Molecular recognition by natural macrocycles. Part II. Esterolytic activity and chiral discrimination of amino acid derivatives by the zwitterionic form of (+)-tubocurarine †

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The esterase and complexation properties of the zwitterionic form of a macrocyclic alkaloid (+)-tubocurarine possessing two phenolic nucleophilic groups are described. Cleavage of 4-nitrophenyl esters of N-protected phenylalanine enantiomers involves reaction paths with one and two alkaloid molecules. Substrate binding enantioselectivity is large and is opposite to the kinetic enantioselectivity, leading to the modest observed enantioselectivity of the reaction, which reaches a maximum ($k_L/k_D \approx 1.5$) at *ca.* 0.002 mol dm^{-3} alkaloid concentration and practically disappears on going to 0.01 mol dm^{-3} alkaloid solution. Addition of boric acid initially enhances the reaction rate and enantiospecificity, but in more concentrated borate solutions the expected inhibition due to blocking of the phenolate groups is observed. For the first time reported for an alkaloid, the esterolytic activity of (+)-tubocurarine towards 4-nitrophenyl acetate falls on a common Brønsted plot together with cyclodextrins and some synthetic macrocycles. Binding of enantiomers of differently charged derivatives of alanine, phenylalanine and β-phenylethylamine to the zwitterionic form of (+)-tubocurarine in aqueous solution was studied by ^{1}H NMR and fluorescence titration. The binding constants vary from <5 dm^3 mol^{-1} for alanine to *ca.* 50 dm^3 mol^{-1} for phenylalanine derivatives and the binding enantioselectivity varies from marginal for *N*-acetylphenylalanine enantiomers to a quite notable, three-fold differentiation between L- and D-phenylalanine. While the enantiospecificity depends primarily on electrostatic interactions, the overall stability is determined by guest hydrophobicity. This conclusion was confirmed by docking calculations for enantiomers of phenylalanine. Addition of amino acid derivatives to solutions containing (+)-tubocurarine and highly fluorescent 8-anilinonaphthalenesulfonate anion leads to enantioselective spectral responses which are indicative of formation of ternary complexes.

Introduction

Macrocycles bearing nucleophilic functional groups have been studied extensively as mimics for hydrolytic enzymes.[1] Within this class of molecules, the natural and modified cyclodextrins have attracted particular attention primarily in the hydrolysis of aryl esters.[1a–c,2] Among synthetic macrocycles, the cyclophanes with phenolic[3a] and imidazole[3b] nucleophiles, and a dimethylamino derivative of calixresorcin[4]arene[4] were tested as artificial enzymes for the cleavage of aryl esters.

We have recently shown that the use of natural cyclophane-like macrocycles offers a viable alternative to the synthetic routes for finding novel systems of molecular, in particular chiral, recognition.[5] It was discovered that the cationic form of the alkaloid (+)-tubocurarine (TC), which dominates in neutral and acidic TC solutions, binds enantioselectively anionic derivatives of alanine and phenylalanine in aqueous solutions. The observed affinity and selectivity were attributed to the combination of ionic and hydrophobic host–guest interactions, although no guest inclusion occurred into the too small host cavity. The macrocycle of TC bears one quaternary ammonium group, one tertiary amino group and two aromatic hydroxys possessing pK_a values of 7.6, 8.65 and 9.65.[6] The first and second pK_a values correspond to the phenolic groups of oxygen O6 and O2, respectively and the third one to the protonated amino group N2 (Chart 1). Evidently TC is capable of existing in various ionic forms, including a zwitterionic form at about pH 9. The presence of two phenolate groups, which potentially can serve as nucleophiles for ester cleavage, prompted us to investigate possible esterolytic activity of the TC zwitterion. Additionally, neutralization of the TC positive charges noticeably changes the conformation of the alkaloid[7] and may therefore affect its recognition properties.

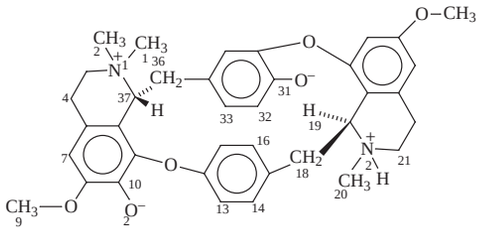
Chiral molecular recognition of amino acids and peptides has attracted much current interest. Among numerous classes of synthetic and natural host compounds, modified cyclodextrins,[8] cyclophanes and cage-like compounds bearing chiral sites have been used recently for chiral discrimination of amino acid derivatives.[9–12] However, with a few exceptions,[13] the creation of artificial chiral macrocycles requires complex synthetic routes. Therefore we consider it of interest to extend the previous study of chiral discrimination of *N*-acylated amino acid derivatives by the dicationic form of TC to its zwitterion. This is due to the presence of both positive and negative charges which may render the TC zwitterion an effective receptor for a larger variety of differently charged guests.

In this paper we present the results of a kinetic study of the esterolytic activity of the TC zwitterion towards activated esters, and the spectral and thermodynamic data on the chiral discrimination of a number of amino acid derivatives.

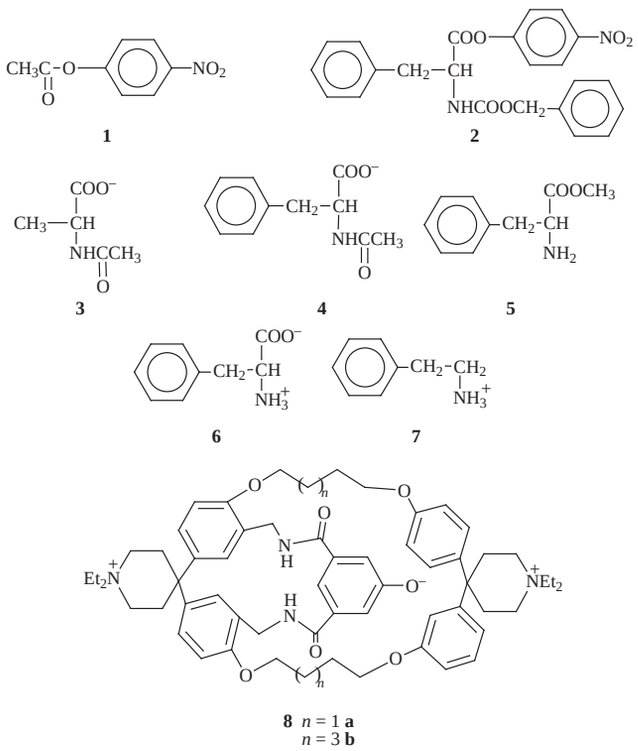
Results

The chemical structures of the TC macrocycle, hydrolysis substrates and guests studied in their dominant ionic forms at pH 9 are shown in Chart 1.

† For Part I, see ref. 5(*b*).



Zwitterionic form of (+)-tubocurarine (TC) macrocycle



8 $n = 1$ **a**
$n = 3$ **b**

Chart 1

Prior to the kinetics and binding studies we performed a standard spectrophotometric titration of TC by using its pH-variable absorbance in the UV region (see Experimental section for details) in order to determine pK_a values of the aromatic hydroxys under our experimental conditions. They were found to be pK_{a1} = 7.16 ± 0.07 and pK_{a2} = 8.67 ± 0.01, in reasonable agreement with published values (see Introduction). Since the protonated amino group of TC has pK_a = 9.65[6] the zwitterionic form of TC should dominate at pH about 9.0.

