed primarily by the cavity effect (molecular recognition within the macrocyclic cavity), whereas the second step appears more sensitive

to the collective aggregate effect, such as micellar-like electrostatic concentration of hydroxide ions. The observed reversal of the substituent effect in C_{12}-C4A-Ch, which is absent in its monomeric analog, could serve as an indication that the macrocyclic cavity plays a role in modulating the reaction pathway for nitro-substituted fluoresceins.

The experimental data for nitro-substituted fluorescein esters in organized media demonstrate a pronounced enhancement in catalytic efficiency, with rate constants increasing systematically across the investigated systems. Specifically, the first hydrolysis step (k_1') shows an acceleration of nearly three orders of magnitude, reaching a 966-fold peak for the 3'-nitro derivative in C_{12}-C4A-Ch. These findings illustrate a consistent hierarchy of catalytic activity (CTAB < C_{12}-Bn-Ch < C_{12}-C4A-Ch), where the complexity of the organized environment appears to correlate with the magnitude of the kinetic response.

Importantly, this acceleration trend across the nitro-substituted series tends to confirm the general catalytic frameworks established in our previous studies for the unsubstituted fluorescein diacetate. The consistency of these results suggests that the presence of the nitro group, while modulating individual rates, does not appear to alter the fundamental catalytic pathway provided by the organized environment. This reinforces the hypothesis that the reaction is governed by an interplay between the individual cavity effect (host-guest recognition) and the collective aggregate effect. The observed patterns suggest that the initial stage of the reaction might be particularly sensitive to the specific microenvironment and orientation within the macrocyclic cavity, whereas the subsequent hydrolysis step is further enhanced by the collective properties of the supramolecular assembly, such as its surface charge density and the resulting electrostatic concentration of hydroxide ions.

When placed in the broader context of supramolecular chemistry, these results could be considered a notable advancement in the study of artificial catalytic systems. Our observed accelerations (10^2–10^3) markedly surpass the 3–10-fold enhancements typical for traditional micellar systems, such as CTAB. Furthermore, the efficiency of the C_{12}-C4A-Ch system exceeds the classical benchmarks for micellar solution by achieving nearly 1000-fold acceleration, these choline-functionalized calixarene assemblies could be positioned among the efficient artificial esterase mimics reported to date [17,18].

Conclusions

The kinetics of the stepwise hydrolysis of 3'-, 4'-, and 5'-mononitrofluorescein diacetates were investigated in organized aqueous solutions of CTAB, C_{12}-Bn-Ch, and the calix[4]arene C_{12}-C4A-Ch. While the influence of the nitro groups in the residue of the phthalic acid on the reaction rate in homogeneous water is relatively modest due to the lack of direct conjugation through the sp3-hybridized C9 bridge, the organized microenvironments were found to markedly modulate these intrinsic electronic effects. The catalytic efficiency followed a consistent hierarchy: CTAB < C_{12}-Bn-Ch < C_{12}-C4A-Ch, with the calixarene-based system achieving a nearly 1000-fold acceleration for the first hydrolytic step of the 3'-nitro derivative.

The structural rigidity of the calixarene macrocycle introduces a confinement effect, which likely leads to substrate-selective binding and specific kinetic patterns for the monoacetate intermediates. Overall, these findings suggest that structured supramolecular assemblies can approach the catalytic efficiency and selectivity characteristic of enzymatic systems through substrate pre-organization and multivalent interactions within specific microenvironments.

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Conflict of Interest: The authors declare no conflict of interest

Authors Contributions: D.V. Kharchenko: Investigation, Visualization, Writing – original draft. T.A. Cheipesh: Conceptualization, Supervision, Writing – review & editing. S.V. Shekhovtsov: Investigation, Visualization. R.V. Rodik: Investigation, Visualization, Formal analysis. V.I. Kalchenko: Validation, Supervision, Resources.

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Д. В. Харченко*, С. В. Шеховцов*, Т. А. Чейпеш*, Р. В. Родік†, В. І. Кальченко†. Кінетика стадійного гідролізу діацетатів мононітрофлуоресцеїну в організованих розчинах.

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Досліджено кінетику стадійного лужного гідролізу трьох ізомерних діацетатів мононітрофлуоресцеїну, що містять нітрогрупу в залишку фталевої кислоти, у воді та організованих водних розчинах катіонних поверхнево-активних речовин і катіонного калікс[4]арену. Реакція перебігає за послідовним двостадійним механізмом із утворенням моноацетатного проміжного продукту, що чітко ідентифікується спектрофотометрично. Моніторинг методом спектрофотометрії у видимій області дозволив окремо визначити константи швидкості псевдопершого порядку для обох стадій гідролізу. Встановлено, що каталітична ефективність організованих систем зростає у ряду: цетилтриметиламоній бромід (CTAB) < N,N-диметил-N-гідроксіетил-4-додецилоксибензиламоній хлорид (C_{12}-Bn-Ch) < тетрахлорид 5,11,17,23-тетра(N,N-диметил-N-гідроксіетиламмоній)метилен-25,26,27,28-тетрадодецилоксикалікс[4]арену (C_{12}-C4A-Ch), причому фактори прискорення для 3'-нітропохідної досягають майже трьох порядків. Положення нітрозамісника суттєво впливало на відносні швидкості двох стадій гідролізу, де співвідношення визначалося поєднанням внутрішніх електронних ефектів та природи організованого середовища. Отримані результати свідчать про те, що супрамолекулярні ансамблі на основі похідних каліксаренів створюють високоефективне мікросередовище для гідролізу естерів, імовірно, завдяки синергії між специфічним розпізнаванням «гість-господар» усередині макроциклів та колективними електростатичними ефектами на межі поділу агрегатів.

Ключові слова: *мононітрофлуоресцеїн, гідроліз естерів, міцелярний каталіз, катіонні каліксарени, організовані розчини, розпізнавання господар-гість.*

Конфлікт інтересів: *Автори повідомляють про відсутність конфлікту інтересів.*

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