Journal of Inclusion Phenomena and Macrocyclic Chemistry **39:** 339–346, 2001.

1,3-Disubstituted p-tert-Butylcalix[4]arenes as Cholinesterase Inhibitors

E. E. STOIKOVA, G. A. EVTUGYN*, S. V. BELYAKOVA, A. A. KHRUSTALEV, I. I. STOIKOV, I. S. ANTIPIN, H. C. BUDNIKOV and A. I. KONOVALOV
Kazan State University, 420008, Kazan, Russian Federation

(Received: 31 May 2000; in final form: 26 September 2000)

Key words: calix[4]arene, cholinesterase, inhibition, ester binding

Abstract

The inhibitory effect of 1,3-substituted *p-tert*-butylcalix[4]arenes on butyrylcholinesterase from horse serum has been discovered and kinetically investigated with photometric microassay techniques. The interaction of calix[4]arene with the enzyme is described in accordance with the formal kinetics of competitive reversible inhibition. The inhibition constants calculated depend on the substituent in the lower rim of the calix[4]arene and vary in the range of (5–110) $\times$ 10^{-6} M. The proposed mechanism of inhibition involves the cooperative interaction of indophenyl acetate used as a substrate, calix[4]arene and the enzyme without any covalent or electrostatic binding of the functional groups in the active site of cholinesterase. This results in the coordination of the calixarene on the enzyme surface in the proximity of the enzyme active site. Such interaction prevents the substrate from entering the enzyme active site.

Introduction

The synthesis of selective molecular receptors, which realise the principles of host guest chemistry, i.e., calixarenes, cyclodextrins and crown ethers, is one of the promising ways in the further progress of organic and bioorganic chemistry. Calixarene derivatives and their analogs have many advantages in the construction of molecular recognition systems, i.e., the variety of steric structure, the diversity of functioning and relative availability. In addition, the calixarene conformation is rigid enough for fixing the desirable coordination of functional groups at the lower and/or upper rims and providing for the multi-point binding of the guest molecules.

Calixarene based complexing agents have been described for the selective binding of metal ions [1–7] and organic compounds [8–14]. They find application in the optical and electrochemical methods of analytical determination of appropriate guest species [4, 15, 16] and their extraction from water media in organic solvents [17, 18]. The complexes of macrocyclic compounds are also used as catalysts in stereoselective organic synthesis [19].

The further extension of the spectrum of guests as well as the improvement of the selectivity of host-guest interaction can be achieved due to the implementation of novel binding sites and optimisation of their complexing features.

Previously, the conditions of regioselective alkylation of the hydroxyl groups of calix[4]arenes have been established [20–22]. On this basis, a series of alkyl derivatives of *tert*-butylcalix[4]arenes were synthesized and examined for the complexation of α-amino and α-hydroxy acids. The solutions of *tert*-butylcalixarenes synthesized were introduced into the liquid lipophilic membranes and a selective transfer of glycolic, d,l-tartaric and amygdalic acids through these membranes has been kinetically investigated [23, 24]. In this work, the specific interaction of dialkyl derivatives of *tert*-butylcalix[4]arenes with butyrylcholinesterase was explored and their inhibiting effect quantified.

* Author for correspondence: E-mail: Gennady.Evtugyn@ksu.ru

Experimental

Preparation of substituted calix[4]arenes

Substituted calix[4]arenes (**1–7**, **9**, Figure 1) were obtained from *p-tert*-butylcalix[4]arene and alkyl halides by selective 1,3-alkylation in the presence of potassium carbonate in acetonitrile. The yields obtained were from 60 to 80%. Compound (**8**) was obtained by the nitration of (**5**) with nitric acid in the presence of acetic acid/methylene chloride at low temperature [24]. Compound (**10**) was obtained and characterised in accordance with [25]. Calixarenes (**11** and **12**) were obtained from compounds (**9**) and (**10**), respectively, as described below. The structures of (**1–12**) were proved by ^{1}H-NMR spectroscopy, mass-spectrometry and elemental analysis. Two doublet ^{1}H-NMR signals of bridging methylene protons in a macrocyle confirm the cone conformation of the calix[4]arenes investigated.

The nonsubstituted 5,11,17,23-tetra-*p-tert*-butyl-25,26,27,28-tetrahydroxycalix[4]arene was synthesized in accordance with [24], melting point 342–344 °C.

Synthesis of 26,28-disubstituted derivatives of 5,11,17,23-tetra-*p-tert*-butyl-25,26,27,28-tetrahydroxycalix[4]arene (1–7, 9). A mixture of 1.54 mmol of *p-tert*-

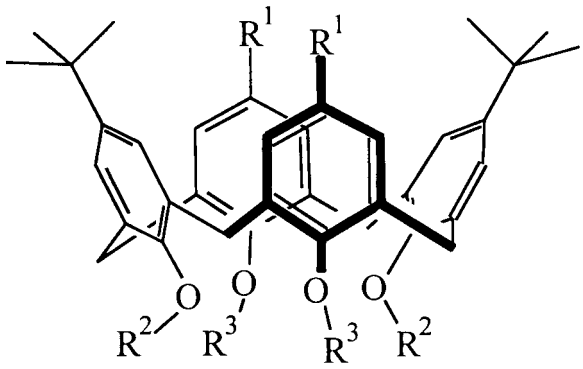


Figure 1. The stucture of the 1,3-substituted *p-tert*-butylcalix[4]arenes investigated.

butylcalix[4]arene, 3.24 mmol of the appropriate alkyl bromide and 1.7 g (0.01391 mol) of anhydrous potassium carbonate in 30 mL of acetonitrile was boiled for 12 hours. Then the solvent was evaporated, the residue was treated with 10 mL of 5 M HCl and extracted with 50 mL of $CHCl_3$. The chloroform fraction was dried with 4 Å molecular sieves. After the evaporation of chloroform the residue was recrystallized from a chloroform/methanol mixture.

Calix[4]arene (1). Yield 0.9 g (60%), m.p. 210211 °C. ^{1}H NMR ($CDCl_3$) δ, ppm: 0.96 [18H, s, $C(CH_3)_3$], 1.26 [18H, s, $C(CH_3)_3$], 3.17 [4H, d, $^2J_{HH}$ = 13.20 Hz, Ar*CH*$_{2eq}$Ar], 4.24 [4H, d, $^2J_{HH}$ = 13.20 Hz, Ar*CH*$_{2ax}$Ar], 5.97 [4H, s, O-*CH*$_2$], 6.81 [4H, s, ArH], 6.85 [2H, s, -OH] 7.00 [4H, s, ArH], 7.12–7.18 [2H, m, FlnH], 7.40–7.46 [4H, m, FlnH], 7.68–7.70 [2H, m, FlnH]. MS (CI): 1072,73 (M^+).