Kinetics of ester cleavage by TC

Two esters, 4-nitrophenyl acetate (**1**) and L- and D-4-nitrophenyl *N*-(benzyloxycarbonyl)phenylalaninate (**2**) were used as substrates. Like in other related systems,[1–3] ester cleavage proceeds as a stoichiometric reaction with TC rather than a catalytic process. In the case of **1** simple second-order reaction kinetics, first order in both TC and **1**, were observed. Fig. 1 shows the dependence of the observed second-order rate constant $k_{2,obs}$ on pH, which allows one to identify the catalytically active groups in the TC molecule. The reaction rate becomes detectable at pH above 6 and then rapidly increases in the pH-interval where deprotonation of the TC phenolic groups occurs in agreement with the assumption that the phenolates are the active nucleophiles in the TC zwitterion. At pH ⩾ 9.0 deprotonation of the ammonium group becomes appreciable.

The results in Fig. 1 were fitted by non-linear regression to eqn. (1) which was derived on the assumption that **1** reacts with

$$k_{2,obs} = ((k_1 + k_2) + k_1([H^+]/K_{a2}))/(1+[H^+]/K_{a2} + [H^+]^2/K_{a1}K_{a2}) \quad (1)$$

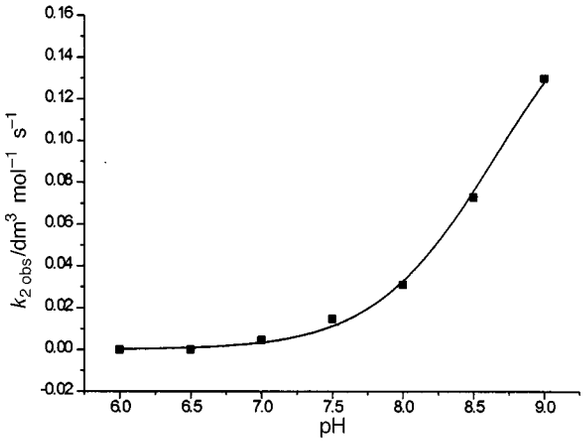


Fig. 1 Second-order rate constants of the cleavage of **1** (40 μmol dm^{-3}) by TC (5 mmol dm^{-3}) as a function of pH.

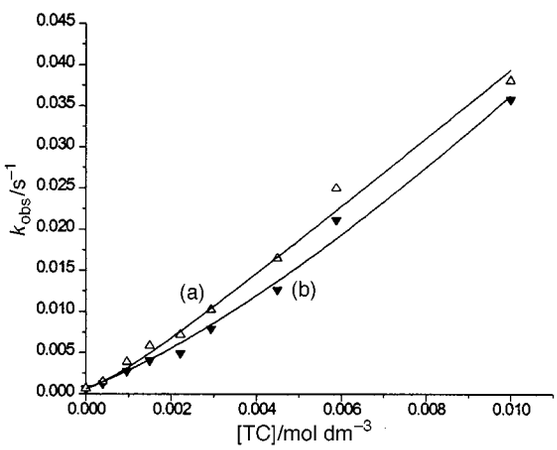


Fig. 2 Pseudo-first-order rate constants of the cleavage of enantiomers of **2** (40.2 μmol dm^{-3}) (a) L- and (b) D-enantiomer by TC at pH 9.0 and 25 °C as a function of macrocycle concentration.

both phenolates of TC independently with second-order rate constants k_1 and k_2 for less and more basic phenolate groups respectively, where K_{a1} and K_{a2} are the acidity constants of the phenolic groups. Obtained rate constants are k_1 = 0.0045 dm^3 mol^{-1} s^{-1} and k_2 = 0.186 dm^3 mol^{-1} s^{-1} for phenolates O6 and O2, respectively. No contribution of the free amino group was detected.

The hydrolysis kinetics of enantiomers of **2** were more complicated. Under pseudo-first-order conditions with a high excess of TC over **2** the reaction was first-order in the ester, but the plots of observed pseudo-first-order rate constants (k_{obs}) *vs.* TC concentration for both enantiomers of **2** showed a small, but quite reliable, downward curve, Fig. 2. A noticeable enantioselectivity is observed at TC concentrations around 0.002 mol dm^{-3} ($k_L/k_D \approx 1.5$), but it disappears at higher macrocycle concentrations, Fig. 3. Such behavior indicates that more than one TC molecule participates in the hydrolysis of **2**. Assuming the simplest reaction scheme, in which two reactive complexes of stoichiometries 1:1 and 1:2 are formed [eqns. (2)–(5)] one

$$S + TC \rightleftharpoons TC{\cdot}S \ (K_1) \quad (2)$$

$$TC{\cdot}S + TC \rightleftharpoons TC_2{\cdot}S \ (K_2) \quad (3)$$

$$TC{\cdot}S \longrightarrow \text{Products} \ (k_{1C}) \quad (4)$$

$$TC_2{\cdot}S \longrightarrow \text{Products} \ (k_{2C}) \quad (5)$$

obtains for the observed rate constant expression (6) where k_0 is

$$k_{obs} = (k_0 + k_{1C}K_1[TC] + k_{2C}K_1K_2[TC]^2)/(1 + K_1[TC] + K_1K_2[TC]^2) \quad (6)$$

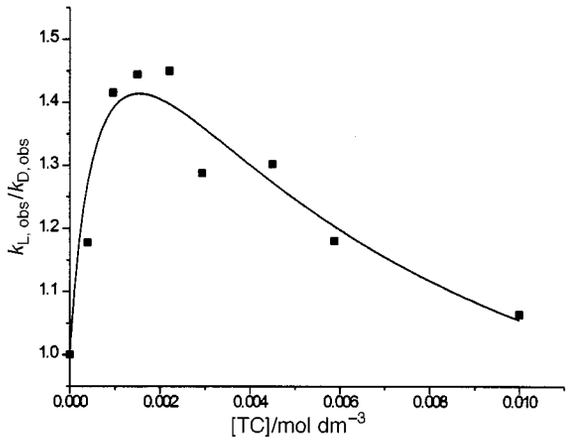


Fig. 3 The ratio of pseudo-first-order rate constants of the cleavage of enantiomers of **2** (40.2 μmol dm^{-3}) by TC at pH 9.0 and 25 °C as a function of macrocycle concentration.

the rate constant of spontaneous hydrolysis. Fitting of the results in Fig. 2 to eqn. (6) allowed us to determine reliably only three parameters: k_{1C}, K_1 and $k_{2C}K_1K_2$. The contribution of K_1K_2[TC]^2 in the denominator was too small. This means actually that the ternary complex TC_2·S is insignificant and steps (3) and (5) can be substituted by a single bimolecular step (7),

$$TC{\cdot}S + TC \longrightarrow \text{Products } (k_{3C}) \qquad (7)$$

where $k_{3C} = k_{2C}K_2$. For L-**2** the rate and equilibrium parameters are $k_{1C} = 0.0052$ s^{-1}, $K_1 = 610$ dm^3 mol^{-1}, $k_{2C}K_1K_2 = 2850$ dm^6 mol^{-2} s^{-1}, and $k_{3C} = 4.7$ dm^3 mol^{-1} s^{-1}; for D-**2**: $k_{1C} = 0.031$ s^{-1}, $K_1 = 65$ dm^3 mol^{-1}, $k_{2C}K_1K_2 = 490$ dm^6 mol^{-2} s^{-1} and $k_{3C} = 7.5$ dm^3 mol^{-1} s^{-1}.

A typical feature of serine hydrolase enzymes is the inhibitory effect of boric acid which blocks the reactive serine hydroxy group.[14] In order to test whether TC, which utilizes a phenolic hydroxy, mirrors this feature, the effect of boric acid on the kinetics of hydrolysis of the ester **2** was studied. Addition of boric acid enhanced the rate of spontaneous hydrolysis, Fig. 4, but the effect on the TC catalyzed hydrolysis was different: small concentrations of boric acid caused acceleration, more pronounced for L-**2**, while at high concentrations the expected inhibition was observed with both substrates. It is worth noting that addition of low borate concentrations leads to an increase in the enantioselectivity of hydrolysis.

No attempt to fit the profiles in Fig. 5 to any theoretical equation was made since under the conditions employed both 1 : 1 and 1 : 2 complexes contribute to the observed rate making the reaction scheme too complicated.

Binding of amino acid derivatives to TC zwitterion

Chiral amino acid derivatives which exist as anionic (**3** and **4**), neutral (**5**) or zwitterionic (**6**) species and the achiral cation **7** were studied as guest compounds for the TC zwitterion.

The complexation studies of the zwitterionic form of TC with guest amino acids were performed by titrations of the host with the guest monitored by ^{1}H NMR, direct and competition fluorescent spectroscopy, and UV-visible spectroscopy. NMR proved to be the most informative technique, both in providing some structural details and in the determination of the association constants. Fig. 6 shows the representative changes in the host signal chemical shifts ($\Delta\delta_{obs}$) on titration by various guest compounds. Fitting of the titration results in eqn. (8), which is

$$\Delta\delta_{obs} = \Delta\delta_\infty K\,[\text{guest}]/(1 + K\,[\text{guest}]) \qquad (8)$$

derived for the 1 : 1 complexation under conditions of a high excess of the guest.[15] The non-linear regression allowed us to calculate the values of binding constants K (Table 1) as well as

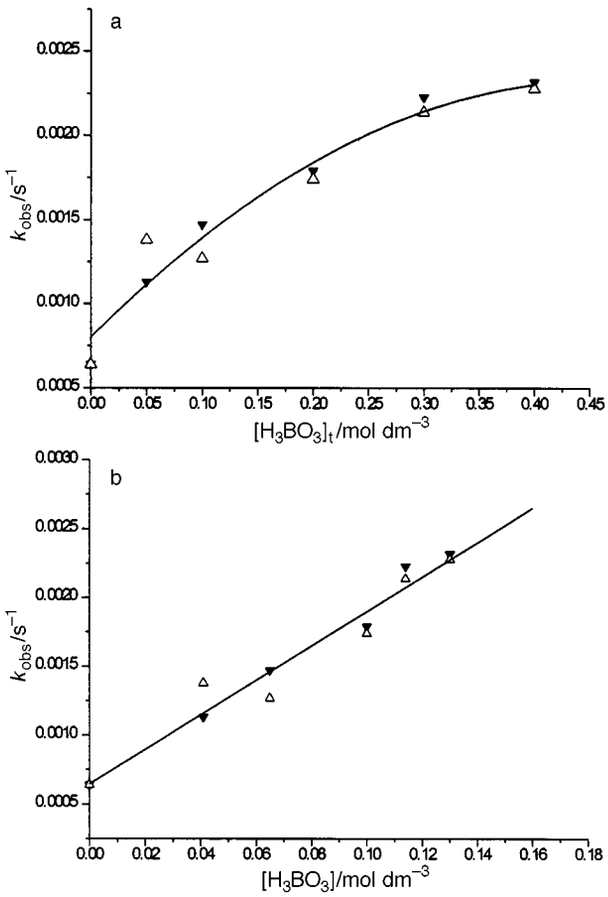


Fig. 4 (a) Effect of boric acid on the rate of spontaneous hydrolysis of **2** (42 μmol dm^{-3}, solid triangles D- and open triangles L-enantiomer). (b) The same data plotted *vs.* concentration of the monomeric form calculated from the published stability constants of trimer and pentamer species.[22]

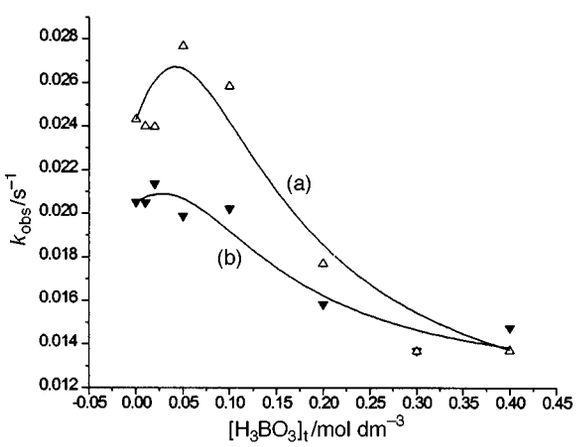


Fig. 5 Effect of boric acid on the rate of hydrolysis of enantiomers of **2** (40.4 μmol dm^{-3}), (a) L- and (b) D-enantiomer in the presence of 6 mmol dm^{-3} TC at pH 9.0 and 25 °C.

the limiting values of the complexation-induced chemical shifts ($\Delta\delta_\infty$) (Table 2). The directions of the induced shifts varied for different host protons, as determined by the anisotropic shielding effect of the guest aromatic moieties. The values of binding constants were calculated from the data for several different signals and then averaged.

Direct fluorescent titrations were also attempted in order to provide an alternative method for the binding constant determinations. The intrinsic fluorescence of TC (excitation wavelength 280 nm, emission wavelength 337 nm)[16] was expected to change on complexation with the hydrophobic guests. The titrations however, led to highly scattered curves