Calaix[4]arene (2). Yield 1.09 g (78%), m.p. 280 °. ^{1}H NMR ($CDCl_3$) δ, 0.96 [18H, s, $C(CH_3)_3$], 1.30 [18H, s, $C(CH_3)_3$], 3.35 [4H, d, $^2J_{HH}$ = 13.15 Hz, Ar*CH*$_{2eq}$Ar], 4.24 [4H, d, $^2J_{HH}$ = 13.15 Hz, Ar*CH*$_{2ax}$Ar], 5.17 [4H, s, O-*CH*$_2$Ar], 7.83 [4H, s, ArH], 7.08 [4H, s, ArH], 7.15 [2H, s, –OH], 7.93 [4H, d, $^3J_{HH}$ = 8.37 Hz, BzH-ortho], 8.17 [4H, d, $^3J_{HH}$ = 8.37 Hz, BzH-meta]. MS (CI): 918,48 (M^+).

Calix[4]arene (3). Yield 0.92 g (68%), m.p. 246–247 °C. ^{1}H NMR ($CDCl_3$) δ, ppm: 0.95 [18H, s, $C(CH_3)_3$], 1.30 [18H, s, $C(CH_3)_3$], 3.31 [4H, d, $^2J_{HH}$ = 13.15 Hz, Ar*CH*$_{2eq}$Ar], 4.21 [4H, d, $^2J_{HH}$ = 13.15 Hz, Ar*CH*$_{2ax}$Ar], 5.12 [4H, s, O-*CH*$_2$Ar], 6.80 [4H, s, ArH], 7.02 [2H, s, –OH] 7.07 [4H, s, ArH], 7.67 [4H, d, $^3J_{HH}$ = 8.62 Hz, BzH-ortho], 7.82 [4H, d, $^3J_{HH}$ = 8.62 Hz, BzH-meta]. MS (CI): 878.50 (M^+).

Calix[4]arene (4). Yield 0.76 g (56%), m.p. 190–191 °C. ^{1}H NMR ($CDCl_3$) δ, ppm: 0.94 [18H, s, $C(CH_3)_3$], 1.30 [18H, s, $C(CH_3)_3$], 3.32 [4H, d, $^2J_{HH}$ = 13.16 Hz, Ar*CH*$_{2eq}$Ar], 4.37 [4H, d, $^2J_{HH}$ = 13.16 Hz, Ar*CH*$_{2ax}$Ar], 4.72 [4H, d, $^3J_{HH}$ = 6.14 Hz, O-*CH*$_2$], 6.57 [2H, d.t, $^3J_{HH}$ = 6.14 Hz, $^3J_{HH\,trans}$ = 15.91 Hz, CH_2*CH*=CH–C_6H_5], 6.77 [4H, s, ArH], 6.84 [2H, d, $^3J_{HH\,trans}$ = 15.91 Hz, CH_2CH=*CH*-C_6H_5], 7.06 [4H, s, ArH], 7.20 [2H, s, -OH], 7.24–7.30 [6H, m, ArH], 7.38–7.43 [4H, m, ArH]. MS (CI): 880.54 (M^+).

Calix[4]arene (5). Yield 1.0 g (79%), m.p. 202–203 °C. ^{1}H NMR ($CDCl_3$) δ, ppm: 0.96 [18H, s, $C(CH_3)_3$], 1.30 [18H, s, $C(CH_3)_3$], 3.28 [4H, d, $^2J_{HH}$ = 13.35 Hz, Ar*CH*$_{2eq}$Ar], 4.29 [4H, d, $^2J_{HH}$ = 13.35 Hz, Ar*CH*$_{2ax}$Ar], 5.11 [4H, s, O-*CH*$_2$], 6.81 [4H, s, ArH], 6.96 [4H, s, ArH], 7.34 [2H, s, -OH], 7.37–7.39 [6H, m, ArH], 7.65–7.69 [4H, m, ArH]. MS (CI): 828.51 (M^+).

Calix[4]arene (6). Yield 0.88 g (59%), m.p. 110 °C. ^{1}H NMR ($CDCl_3$) δ, ppm: 0.93 [18H, s, $C(CH_3)_3$], 1.26 [18H, s, $C(CH_3)_3$], 1.36 [6H, t, $^3J_{HH}$ = 7.17, O-CH_2-*CH*$_3$], 3.28 [4H, d, $^2J_{HH}$ = 13.22 Hz, Ar*CH*$_{2eq}$Ar], 4.25 [4H, d, $^2J_{HH}$ = 13.22 Hz, Ar*CH*$_{2ax}$Ar] 4.37 [4H, q, $^3J_{HH}$ = 7.17, O-*CH*$_2$-CH_3], 5.12 [4H, s, O-*CH*$_2$Ar], 6.78 [4H, s, ArH], 7.04 [4H, s, ArH], 7.26 [2H, s, -OH], 7.76 [4H, d, $^3J_{HH}$ = 8.39 Hz, BzH-ortho], 8.02 [4H, d, $^3J_{HH}$ = 8.39 Hz, BzH-meta]. MS (CI): 972,55 (M^+).

Calix[4]arene (7). Yield 0.85 g (59%), m.p. 206–207 °C. ^{1}H NMR ($CDCl_3$) δ, ppm: 0.97 [18H, s, $C(CH_3)_3$], 1.29 [18H, s, $C(CH_3)_3$], 3.29 [4H, d, $^2J_{HH}$ = 13.35 Hz, Ar*CH*$_{2eq}$Ar], 4.36 [4H, d, $^2J_{HH}$ = 13.35 Hz, Ar*CH*$_{2ax}$Ar], 5.21 [4H, s, O-*CH*$_2$], 6.84 [4H, s, ArH], 7.05 [4H, s, ArH], 7.30–7.35, 7.42–7.48, 7.63–7.83 [14H, m, ArH], 8.4 [2H, s, -OH]. MS (CI): 928.54 (M^+).

Calix[4]arene (9). Yield 0.88 g (60%), m.p. 110 °C. ^{1}H NMR ($CDCl_3$) δ, ppm: 0.99 [18H, s, $C(CH_3)_3$], 1.27 [18H, s, $C(CH_3)_3$], 1.34 [6H, t, $^3J_{HH}$ = 7.14, C(O)-CH_2-*CH*$_3$], 3.33 [4H, d, $^2J_{HH}$ = 13.10 Hz, Ar*CH*$_{2eq}$Ar], 4.31 [4H, q, $^3H_{HH}$ = 7.14, C(O)-*CH*$_2$-CH_3], 4.47 [4H, d, $^2J_{HH}$ = 13.10 Hz, Ar*CH*$_{2ax}$Ar], 4.73 [4H, s, O-*CH*$_2$Ar], 6.82 [4H, s, ArH], 7.02 [4H, s, ArH], 7.06 [2H, s, -OH]. MS (CI): 822.51 (M^+).