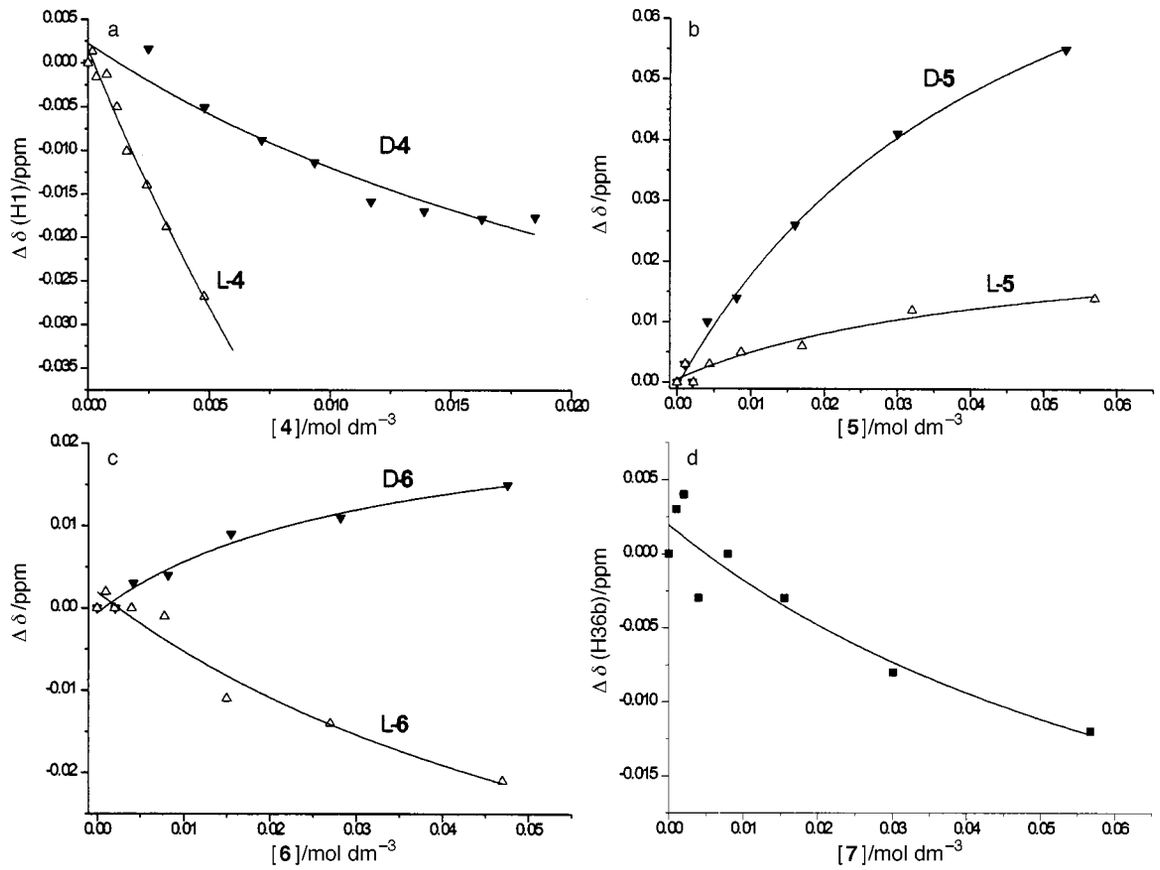


Fig. 6 Representative ^{1}H titration curves of TC (0.5 mmol dm^{-3} of TC for **4** and 2 mmol dm^{-3} of TC for **5**–**7**) in D_2O with L- and D-enantiomers of **4**–**6** and with **7** at pD 9.0. Protons of TC monitored are indicated on the curves. (a) L-**4** and D-**4**, (b) L-**5** and D-**5**, (c) L-**6** and D-**6**, (d) **7**.

Table 1 Binding constants for the TC zwitterion with amino acid derivatives at pH 9.0 and 25 °C

Guest	K/dm^3 mol^{-1}
D-**3**	<5 (85 ± 20)[a]
L-**3**	<5 (<20)[a]
D-**4**	32 ± 1 (270 ± 70)[a]
L-**4**	56 ± 20 (<20)[a]
D-**5**	39 ± 14
L-**5**	22 ± 13
D-**6**	30 ± 9
L-**6**	12 ± 2
7	5 ± 3
D-**2**	65 ± 17[b]
L-**2**	610 ± 50[b]

[a] pH 6.0, data from ref. 5*b*. [b] K_1 calculated from kinetic results, Fig. 2, by fitting to eqn. (6).

with an overall change in the fluorescence being within 10% of the original intensity. A slight decrease in the fluorescence for the first portions of the titrant could be attributed to quenching by minor impurities.

Besides direct fluorescent titrations, we used a competitive titration by the amino acid-containing guests on the TC complex with 8-anilinonaphthalenesulfonate anion (ANS), a well-known fluorescent label.[17] Prior to the experiments with the amino acid derivatives, the complexation of ANS with TC was studied by monitoring the ANS fluorescence upon increasing the concentration of the macrocycle. As shown in Fig. 7a, the intensity of the ANS fluorescence increases nearly by an order of magnitude within the TC concentration range of 0–20 mmol^{-1} dm^3, and the emission maximum shifts to shorter wavelengths which is indicative of hydrophobic interactions with the macrocycle.[17] The linearity of the concentration dependence of fluorescence intensity at a fixed wavelength (Fig. 7b) demon-

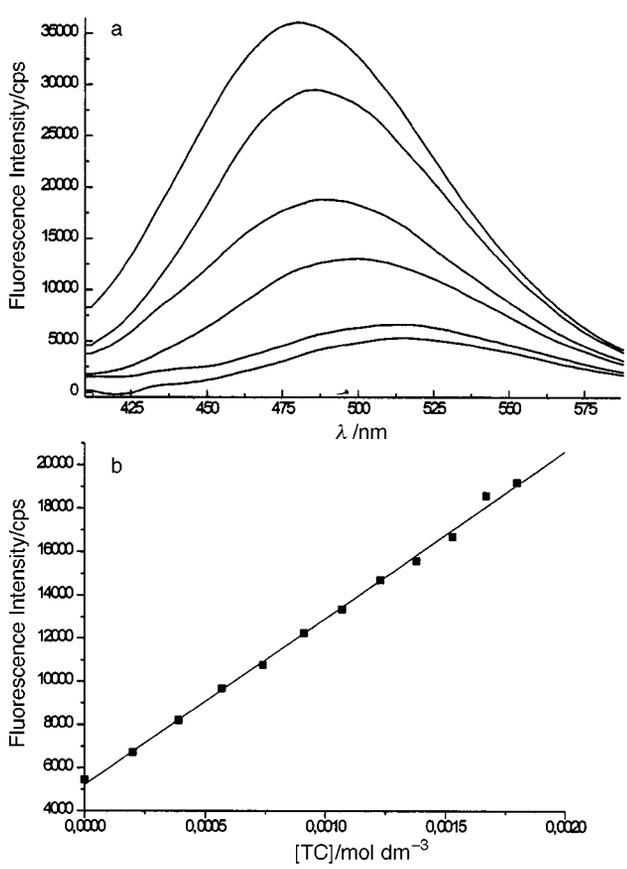


Fig. 7 (a) Emission spectra of 5.04 μmol dm^{-3} ANS (excitation wavelength 367 nm) at increased concentrations of TC at pH 9.0. (b) Effect of TC addition on the ANS fluorescence intensity (excitation wavelength 367 nm, emission wavelength 516 nm).

Table 2 Selected CIS values determined from the curve fitting for different TC protons[a]

Proton	D-**5**	L-**5**	D-**6**	L-**6**
H7	0.068 (0.014)	0.127 (0.051)		
H13	0.110 (0.016)	0.205 (0.049)	0.026 (0.007)	0.040 (0.009)
H18				−0.033 (0.008)
H19			−0.014 (0.006)	−0.022 (0.005)
H21			−0.017 (0.008)	−0.022 (0.005)
H29	0.017 (0.006)			
H32	0.108 (0.027)	0.230 (0.132)	0.089 (0.018)	0.103 (0.023)
H37		−0.032 (0.014)	−0.020 (0.010)	

[a] Errors between parentheses.

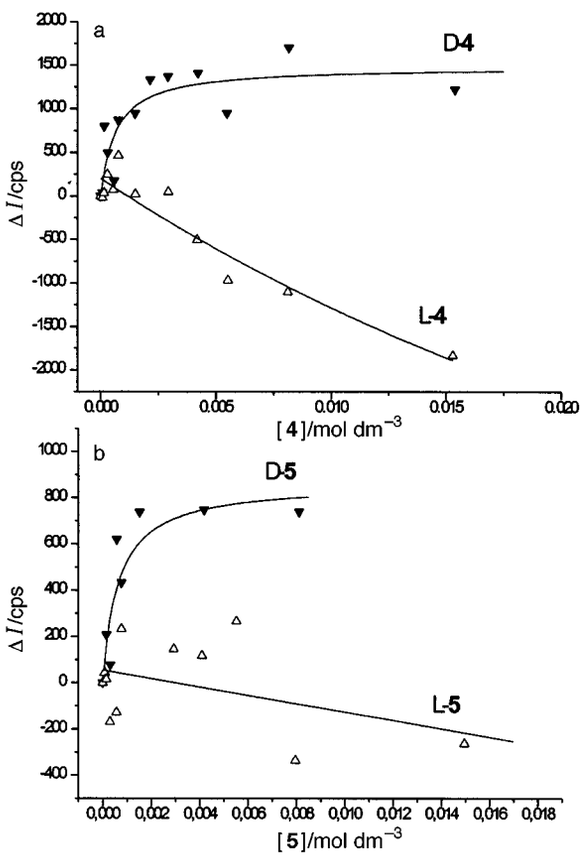


Fig. 8 Competitive titration of the TC–ANS system with enantiomers of **4** and **5**. (a) D-**4** and L-**4**, (b) D-**5** and L-**5**, both with concentrations of 0.91 mmol dm^{-3} and 5.04 μmol dm^{-3} for TC and ANS, respectively.