5,17,dinitro-11,23-di-*tert*-butyl-25,27-dihydroxy-26, 28-bis(benzyloxy)calix[4]arene (8). 0.5 mmol of calix[4]-arene (**1**) in 50 mL of dichloromethane was mixed with 2.9 mL of glacial acetic acid and 5.6 mL (80 mmol) of 65% HNO_3. The solution was stirred for 30 min at room temperature and then 50 mL of water were added. The organic layer was separated, washed twice with water and dried with 5 Å molecular sieves. The solvent was evaporated and the residue was recrystallized from ethanol/dichloromethane. Yield 0.25 g (62.5%), m.p. 294 °C. ^{1}H NMR ($CDCl_3$) δ, ppm: 1.03 [18H, s, $C(CH_3)_3$], 3.45 [4H, d, $^2J_{HH}$ = 13.18 Hz, Ar*CH*$_{2eq}$Ar], 4.27 [4H, d, $^2J_{HH}$ = 13.38 Hz, Ar*CH*$_{2ax}$Ar], 5.08 [4H, s, O-*CH*$_2$Ar], 6.91 [4H, s, ArH], 7.42–7.44 [6H, m, ArH], 7.58–7.62 [4H, m, ArH], 8.05 [4H, s, ArH], 8.90 [2H, s, -OH]. MS (CI): 972.552 (M^+).

5,11,17,23-*tert*-butyl-25,27-bis(N-benzyl-2-carbamoyl-methoxy)-26,28-didroxy calix[4]arene (11). A mixture of 0.68 mmol of calix[4]arene (**9**) and 0.93 mmol of ammonium chloride in 3 mL of benzyl amine was boiled for 1 hour. The reaction mixture was washed once with water and then with several portions of dilute HCl. The white precipitate was recrystallized from the dichloromethane/hexane mixture. Yield 0.2 g (38%), m.p. 266 °. ^{1}H NMR ($CDCl_3$) δ, ppm: 1.23 [18H, s, $C(CH_3)_3$], 1.53 [18H, s, $C(CH_3)_3$], 3.54 [4H, d, $^2J_{HH}$ = 11.78 Hz, Ar*CH*$_{2eq}$Ar], 4.06 [4H, d, $^2J_{HH}$ = 11.78 Hz, Ar*CH*$_{2ax}$Ar], 4.61 (4H, s, O-CH_2), 4.78 94H, d, $^3J_{HH}$ = 4.4 Hz, N-CH_2), 7.06 [4H, s, ArH], 7.52, [4H, s, ArH], 7.43–7.55 (10H, m, ArH), 9.17 [2H, t, $^3J_{HH}$ = 4.4 Hz, N-H]. MS (CI): 942.55 (M^+).

5,11,17,23-*tert*-butyl-25,27-dihydroxy-26,28-bis-(N-benzoyl-2-amidoethoxy calix[4]arene (12). A mixture of 4

g (5.78 mmol) calix[4]arene (**10**) and 2.61 g (11,56 mmol) of benzoic acid anhydride in 30 mL of benzene was boiled for 2 hours. Then the solvent was evaporated, the precipitate was treated with a saturated solution of sodium carbonate, washed with water, dried and washed with hexane. The light-yellow crystals of (**12**) were finally dried in vacuum. Yield 2.7 g (59%), m.p. 135–6 °. ^{1}H NMR ($CDCl_3$) δ, ppm: 1.12 [18H, s, $C(CH_3)_3$], 1.23 [18H, s, $C(CH_3)_3$], 3.39 [4H, d, $^2J_{HH}$ = 14.11 Hz, Ar*CH*$_{2eq}$Ar], 3.62 (4H, dd, $^3J_{HH}$ = 4.53 Hz, $^3J_{HH}$ = 4.94 Hz, CH_2-N), 4.03 (4H, t, $^3J_{HH}$ = 4.53 Hz, O–CH_2), 4.18 [4H, d, $^2J_{HH}$ = 14.11 Hz, Ar*CH*$_{2ax}$Ar], 7.02 [4H, s, ArH], 7.07 [4H, s, ArH], 7.31 [4H, dt, $^3J_{HH}$ = 7.40 Hz, $^3J_{HH}$ = 7.11 Hz, ArH], 7.43 [2H, tt, $^3J_{HH}$ = 7.40 Hz, $^4J_{HH}$ = 1.28 Hz, ArH], 7.95 [4H, dd, $^3J_{HH}$ = 7.11 Hz, $^4J_{HH}$ = 1.28 Hz, ArH], 8.21 [2H, t, $^3J_{HH}$ = 4.94 Hz, N-H], 8.39 [2H, s, -OH]. MS (CI): 957.57 (M^+).

IR and ^{1}H NMR spectra of the calix[4]arenes and of their complexes with benzoic acid were measured at 25 ° with a Specord M-80 spectrometer and a Varian XL-300 spectrometer (300 MHz), respectively. ^{1}H NMR spectra were recorded for 8×10^{-2} M solutions of calix[4]arenes, benzoic acid and tetrabutylammonium benzoate in CCl_4 containing 5% of deuteroacetone $(CD_3)_2CO$. For IR spectroscopic investigation, solid complexes of calix[4]arene and benzoic acid were isolated from 50% ethanol. IR spectra of solid complexes and appropriate initial compounds were recorded in vaseline oil in the range of 3600–600 cm^{-1}.

Measurements of enzyme activity

Butyrylcholinesterase from horse serum (ChE, EC 3.1.1.8)), specific activity 500 U mg^{-1} of peptide, indophenyl acetate and butyrylcholine iodide were purchased from Sigma Chemical Company (St. Louis, USA). All the other reagents used were of analytical grade (Reakhim, Russia, and Fluka, Neu-Ulm, Switzerland).

Calix[4]arenes were first diluted in acetone and then mixed 1 : 4 (v/v) with 0.04% *N*-phthalylchitozan solution used as a ChE stabilizer [26]. As was previously established, the final concentration of organic solvents as well as the stabilizer do not affect the ChE activity. The maximum concentration of calix[4]arenes used for measurements was limited by their solubility in the organic-water mixture. Before contact with the ChE, calix[4]arene solutions were equilibrated for at least 24 hours to avoid their heterogeneity after dilution. The solutions obtained did not change their inhibitory effect for at least four days if stored at room temperature. No opalescence or sediment formation were observed for the working solutions for the whole period of their use in kinetic measurements.

For the determination of the enzyme activity and the quantitative estimation of the inhibitory effect, the modified photometric techniques described in [26] were used. For this purpose, 40 μL of ChE solution in 0.002 M phosphate or *Tris* (tris-hydroxymethylaminomethane) buffer solution was placed in the cell of a standard plate for an immunochemical assay. Equal volumes of the calix[4]arene solution in aqueous ethanol or acetone and of 1.7×10^{-4} M indophenyl acetate or 2.3×10^{-5} M butyrylcholine iodide were then injected into the same cell and the initial rate of the enzymatic reaction was monitored with a AKI-C-01 miniphotometer (JSC "Biomashpribor", Russia) at 530 nm (indophenyl acetate) or 495 nm (butyrylcholine iodide in the presence of Bromothymol Blue as a pH indicator). The slope of a linear portion of the kinetic curve (see Figure 2) was calculated as a measure of the initial rate of reaction.

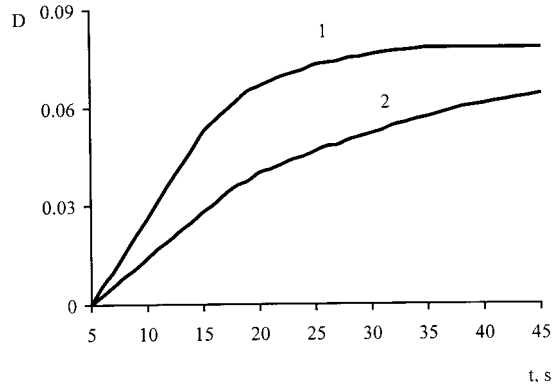
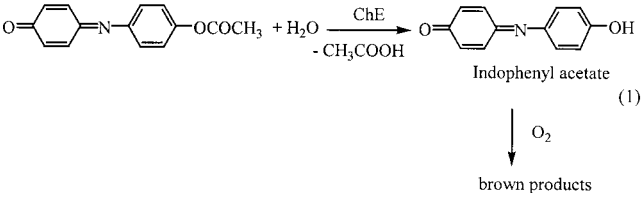


Figure 2. Kinetic curves of the ChE hydrolysis of indophenyl acetate prior to (1) and after (2) contact of the enzyme with calix[4]arene. D – optical density of the solution measured at λ 530 nm.



The kinetic parameters of inhibition were determined from the dependence of the relative shift of the ChE activity on the concentration of the substrate and calix[4]arene. The dependence of the initial rate of an enzymatic reaction on calix[4]arene concentration is linear in the plots of (v_o/v_i) vs. C_I for the concurrent addition of the substrate and inhibitor (reversible inhibition measurement) and in the plots of $\ln(v_o/v_i)$ vs. C_I if the enzyme was preincubated with calix[4]arene solution for 10 min before the addition of a substrate. vo and vi are the initial rates of enzymatic reactions before and after the contact of an enzyme with an inhibitor, respectively, and C_I is the calix[4]arene concentration [27, 28].

In some experiments, the ChE was immobilised on tracing paper by a 3 min treatment with 3% glutaraldehyde as described in [29]. The resulting specific activity of the immobilised enzyme was found to be 0.01 E cm^{-2}. The activity of the immobilised enzyme and the influence of calix[4]arenes on the ChE immobilised on paper were determined by microtitration of butyric acid formed in the ChE hydrolysis of butyrylcholine with 0.1% NaOH.

Results

The inhibitory effect of calix[4]arenes

Tert-butylcalix[4]arenes (**1–10**) with aromatic substitutents in the lower rim as well as the ester derivative **9** showed

Table 1. Inhibitory effect of 1,3-substituted calix[4]arenes toward ChE (v_o and v_i are initial reaction rates prior to and after contact of the enzyme with the inhibitor, respectively). For compound **10**, the parameters obtained with butyrylcholine as a substrate are presented in brackets. Reversible inhibition, $v_o/v_i = a + b \times (C_I, \mu M)$, for 6 repeated measurements

No.	*a*	*b*	*r*	Concentration range, C_I, μM
1	0.90 ± 0.09	0.0160 ± 0.0013	0.9820	12–100
2	1.06 ± 0.14	0.0093 ± 0.0008	0.9846	20–320
3	1.05 ± 0.34	0.0083 ± 0.0019	0.9310	80–180
4	0.93 ± 0.03	0.0084 ± 0.0002	0.9990	60–280
5	1.02 ± 0.04	0.014 ± 0.003	0.9809	13–120
6	0.82 ± 0.10	0.021 ± 0.002	0.9656	10–46
7	1.07 ± 0.02	0.0148 ± 0.0007	0.9922	10–55
8	0.79 ± 0.03	0.0193 ± 0.0067	0.9207	15–65
9	1.04 ± 0.06	0.022 ± 0.002	0.9832	15–50
10	0.96 ± 0.02	0.0029 ± 0.0002	0.9950	14–100
	(1.11 ± 0.03)	(0.0056 ± 0.0004)	(0.9946)	(10–70)

a reversible inhibiting effect on ChE after 10 min incubation both in *Tris*- and phosphate buffer solutions in the presence of indophenyl acetate used as a substrate. Amino derivative **10** affects the enzyme activity irrespective of the substrate used, i.e., both for indophenyl acetate and butyrylcholine used as ChE substrate. Calix[4]arenes **11** and **12** exert neither a reversible nor an irreversible inhibiting effect on free and immobilised ChE. The results of inhibition measurements are presented in Table 1. The calix[4]arene solutions show a highly reproducible inhibiting effect during the whole period of their storage, i.e., during at least four days after preparation.

In the case of the pre-incubation of ChE in a calix[4]arene solution with no substrate (incubation stage), the variation in the incubation time does not lead to the corresponding decay of enzyme activity as predicted in accordance with the Aldridge Equation (2) [27].

$$\ln[v_o/v_i] = k_{II}\tau C_I, \qquad (2)$$

where k_{II} is the bimolecular inhibition constant, M^{-1} min^{-1}, τ is the incubation time, min, and C_I is the concentration of irreversible inhibitor, M. When the incubation time exceeds 15 min, the shift of enzyme activity is irregular, and a small irreproducible activation ($v_o/v_i \approx 0.8$–0.9) is observed for $\tau > 30$ min.

In experiments with butyrylcholine as the ChE substrate the inhibitory effect was found to be 10–100 times lower than that observed with indophenyl acetate under the same measurement conditions. The only exception is amino derivative **10**. The enzymatic hydrolysis of butyrylcholine was found to be twice as sensitive to this inhibitor than that of indophenyl acetate (see Table I).