strates, however, that the degree of complexation at these host concentrations is relatively low. Nevertheless, this highly fluorescent complex can be used for competitive studies of TC with other guest compounds. Thus, the solution containing 5.04 μmol^{-1} dm^3 ANS and 0.91 $mmol^{-1}$ dm^3 TC, with the fluorescence intensity *ca.* 250% of the pure ANS, was titrated with the enantiomers of **4** and **5** (see Fig. 8a,b). Although the titration revealed remarkable enantioselective spectral responses discussed below, the interaction probably involved the formation of ternary complexes and did not provide any reliable binding constant values. Indeed, displacement of ANS from its complex with TC must lead to a restoration of the fluorescence intensity observed for ANS in the absence of TC, that is a *ca.* 2.5-fold decrease in the fluorescence intensity should be observed. Instead of this, addition of D-**4** and D-**5** caused an increase in the fluorescence intensity, Fig. 8. Addition of L-**4** caused the expected decreasing effect, Fig. 8a, but the fluorescence intensity tended to "saturate" at a much higher level than that of free ANS.

The titrations monitored by UV-visible spectroscopy showed very subtle changes of the host and guest spectra upon complexation and did not allow us to get reliable binding constant values.

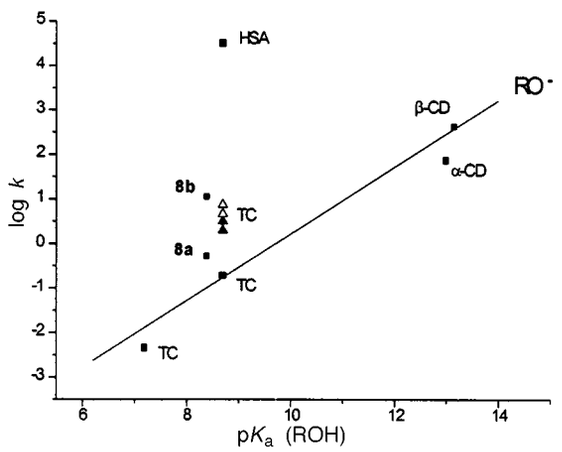


Fig. 9 Logarithms of rate constants for the cleavage of **1** by TC (present study), α- and β-cyclodextrins (CD) (ref. 2*a*) and human serum albumin (HSA) (ref. 17*e*), of 4-nitro-1-naphthyl acetate by cyclophanes **8a**,**b** (ref. 3*a*) and enantiomers of **2** by TC (present study, solid triangles —rate constants $k_{1C}K_1$ for the reaction with one TC zwitterion, open triangles—rate constants k_{3C} for the reaction with two TC zwitterions) *vs.* their pK_a (statistically corrected pK_a values of α- and β-cyclodextrins for 6 and 7 equivalent carbohydrate monomeric units, respectively, are used). The line represents the Brønsted eqn. (9) for phenolate and alcoholate nucleophiles.

Discussion

Esterolysis

Esterolytic activity towards activated substrates like nitrophenyl esters was reported for several biological molecules, which are not hydrolytic enzymes by their biological function, *e.g.* liver alcohol dehydrogenase,[18a] several aldehyde dehydrogenases,[18b–d] serum albumin.[18e] In all cases a nucleophilic group such as cysteine sulfhydryl[18a] or tyrosine phenolate[18e] functions as a reactive site. To our knowledge, the esterolytic activity of alkaloids was not reported previously.

Kinetic results for the hydrolysis of widely employed ester **1** allow us to compare the reactivity of TC with other similar systems. It is known that the reactivity of oxygen nucleophiles towards **1** perfectly follows the Brønsted correlation for the pK_a of their conjugated acids for moderately basic nucleophiles.[19] Williams *et al.*[19b] found for substituted phenolate nucleophiles eqn. (9), which is valid in the pK_a range from 5.5 to 10.6. Actu-

$$\log k_{ArO} = 0.75\ pK_a^{ArOH} - 7.28 \qquad (9)$$

ally, it is valid for a wider range of pK_a up to approximately 13 since data for substituted aliphatic alcohols such as trifluoroethanol and propargyl alcohol (propargyl = prop-2-ynyl) follow the same correlation as phenols.[19a] The line in Fig. 9 corresponds to eqn. (9) and the points show the rate constants for the

cleavage of **1** by TC (two constants correspond to two phenolate groups of different basicity), cyclodextrins and cyclophanes **8a**,**b** (with 4-nitro-1-naphthyl acetate, an ester of similar reactivity, as the substrate). Evidently, TC as well as cyclodextrins and cyclophane **8a** possess the reactivity expected from their basicities and therefore complexation of the substrate does not lead to a transition state stabilization for these catalysts. In other words, substrate binding here is non-productive, as was shown for cyclodextrins on the basis of other arguments.[2]

Cyclophane **8b** possessing a larger cavity than **8a** shows considerable positive deviation from the Brønsted plot indicative of a transition-state stabilization by inclusion in this macrocycle. Productive binding was observed also for the cleavage of **1** by a dimethylamino derivative of calixresorcin[4]arene,[4] but unsubstituted calixarenes, possessing like TC or cyclophanes **8a**,**b** a phenolic nucleophilic group, did not affect or even inhibited the hydrolysis of **1**.[20] In the case of ester **2** the second-order rate constant for the interaction with the first TC zwitterion is given by the product $k_{1C}K_1$ which equals 3.2 $dm^3\ mol^{-1}\ s^{-1}$ and 2.0 $dm^3\ mol^{-1}\ s^{-1}$ for L- and D-**2** respectively. These rate constants, as well as k_{3C} values for step (7), show positive deviations from the Brønsted plot, Fig. 9.

The point for hydrolysis of **1** by human serum albumin (HSA)[18e] is included for comparison. The hydrolytic active site of this protein is a tyrosine residue with anomalously low pK_a 8.7. Evidently, all "artificial hydrolases" utilizing an oxygen nucleophile are still not as efficient as a biological molecule possessing a highly specific recognition site. Notably, the comparisons of esterase activity of "artificial enzymes" towards **1** with a natural catalyst always refer to α-chymotrypsin as supposedly being the best natural hydrolaze. This enzyme, however, is not specific for **1** and has 10 times lower activity with this substrate than HSA (second-order rate constants 2.8×10^3 and 3×10^4 $dm^3\ mol^{-1}\ s^{-1}$, respectively).[18e,f]

Participation of the second macrocyclic molecule in the ester cleavage, Schemes (2)–(5), was reported for cyclodextrins also.[21] The importance of this route increased for larger, more hydrophobic, substrates capable of being in contact with two cyclodextrin molecules. The same tendency is observed for TC: its reaction with small ester **1** involves only one macrocycle molecule, but with large **2** the contribution of the second molecule becomes important.

Hydrolysis of **2** in the presence of TC possesses a noticeable enantioselectivity. Binding enantioselectivity is large, $(K_1)_L/(K_1)_D = 9.4$, and is opposite to the kinetic enantioselectivity, $(k_{1C})_L/(k_{1C})_D = 0.17$. Expressing enantioselectivity as the ratio of second-order rate constants one obtains $(k_{1C}K_1)_L/(k_{1C}K_1)_D = 1.6$. This ratio can be interpreted as the ratio of the "binding constants" of the respective transition states.[2b] Evidently, the binding enantioselectivity of TC towards the neutral substrate practically disappears on passing to anionic transition states. This observation indicates the importance of polar interactions in determining the binding selectivity (see below). Interestingly, a reversal of enantioselectivity occurs on going from the reaction with the first to the reaction with the second macrocycle molecule: $(k_{3C})_L/(k_{3C})_D = 0.6$. In the range of TC concentrations employed, this reversal is not observed experimentally because even at the highest TC concentration the contribution of step (7) is not dominant yet. This leads only to nearly complete compensation of enantiospecificity of the hydrolysis catalyzed by the first TC molecule.

The catalytic effect of boric acid on the ester hydrolysis is well documented,[22a,b] and is attributed to nucleophilic reactivity of $B(OH)_4^-$. Apparent "saturation" of the plot in Fig. 4a is due to polymerization of borate anions at high concentrations as is evident from the linear dependence of k_{obs} on monomeric H_3BO_3 concentration, Fig. 4b. In the case of the TC-catalyzed reaction, one would expect to observe an inhibitory effect of boric acid due to its complexation with reactive phenolate groups in a manner analogous to serine proteases[14] and some model systems.[22c] However, at low borate concentrations an acceleration effect of H_3BO_3 was observed, Fig. 5. Most likely it is of the same origin as mentioned above for the catalytic effect, namely, complexation of boric acid with one of the TC phenolates producing a new nucleophile of the type $(RO)B(OH)_3^-$ which possesses higher reactivity than the parent phenolate. In more concentrated borate solutions more H_3BO_3 molecules become involved in complexation and the resulting complex possesses a reduced reactivity. Obviously, the second complexation must affect the reactivity of the initially formed $(RO)B(OH)_3^-$ anion. Taking into account the known tendency of borate anions to polymerize in solution, this second complexation can be viewed as the formation of an unreactive polymeric anion, probably bridging two phenolate groups at TC.

Complexation

As mentioned previously, the ionization state of the TC molecule is crucial for its recognition properties. The distribution curves for different ionization states calculated with the pK_a values of the TC aromatic hydroxys (7.16 and 8.67) and the tertiary amino group (9.65) predict that the fraction of the bis-zwitterionic structure (Chart 1) reaches the maximum of *ca.* 65% of the total TC concentration in the pH range 9.0–9.3. Unfortunately at no pH can the fraction of zwitterion be higher than this figure, and one should realize that the observed binding constants involve some contributions from the guest binding to the monocationic and monoanionic forms of TC. As shown later, the main driving force of complexation at pH 9 is the guest hydrophobicity, and minor fractional variations in TC charge are insignificant.

Since the recognition properties of the macrocycle are primarily determined by the proper balance of electrostatic and hydrophobic interactions, it is reasonable to explore its binding with the amino acid derivatives containing different charges as well as hydrophobic moieties. The set of guest compounds that has been chosen for the binding studies (Chart 1) includes the enantiomers of phenylalanine zwitterions (**6**), whose phenyl rings were proven to complement the TC groove-like structure,[5b] two protected phenylalanine derivatives bearing the anionic carboxylate (**4**), neutral but highly polar amino group (**5**), or less polar N- and O-protected groups (**2**), cationic β-phenylethylamine (**7**) and a less hydrophobic anionic *N*-acetylalanine (**3**).

Complexation thermodynamics

The binding constants of amino acid derivatives with the TC zwitterion determined by NMR and kinetically are summarized in Table 1. A comparison with the previously obtained data for the cationic form shows that the general binding affinity of the host toward anionic guests decreases, but does not disappear with the introduction of two negative charges. The observed decrease of the K values by at least an order of magnitude for L-**3**, D-**3** and D-**4** is readily explained by the electrostatic repulsion between the host and guest anionic groups. At the same time, the binding of L-**4** noticeably improves at pH 9 thereby inverting the enantioselectivity of recognition as compared to pH 6. This effect should probably be attributed to the change in the macrocycle conformation in the zwitterionic state.

On the other hand, the appearance of two negative charges in the host molecule does not lead to any significant binding of cationic guests, as one can conclude from the small binding constant for **7**. Comparison of the binding constants for **4**, **5** and **6** shows that the guest charge is insignificant for the binding of D-enantiomers, but for L-enantiomers the presence of the negative charge improves binding whereas the positive charge weakens it. All three guests are expected to have similar hydrophobic contributions to binding due to the presence of one benzyl group and this contribution seems to be dominant.

Indeed, the binding free energies of all these guests differ by less than 3.8 kJ mol^{-1}. On going to a considerably more hydrophobic guest **2** the binding constants increase nearly 30-fold for the L-enantiomer and by 50% for the D-enantiomer, as compared to the neutral, but more polar **5**, again demonstrating the importance of the hydrophobic contribution. Together with the low binding constant of **7** these observations indicate a conformation of the host–guest complexes which allows a maximum contact between hydrophobic areas of TC and guest molecules, a close contact of the guest carboxylate with the host ammonium sites for L-enantiomers but no attraction of guest ammonium groups to TC anionic sites.

Somewhat unexpectedly the general binding affinity for guests **4**–**6** is modest and almost independent of the ionic state of the guest. Indeed, multiple salt bridges, that might be formed between the moieties bearing several charges, would be expected to bring about much stronger complexation.[24] The most probable explanation for such behavior is the inability to form the optimal number of ion pairs between the host and the guest due to a steric hindrance and/or electrostatic repulsions between similar charges. As a result, it appears that the primary driving force for the complexation is the surface hydrophobic interaction. Also a contribution from cation–π interactions[25] involving one (or both) of the ammonium TC groups and the guest phenyl moiety can be significant for the binding, however, the available results do not allow us to discuss this aspect in detail.

Among guests **4**–**6** the binding enantioselectivity varies from marginal and close to experimental error for L- and D-**4** to quite notable, three-fold differentiation between L- and D-**6**. The highest discrimination is observed for the isomers of the unprotected phenylalanine zwitterion which possess the lowest absolute affinity. This may indicate that the selectivity is due to weak ionic and other polar interactions which are maximized in the presence of several charges. The total binding energy is, on the contrary, contributed to by the hydrophobic interactions. High binding enantioselectivity of **2** is unexpected and at present we cannot rationalize it. Enantioselectivity of "binding" of the anionic transition state evaluated above as $(k_{1C}K_1)_L/(k_{1C}K_1)_D = 1.6$ is modest and close to that for anionic guest **4**.

Structure of the complexes

In order to visualize some of the possible complex structures, we performed docking calculations on the zwitterions of L- and D-**6** with TC bis-zwitterion with the aid of the SYBYL/TRIPOS force field. The simulation is complicated by the fact that the macrocycle can accept numerous conformations, as can be seen from a Corey–Pauling–Koltun (CPK) model. The evaluation of the energy differences between the conformations by molecular modeling cannot take into account the solvation effects and therefore would be unreliable. For this reason, we used as an initial approximation of the conformation of TC a reported crystal structure.[26] As shown in Fig. 10, the macrocycle in this conformation forms a concave surface with the aromatic rings open for contact with the guest phenyl moiety. As found previously,[5] the recognition of aromatic guests by the TC macrocycle occurs without formation of inclusion complexes due to the small cavity size of the host. More likely, the guest is positioned inside the groove of the concave macrocycle.