The addition of other compounds which can form complexes with calix[4]arenes results in a decrease of the inhibitory effect observed for calix[4]arenes alone. Thus, the addition of sodium benzoate in the concentration range of

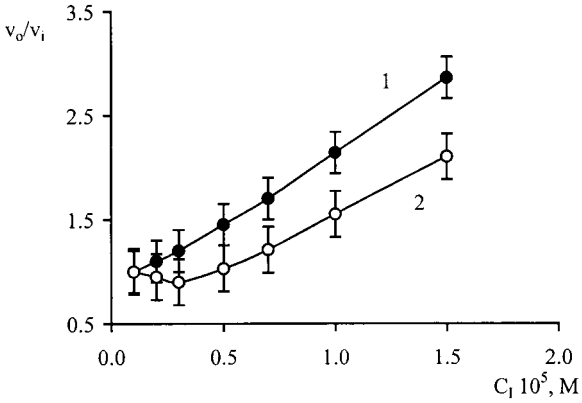


Figure 3. The influence of benzoic acid on the inhibitory effect of calix[4]arene **2**. The inhibition curves obtained prior to (1) and after addition of 5.5×10^{-4} M benzoic acid (2).

(2–6) $\times$ 10^{-5} M reduces the inhibitory effect by 40–50% for different calix[4]arenes (see Figure 3). Meanwhile, the presence of up to 5×10^{-4} M of benzoate salt alone did not alter ChE activity. In these experiments, sodium benzoate was first added to the buffered calix[4]arene solution, pH 7.5. Then after 1 hour incubation the mixture was injected into the enzyme solution and the rate of indophenyl acetate hydrolysis was measured as described earlier. The effect of benzoate anion, which is well pronounced in weakly basic media, becomes lower and less reproducible with the decrease of pH value, i.e., in neutral and weakly acidic media. Unfortunately, the narrow range of the v_o/v_i variation did not contribute to discussing the influence of calix[4]arene structure on the effect of benzoate additives.

ChE immobilised on paper by cross-linking with glutaraldehyde is insensitive towards the calix[4]arenes until their saturated concentration in a 30% aqueous ethanol is reached irrespective of the nature of the substrate used.

Determination of inhibition kinetics

The mechanism of inhibition was established on the basis of the kinetic analysis of the dependence of the relative decay of the rate of enzymatic reaction on the concentrations of a substrate and calix[4]arene. The measurement data obtained for the same substrate and different calix[4]arene concentrations are linear in the plots of v_o/v_i vs. C_I (Figure 4 and Table 1). All of these curves obtained with different substrate concentrations intersect at one point. This refers to the case of competitive inhibition [28] and the x coordinate of intersection equals the opposite value of the inhibition constant K_I^{-1}. The average values of the inhibition constants calculated from the curves obtained for six concentrations of indophenyl acetate and for five concentrations of calix[4]arene are summarised in Table 2. The experimental values of the bimolecular inhibition constant k_{II} calculated for a 10 min incubation from the Aldridge equation (2) are also presented for comparison.

Complexation investigation

Sodium benzoate was chosen for the investigation of the mechanism of calix[4]arene inhibition because its compl-

Table 2. Inhibition constants of the 1,3-substituted calix[4]arenes

No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Ki, μM	5	55	83	110	2.5	26	22	6	12	2
K_{II}, μM^{-1} min^{-1}	–	0.06	0.04	0.03	0.5	0.07	0.07	0.05	0.08	0.02

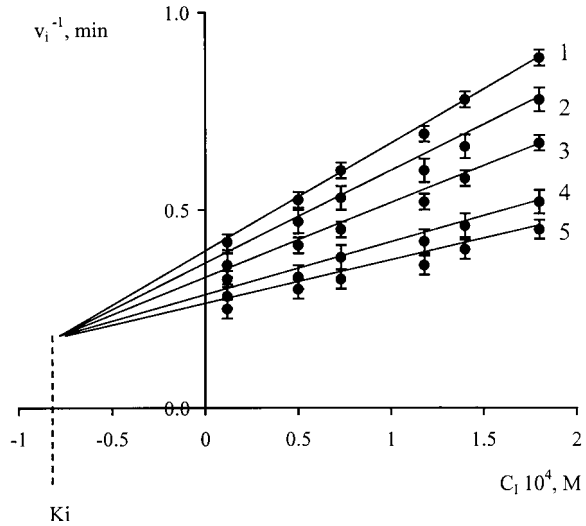


Figure 4. The determination of the inhibition constants K_i from the dependence of the rate of enzymatic reaction v_i on the inhibitor concentration C_I (calix[4]arene **5**). The concentration of indophenyl acetate is: 0.41 (1), 0.52 (2), 0.62 (3), 0.73 (4) and 0.83 (5) mM.

exation with the diaryl substituted p-tert-butylcalix[4]arenes has been established previously [23]. The complexes of benzoic acid and calix[4]arenes **1**, **5** and **7** were investigated with ^{1}H NMR and IR spectroscopy.

The addition of benzoic acid to the calix[4]arene solution does not change the calixarene spectrum. Under these conditions, benzoic acid forms a stable and inert dimer. In contrast, tetrabutylammonium benzoate causes changes of appropriate signals. Thus, for calix[4]arene **5** the signals of aromatic protons are shifted to lower field by 0.1 ppm and the signals of methyl and methylene protons of tetrabutylammonium cation by 0.05–0.1 ppm. The incorporation of benzoate into the molecular cleft formed by two aryl substituents of calix[4]arene probably leads to the screening of the aromatic ring in the benzoate anion by benzyl substituents due to π-staking interactions. This process is followed by the transformation of a close tetrabutylammonium – benzoate ion pair into one separated by solvent.

On the other hand, a higher field shift was observed for OH-protons of calix[4]arene **5** and **7** due to their hydrogen bonding with the carboxylate moiety. For the fluorenyl derivative **1** no shift of OH protons signals was observed.

The interaction of benzoic acid with aryl substituents of calix[4]arene was also confirmed with IR spectrometry. The complexation results in significant changes of the absorbance characteristics of the initial spectra. Thus, the 3000–2500 cm^{-1} absorbance band corresponding to the valency vibrations of hydroxyl groups in a dimer as well as the absorption bands of a six-membered ring at 1420 and 1300 cm^{-1} related to the dimer of benzoic acid, disappeared completely. These changes as well as the shift of the carboxylate group from 1690 cm^{-1} in the dimer to 1700 cm^{-1} in the complex indicate the destruction of the initial dimer structure of benzoic acid compensated by the strong interactions in the complex with calix[4]arene. The strong complexation of benzoic acid can be related to the well known macrocyclic effect (i.e., entropy factor). The diaryl substituted calix[4]arene is a rigid pre-organized system with two hydroxyl groups well arranged for hydrogen bonding with the carboxyl group of a guest molecule.

The existence of π-stacking interactions of aromatic rings of the host and guest are also confirmed by the sharp decreae of absorbance intensity at 940 and 710 cm^{-1} corresponding to non-planar deformation oscillations of the carboxyl OH-group and C–H bonds of the aromatic ring of benzoic acid in a complex with calixarene **5**.