Qualitative information about the location of the guest can be obtained from the values of the ^{1}H NMR chemical shifts (see Table 2). Thus, both enantiomers of **5** and **6** cause the greatest changes in the signals of H13 and H32 located on the opposite aromatic rings of TC. Since these changes are most likely attributable to the deshielding effects of the guest phenyl groups, the latter was positioned close to the corresponding host protons in the docked complex structure.

The minimized docked structures of the complexes with L-**6** and D-**6** are shown in Figs. 10a and 10b, respectively. The

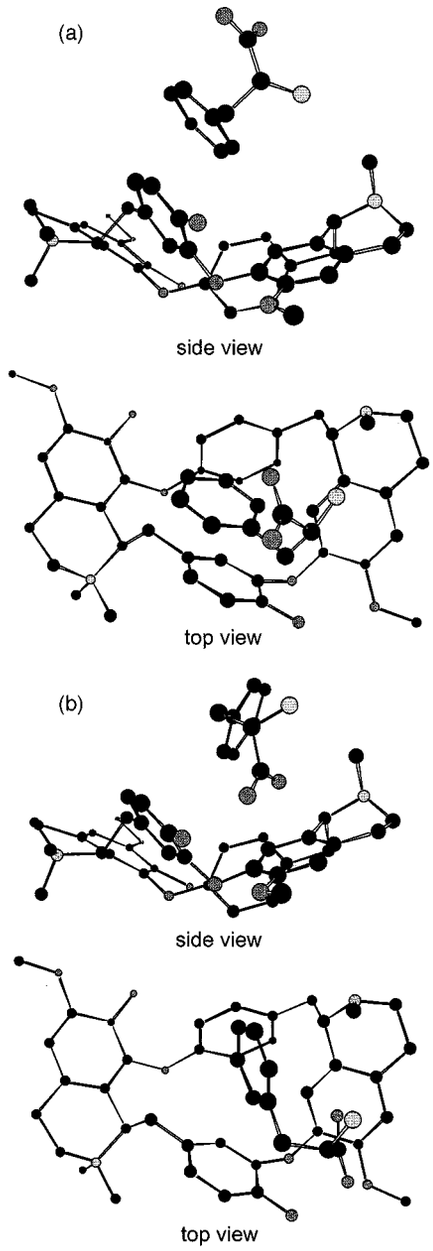


Fig. 10 Simulated structures of the TC complexes with (a) D-**6** and (b) L-**6**.

relative position of the L- and D-enantiomers in the minimized structures is substantially different. Phenyl rings of host and guest molecules are coplanar for the D-enantiomer providing a larger contact surface at one side of the guest phenyl, but no contact at all at the other side. In the binding conformation of the L-enantiomer, both sides of the guest phenyl are in partial contact with the hydrophobic areas of TC resulting in approximately equal total contact surface for both enantiomers. The most notable difference between the binding conformations of the two enantiomers is the much shorter distance between the oxygen atoms of the guest carboxylate and phenolate oxygen at C31 of TC for L-**6** (0.27 and 0.40 nm) than for D-**6** (0.69 and 0.67 nm). Since the Coulomb energy is proportional to r^{-1}, this difference in distances leads to the approximate doubling of the repulsion energy for the L-enantiomer. This effect is partly compensated for by the shorter distance of the same carboxylate oxygens to the ammonium positively charged site at N2 of TC for the L-enantiomer (0.41 and 0.62 nm) as compared to the D-enantiomer (0.69 and 0.85 nm), but this leads to only a *ca.* 40% increase in the attractive energy. In addition, there is practically no difference in the position of the guest ammonium

group with respect to the phenolate and ammonium groups of TC: 0.57 nm to both groups for L-**6** and 0.54 (phenolate at C31) and 0.51 nm (N2 ammonium) for D-**6**. In total, an increased repulsion with the guest carboxylate for L-**6** lowers the binding constant of this enantiomer and leads to the observed enantioselectivity.

Admittedly, the minimized structures represent only one of the possible complex geometries for each enantiomer. However, these structures agree with the spectral data and qualitatively reflect the general mechanisms of recognition by the TC zwitterion.

Use of chiral recognition for spectroscopic differentiation of enantiomers

One of the most valuable applications of artificial and natural chiral recognition systems is selective analytical detection and determination of enantiomers. While extensively applied in such techniques as HPLC[27] and capillary electrophoresis,[28] molecular receptors have found relatively limited use as chiral sensor compounds.[29] We discovered that, besides thermodynamic discrimination of the amino acid derivatives, the zwitterionic form of TC is capable of generating distinctly different spectral responses to the presence of different guest enantiomers. Fig. 8a shows the change in the fluorescence intensity of the ANS–TC complex upon titration with L-**4** and D-**4**. One can see that the association with the L- and D-isomers has an opposite effect on the fluorescence intensity. The effect of L-**4** corresponds to the expected displacement of the ANS fluorophore from the complex due to the competition of **4** for the host molecule. The opposite effect of D-**4** may be explained by the formation of a stronger ternary complex with ANS. Whatever the reason, the ANS–TC system behaves in this case as a sensing system that provides a simple qualitative spectral differentiation between the guest enantiomers. A similar, but weaker effect was observed for D-**5** and L-**5** (Fig. 8b).

Another example of the chiral spectral differentiation is provided by the NMR chemical shifts of TC in the presence of **4**. For example, the strong 1H singlet in the TC spectrum experiences a much higher shift upon addition of L-**4** than it does with D-**4** (Fig. 6a). In this case, a more than 5-fold difference in the induced shift (absolute value of 0.02 ppm) is observed in the presence of 5 mmol dm^{-3} guests.

It is important to mention that considerable spectral response is observed at the guest concentrations that correspond to low degrees of complex formation. Therefore, the absolute affinity of the receptor is of less importance for the analytical applications than the selectivity of binding and the magnitude of physical response.

Experimental

Materials

The compounds studied as guest molecules, inorganic salts used as components of the buffer solutions or used for adjusting the pH of the solutions, 4-nitrophenyl acetate, the 4-nitrophenyl ester of *N*-benzoylphenylalanine, and (+)-tubocurarine chloride ((+)-tubocurarine chloride pentahydrate, $[TCH]Cl_2$) were purchased from Sigma and used without further purification. All solutions were prepared in purified (Milli-Q Reagent Water System) water or in D_2O for NMR studies.

Instrumentation

Ultraviolet/Visible spectra were obtained with a Hewlett Packard 8452A spectrophotometer and fluorescence spectra were recorded on a FluoroMax SPEX spectrofluorometer. ^1H NMR spectra were recorded on 300 MHz Varian Gemini and 400 MHz Varian Unity spectrometers.

Methodology

Spectrophotometric pH-titration of TC was performed with 35.7 μmol dm^{-3} TC in the mixture of acetate, phosphate and borate buffers. UV spectra were recorded in the pH range 4–11. Two isosbestic points at 233 and 276 nm were observed and the values of pK_a were calculated from a non-linear least-squares fit of the absorbance *vs.* pH profiles at several wavelengths around 250 and 330 nm to the respective theoretical equation for two successive deprotonation equilibria.[30]

Kinetics of the cleavage of esters **1** and **2** were studied spectrophotometrically by the appearance of the 4-nitrophenolate anion at 400 nm at 25 °C and ionic strength 0.1 mol dm^{-3} NaCl. Stock solutions of the substrates were prepared in acetonitrile. The reaction was initiated by addition of 0.050 cm^3 of the stock solution to 2.45 cm^3 of TC solution at desired pH placed into the thermostatted cell of a spectrophotometer. Since TC possesses considerable buffer capacity in the pH range studied no additional buffer was added. All experiments were performed under conditions of high excess of TC over the substrate.