The hindrance for non-planar deformation oscillations is probably associated with the incorporation of benzoic acid into the molecular cleft formed by two benzyl substituents of calix[4]arene. Thus, the absorbance intensities corresponding to asymmetrical and symmetrical stretching vibrations of carboxylate anion (1600 and 1400 cm^{-1}) essentially decreased after the complex formation and the band at 700 cm^{-1} corresponding to the scissor oscillations of carboxylate anion disappeared. This agrees with the proposed interaction of carboxylate with two free OH groups of the macrocycle **5**.

Similar shifts of ^{1}H NMR spectra were observed for indophenyl acetate – calix[4]arene mixtures under the same measurement conditions (Figure 5). Thus, the proton signals of indophenyl acetate were shifted to lower field by 0.08–0.1 ppm in the presence of **5**. This corresponds to the complex with the guest molecule oriented outside the calix[4]arene cavity. Otherwise, i.e., for indophenyl acetate included into the calixarene cone, the proton signals would shift to higher field due to the screening effect [30]. The signals of benzyl protons of calix[4]arene susbtituents are also shifted to lower field by about 0.05 ppm and the signal of OH protons to higher field by 0.13 ppm. Unfortunately, the isolation of appropriate complexes from aqueous ethanol solution was followed by partial hydrolysis of indpophenyl acetate. This complicates the interpretation of the IR spectrum because of the intensive absorbance of the products formed in accordance with (1).

Discussion

Various cholinesterases offer a remarkably wide list of chemicals affecting their activity [31–33]. It includes or-

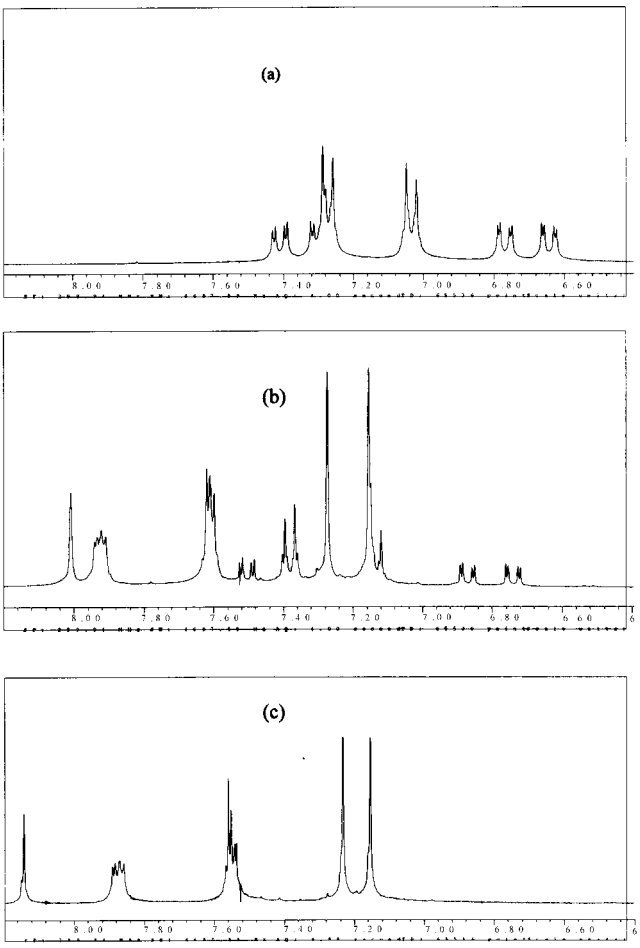


Figure 5. ^{1}H NMR spectra of indophenyl acetate (a), the complex of calix[4]arene **5** with indophenyl acetate (b) and calix[4]arene **5** (c). Concentration 8×10^{-2} M, solvent 5% of deuteroacetone in CCl_4.

ganic esters of organophosphorus, carbamic and sulphonic acids, most metal cations and organic compounds with quaternary nitrogen atoms or amino groups protonated in aqueous solution, including hydrazones. Their influence on the interaction of the substrate with the enzyme active site is usually considered in the context of two different mechanisms. The first accounts for the effect of organophosphorus, carbamate pesticides and sulphonic acid ethers. It involves the formation of a covalent bond between an inhibitor and the serine residue in the active site of cholinesterase [33]. This reaction results in the formation of a stable product, i.e., phosphorylated, sulphonylated or carbamylated cholinesterase, which cannot convert the substrate. The second mechanism common for cationic species is based on the electrostatic interaction of an inhibitor with the enzyme active site followed by a full or partial restriction of substrate access [32]. In a sense, cationic species reveal analogs of the natural substrate acetylcholine. In accordance with the three-dimensional structure of cholinesterase established in 1991, the active site is placed on the bottom of a narrow gorge lined with 14 aromatic residues [34]. The positively charged species are pushed into the active site due to a large dipole moment oriented parallel to the gorge axis and a constantly increasing negative potential inside the gorge itself. Although the first results of structure interpretation were obtained for acetylcholinesterase from *Torpedo californica*, the mechanism of "Nature's vacuum cleaner" accounting for the high substrate specificity of cholinesterase was later confirmed for human and some mutant cholinesterases and is accepted for other cholinesterases [35].

The 1,3-substituted calix[4]arenes investigated do not belong to any of the types of inhibitors described, therefore their inhibitory effect was rather surprising and has never been observed previously. This cannot be related to the probable heterogeneity of the solutions used for kinetic measurements. This is confirmed by the reproducibility of the changes in the ChE activity observed for various calix[4]arene concentrations several days after their preparation as well as by the lack of any changes in the ChE activity in the presence of **11** and **12**. Even assuming the formation of a colloidal system in the dilution of the initial organic solution of calix[4]arene (see Experimental), the rate of reagent transfer is much higher than that of enzymatic reaction and does not cause any changes in the experimental kinetic parameters. The feasibility of applying homogeneous enzymatic kinetics to heterogeneous systems has long been known. Thus, the maximum of the inhibitory effect of beryllium on alkaline phosphatase was observed at pH 9.8, i.e., in the conditions of full hydrolysis of beryllium salt [36]. Moreover, the kinetic control of enzyme inhibition was confirmed even for the enzymes immobilized in thin layers on solid supports [37,38].

The competitive mechanism of inhibition established from kinetic data suggests that an inhibitor and a substrate interact with the same site of an enzyme globule. The inhibition constants consecutively decrease with the increase of the substituent chain or its branches (see Table 2). This allows us to propose another mechanism of interaction which involves the multi-point binding of calix[4]arene with the aromatic residues in the area near the rim of the gorge.

As shown by IR and ^{1}H-NMR spectroscopy, the calix[4]arenes **1**, **5** and **7** form stable host-guest complexes with benzoate anion where carboxylate is coordinated on the lower rim of the calixarene cone (Figure 6). A similar complex structure was proposed for calix[4]arene **5** with indophenyl acetate. The complex structure is additionally fixed by a π-stacking interaction of appropriate aryl substituents of host and guest. Similar complexes can be proposed for other host molecules investigated.

While this host-guest complex draws together the active site of ChE, the *tert*-butylcalix[4]arene will close the jaws of a gorge like a stopper so that the molecule of a substrate is not allowed to be inserted into the gorge of the active site. Indophenyl acetate binding is confirmed by the influence of sodium benzoate on the inhibitory effect of calix[4]arenes. Benzoic acid competes with indophenyl acetate for the same binding site of calixarene, i.e., for the molecular cleft between aryl substituents at the lower rim of the calixarene. As a result, the concentration of the binary complex calix[4]arene – indophenyl acetate becomes lower and hence its influence on the ChE activity diminishes. Butyrylcholine cannot form similar complexes because it does

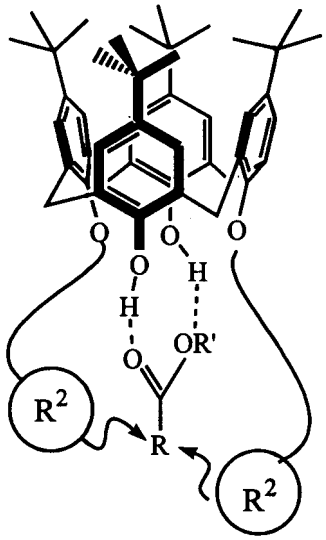


Figure 6. The possible structure of the complex formed in the solution of 1,3-substituted *tert*-butylcalix[4]arene and indophenylacetate (RCOOR′). For R^2 decriptions see Figure 1.

not contain any aromatic rings and is positively charged. Thus, the inhibitory effect of calixarene in the presence of this ChE substrate is not observed.

The realisation of the above mechanism of ChE inhibition is related to the following special features of the calixarene structure:

- the size of the *p-tert*-butylcalix[4]arene platform is close to that of the ChE gorge (about 20 Å);
- the distance between the guest molecule (indophenyl acetate) and calix[4]arene in the complex formed is small enough to establish the close contact of the phenyl rings of the calix[4]arene cone and the aromatic residues of the amino acids lining the gorge while indophenyl acetate is drawn to the active site;
- the calix[4]arene does not form stable complexes with cations introduced into the cavity therefore the electrostatic forces do not disturb the necessary orientation of the calix[4]arene and the gorge of the active site.

From this point of view, both the substrate and the *tert*-butylcalix[4]arene are necessary for the ChE inhibition. The indophenyl acetate provides for the implementation of the complex into the gorge. Due to the hydrophobic interactions and large-sized *tert*-butyl substituents on the upper rim, the calixarene plugs the active site like a cork in a bottle. A similar mechanism of the ChE inhibition was previously proposed for the snake toxin fasciculin [39]. Computer modelling and ChE mutation studies have localised the area of interaction of fasciculin and acetylcholinesterase to the aromatic residues near the rim of the gorge, but not to the specific active site as such.

The established relationships of the kinetic inhibition constants and substituent structures confirm the above mechanism. Thus, the lowest inhibition constants K_i were obtained for *tert*-butylcalix[4]arenes with the planar substituents **1**, **5** and **8**. The replacement of t-butyl radical by the NO_2 group at the upper rim (R^1 substituent, compounds **5** and **8**, Figure 1) does not significantly change the inhibitory effect because it does not alter the π-interactions of aromatic rings. In contrast, the presence of polar groups (NO_2, **2**, CN, **3**, $COOC_2H_5$, **6**) in the R^2 substituents diminishes the inhibitory effect due to the weakening of the indophenyl acetate binding. In a similar way, the separation of an aromatic ring from the calix[4]arene platform by the aliphatic chain in **4** increases the K_I value and decreases the enzyme sensitivity toward the inhibitor as compared with the benzyl derivative **5**. Increase of the distance by additional methylene groups (see **11** and **12** in comparison with **3** and **5**) makes inhibition impossible. The inclusion of indophenyl acetate into the gorge is not followed by the necessary close contact of aromatic rings in the gorge.

The multi-point interaction of the host-guest complex and the ChE active site is kinetically hindered. Similar weak adducts can be formed with other functional groups far away from the location of the active site. Such reversible non-specific interaction can be important at the preliminary stage of drawing together the complex partners. The same interactions complicate their kinetic description. In comparison with the investigations of the traditional ChE inhibitors the plots of v_o/v_i vs. C_I are linear in a narrow concentration range. The minimal concentrations of calix[4]arene inhibiting the ChE differ much less than the calculated K_i values.

The pre-incubation of ChE with *tert*-butylcalix[4]arene does not provide for the effective binding of the reaction partners. This is probably due to the elimination of the key stage of interaction, i.e., inclusion of indophenyl acetate into the active site gorge followed by the optimal mutual orientation of the calixarene cone and the active site. As a result, the effective calix[4]arene concentrations and the deviation of kinetic data are much higher than those obtained for simultaneous addition of both the substrate and inhibitor (zero duration of incubation stage).

The formation of a similar complex between butyrylcholine and *tert*-butylcalix[4]arene is unlikely. The quaternary ammonium cations are involved in the cavity of calixarenes [40, 41]. This provides for the opposite orientation of the host and the guest as compared with the previously described indophenyl complex. As a result, when approaching the enzyme globule, the complex is coordinated with the *tert*-butyl substituents to the gorge of the active site. This coordination does not allow the complex to enter the gorge and hence to interlock the active site of the ChE. Even in case of stable complex formation, the interaction of butyrylcholine and calixarene will result in a negligible decrease of the bulk concentration of the substrate available for the enzyme. As a result, the inhibiting concentrations of calix[4]arenes established in experiments with butyrylcholine as a ChE substrate, are comparable with the concentration range of the substrate, i.e., $n \times (10^{-4}$–$10^{-3})$ M. A similar orientation of the host and the guest is probably attained for calix[4]arene **10**. The protonation of amino groups due to the release of acetic acid in the enzymatic reaction (1) results in the reversible inhibition of ChE caused by the inclusion of a cation in the enzyme active site similarly to other cations. In contrast to the mechanism proposed above,

this interaction involves the functional groups of the ChE active site. Consequently, this can be discovered using all the ChE substrates irrespective of their structure, e.g., both indophenyl acetate and butyrylcholine.

Conclusion

The molecular design of synthetic receptors is one of the most attractive goals of host-guest chemistry. Most interesting in this case is, that the decay of the ChE activity is related to the similarity in the geometry of local sites on the enzyme globule and the cooperative interaction of all the species involved in the reaction. This mechanism of inhibition does not involve any specific chemical binding and can be extended to some other targeting biomolecules. The introduction of various substituents in the upper and lower rims of calixarenes or the change of the cavity size can result in the directional alteration of the biological activity of appropriate calixarenes. Surely, the values of inhibition constants established are small enough in comparison with those obtained for specific ChE inhibitors. On the other hand, the inhibiting effect of the calixarenes investigated is suggestive of the necessity of a toxicity assay for the compounds recommended for industrial use, for example, for solid extraction or waste water treatment.