Fluorescence and UV absorbance were measured in 0.03 mol dm^{-3} borate buffer solution pH 9.0. For NMR studies TC and amino acid solutions were adjusted to pD 9.0 with concentrated solutions of sodium carbonate or sodium deuteroxide and trifluoroacetic acid in D_2O.

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References

1 For reviews: (*a*) M. L. Bender and M. Komyiama, *Cyclodextrin Chemistry*, Springer-Verlag, Berlin, 1978; (*b*) R. Breslow, *Adv. Enzymol.*, 1986, **58**, 1; (*c*) I. Tabushi, *Acc. Chem. Res.*, 1982, **15**, 66; (*d*) H.-J. Schneider, R. Bush, R. Kramer, U. Schneider and I. Theis, *ACS Monogr.*, *Nucleophilicity*, 1987, 457; (*e*) Y. Murakami, J. Kikuchi, Y. Hisaeda and O. Hayashida, *Chem. Rev.*, 1996, **96**, 721.
2 (*a*) O. S. Tee, M. Bozzi, J. J. Hoeven and T. A. Gadosy, *J. Am. Chem. Soc.*, 1993, **115**, 8990; (*b*) O. S. Tee, *Adv. Phys. Org. Chem.*, 1994, **29**, 1.
3 (*a*) F. Diederich, G. Shürmann and I. Chao, *J. Org. Chem.*, 1988, **53**, 2744; (*b*) I. Chao and F. Diederich, *Recl. Trav. Chim.*, 1993, **112**, 335.
4 N. Pirrincioglu, F. Zaman and A. Williams, *J. Chem. Soc.*, *Perkin Trans. 1*, 1996, 2561.
5 (*a*) C. G. Alcántar, A. V. Eliseev and A. K. Yatsimirsky, *Bioorg. Med. Chem. Lett.*, 1995, **5**, 2993; (*b*) C. G. Alcántar, A. V. Eliseev and A. K. Yatsimirsky, *J. Mol. Recognit.*, 1996, **9**, 54.
6 R. B. Barlow, *Br. J. Pharmacol.*, 1982, **75**, 503.
7 Y. Fraenkel, J. M. Gershoni and G. Navon, *Biochemistry*, 1994, **33**, 644.
8 T. Kitae, T. Nakayama and K. Kano, *J. Chem. Soc.*, *Perkin Trans. 2*, 1998, 207 and references therein.
9 (*a*) T. H. Webb and C. S. Wilcox, *Chem. Soc. Rev.*, 1993, **20**, 383; (*b*) H.-J. Schneider, *Angew. Chem.*, *Int. Ed. Engl.*, 1993, **32**, 848.
10 Y. Murakami, O. Hayashida and Y. Nagai, *J. Am. Chem. Soc.*, 1994, **116**, 2611.
11 K. Araki, K. Inada and S. Shinkai, *Angew. Chem.*, *Int. Ed. Engl.*, 1996, **35**, 72.
12 (*a*) J. Dowden, P. D. Edwars and J. D. Kilburm, *Tetrahedron Lett.*, 1997, **38**, 1095; (*b*) Ch. P. Waymark, J. D. Kilburn and I. Gillies, *Tetrahedron Lett.*, 1995, **36**, 3051.
13 S. S. Yoon and W. C. Still, *J. Am. Chem. Soc.*, 1993, **115**, 823.
14 (*a*) M. Philipp and M. L. Bender, *Proc. Natl. Acad. Sci. USA*, 1971, **68**, 478; (*b*) J. C. Powers and J. W. Harper, in *Proteinase Inhibitors*, eds. A. J. Barrett and G. Salvesen, Elsevier, New York, 1986, p. 69.
15 K. A. Connors, *Binding Constants*, Wiley, New York, 1987.
16 E. P. Gibson and J. H. Tumbult, *Analyst* (*London*), 1979, **104**, 582.
17 Y. Kanaoka, *Angew. Chem.*, *Int. Ed. Engl.*, 1977, **16**, 137.
18 (*a*) C. S. Tsai, *Arch. Biochem. Biophys.*, 1982, **213**, 635; (*b*) J. H. Park, B. P. Meriwerther, P. Clodfelder and L. W. Cunningham, *J. Biol. Chem.*, 1961, **236**, 131; (*c*) R. I. Feldman and H. Weiner, *J. Biol.*

Chem., 1972, **247**, 267; (*d*) R. S. Sidhu and A. H. Blair, *J. Biol. Chem.*, 1975, **250**, 7894; (*e*) G. E. Means and M. L. Bender, *Biochemistry*, 1975, **14**, 4989; (*f*) M. J. Hardmna, P. Valenznela and M. L. Bender, *J. Biol. Chem.*, 1971, **246**, 5907.
19 (*a*) D. J. Hupe and W. P. Jencks, *J. Am. Chem. Soc.*, 1977, **99**, 451; (*b*) S. Ba-Saif, A. K. Luthra and A. Williams, *J. Am. Chem. Soc.*, 1987, **109**, 6362.
20 H. M. Chawla and M. Pathak, *Bull. Soc. Chim. Fr.*, 1991, 232.
21 (*a*) O. S. Tee and X.-X. Du, *J. Org. Chem.*, 1988, **53**, 1837; (*b*) O. S. Tee and X.-X. Du, *J. Am. Chem. Soc.*, 1992, **114**, 620; (*c*) T. A. Gadosy and O. S. Tee, *J. Chem. Soc.*, *Perkin Trans. 2*, 1994, 715.
22 (*a*) B. Capon and B. C. Ghosh, *J. Chem. Soc. B*, 1966, 472; (*b*) M. N. Khan, *J. Mol. Catal.*, 1987, **40**, 195; (*c*) A. K. Yatsimirsky, K. Yu. Bezsoudnova and I. K. Sakodinskaya, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 635.
23 R. M. Smith and A. E. Martell, *Critical Stability Constants*, Plenum Press, USA, 1975, vol. 4, p. 25.
24 (*a*) H.-J. Schneider, *Chem. Soc. Rev.*, 1994, **23**, 227; (*b*) H.-J. Schneider and I. Theis, *Angew. Chem.*, *Int. Ed. Engl.*, 1989, **28**, 753.
25 J. C. Ma and D. A. Dougherty, *Chem. Rev.*, 1997, **97**, 1303.
26 C. D. Reynolds and R. A. Palmer, *Acta Crystallogr.*, *Sect. B*, 1976, **32**, 1431.
27 (*a*) F. Bressolle, M. Audran, T. N. Pham and J. J. Vallon, *J. Chromatogr. B*, 1996, **687**, 303; (*b*) D. W. Armstrong, T. Tang, Sh. Chen, Y. Zhou, C. Bagwill and J.-R. Chen, *Anal. Chem.*, 1994, **66**, 1473.
28 (*a*) S. Fanali, *J. Chromatogr.*, 1996, **735**, 77; (*b*) D. W. Armstrong, K. Rundlett and G. L. Reid III, *Anal. Chem.*, 1994, **66**, 1690.
29 L. Fabrizzi and A. Poggi, *Chem. Soc. Rev.*, 1995, **24**, 197.
30 J. Polster and H. Lachman, *Spectrophotometric Titrations*, VCH Publishers, Weinheim, 1989.

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