Acknowledgement

The financial support of RFBR (grant 00-03-32605) is gratefully acknowledged.

References

1. W.H. Chan, A.W.M. Lee, D.W.J. Kwong, W.L. Tam, and K. Wang: *Analyst* **121**, 531 (1996).
2. Y. Okada, M. Mizutani, F. Ishii, and J. Nishimura: *Tetrahedron Lett.* **40**, 1353 (1999).
3. U. Radius and J. Attner: *Eur. J. Inorg.Chem.* **12**, 2221 (1999).
4. M.T. Cygan, G.E. Collins, T.D. Dunbar, D.L. Allara, C.G. Gibbs, and C.D. Gutsche: *Anal. Chem.* **71**, 3936(1999).
5. X.Y. Chen, M. Ji, D.R. Fisher, and C.M. Wai: *Inorg. Chem.* **38**, 5449 (1999).
6. F. Vögtle, V. Prautzsch, C. Chartroux, and K. Gloe: *Supramol. Chem.* **11**, 93 (1999).
7. L. Lesaulnier, S. Varbanov, R. Scopelliti, M. Elhabiri, and J.C.G. Bunzli: *J. Chem. Soc. Dalton Trans.* 3919 (1999).
8. K. Kurihara, K. Ohto, Y. Tanaka, Y. Aoyama, and T. Kunitake: *J. Am. Chem. Soc.* **113**, 440 (1991).
9. W.H. Chan, K.K. Shiu, and X.H. Gu: *Analyst* **118**, 863 (1993).
10. W.H. Chan, W.M. Lee, and S. Lam: *Analyst* **120**, 2841 (1995).
11. W.H. Chan, Y.L. Wong-Leung, T.F. Lai, and R. Yuan: *Anal. Lett.* **30**, 45 (1997).
12. K. Ito, A. Kida, Y. Ohba, and T. Sone: *Chem. Lett.* **12**, 1221 (1998).
13. W.H. Chan and X.J. Wu: *Analyst* **123**, 2851 (1998).
14. J. Pfeiffer and V. Schurig: *J. Chromatogr. A* **840**, 145 (1999).
15. T.D. Chung and H. Kim: *J. Incl.Phenom.* **32**, 179 (1998).
16. O. Lutze, R.K. Meruva, A. Frielich, N. Ramamurthy, R.B. Brown, R. Hower, and M.E. Meyerhoff: *Fresenius J. Anal. Chem.* **364**, 41 (1999).
17. T.N. Lambert, L. Dasaradhi, V.J. Huber, and A.S. Gopalan: *J. Org. Chem.* **64**, 6097 (1999).
18. D.D. Malkhede, P.M. Dhadke, and S.M. Khopkar: *Anal. Sci.* **15**, 781 (1999).
19. Y.S. Zheng and Z.Q. Shen: *Eur.Pol. J.* **35**, 1037 (1999)
20. C.D. Gutsche, B. Dhawan, J.A. Levine, Kwang Hyun No, and L.J. Bauer: *Tetrahedron* **39**, 409 (1982).
21. L.C. Groenen, B.H.M. Ruel, A. Casnati, P. Timmerman, W. Verboom, S. Harkema, A. Pochini, R. Ungaro, and D.N. Reinhoudt: *Tetrahedron Lett.* **32**, 2675 (1991).
22. A.Casnati: *Gazz. Chim. Ital.* **127**, 637 (1997).
23. I.I. Stoikov, I.S. Antipin, A.A. Khrustalev, and A.I. Konovalov: *Proc. 5th Intern.Conf. on Calixarenes*, September 19–23, 1999, The University of Western Australia, 80 (1999).
24. I.I.Stoikov, A.A.Khrustalev, I.S.Antipin, and A.I.Konovalov: *Proc. 7th All-Russian Conf. on Organometal. Chem.*, Moscow 6–11 Sept. 1999, 95 (1999).
25. S. Smirnov, V. Sidorov, E. Pinkhassik, J. Havlicek, and I. Stibor: *Supramol. Chem.* **8**, 187 (1997).
26. G. A. Evtugyn, E.E. Stoikova, E.B. Nikol'skaya, and H.C. Budnikov: *Zh. Anal. Khim.* **52**, 188 (1997). *Chem.Abstr.* **126**, 126301 n (1997).
27. W.N. Aldridge: *Biochem. J.* **46**, 451 (1950).
28. T. Keleti: *Basic Enzyme Kinetics*, Akadémia Kiadó, Budapest, 1986.
29. H.C. Budnikov and G.A. Evtugyn: *Electroanalysis* **8**, 817 (1996).
30. E.B.Brouwer, J.A.Ripmeester, and G.D.Enright: *J. Incl. Phenom.* **24**, 1 (1996).
31. H.C. Froede and I.B. Wilson: Acetylcholinesterase, in P.D. Boyer (ed.), *The Enzymes*, Academic Press, New York (1971), pp. 87-97.
32. A.P. Brestkin, Yu.G. Zhukovskii, N.A. Kolchanova, E.A. Mirzabaev, and E.V. Rozengart: *Ukr. Biokhim. Zh.* **58**, 26 (1986). *Chem.Abstr.* **104**, 221219 w (1986).
33. P.A.Giang and S.A.Hall: *Anal.Chem.* **23**, 1830 (1951).
34. J.L. Sussman, M. Harel, F. Frolow, C. Oefner, A. Goldman, L. Toker, and I. Silman: *Science* **253**, 872 (1991).
35. D.R. Ripoll, C. Faerman, P.H. Axelsen, I. Silman, and J.L. Sussman: *Proc. Natl. Acad. Sci. USA* **90**, 5128 (1993).
36. G.G. Guilbault, M.H. Sadar, and M. Zimmer: *Anal. Chim. Acta* **44**, 361 (1969).
37. O. Adeyoju, E.I. Iwuoha, and M.R. Smyth: *Talanta* **41**, 1603 (1994).
38. A. Günther and U. Bilitewski: *Anal. Chim. Acta* **300**, 117 (1995).
39. P. Marchot, A. Khelif, Y.H. Ji, P. Mansuelle, and P.E. Bougis: *J. Biol. Chem.* **268**, 12458 (1993).
40. H.-J. Schneider, D. Guttes, and U. Schneider: *J. Am. Chem. Soc.* **110**, 6449 (1988).
41. J.M. Garrowfield, W.R. Richmond, and A.N. Sobolev: *J. Incl. Phenom.* **19**, 257 (1994).