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Synthesis and binding properties of carboxylphenyl-modified calix[4]arenes and cytochrome *c*

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ABSTRACT

Two novel carboxylphenyl-modified calix[4]arenes, *tetrakis*-carboxylphenylcalix[4]arene (TCPC) and 1,3-*bis*-carboxylphenylcalix[4]arene (BCPC), as well as a corresponding analogue for comparison, *tetrakis*-phenylcalix[4]arene (TPC), have been synthesized by palladium-catalyzed Suzuki cross-coupling of arylboronic acid and tetrabromocalix[4]arene as a key step. The binding properties of these calix[4]arene derivatives with bovine heart cytochrome *c* (cyt *c*) in dimethylformamide (DMF) was investigated by fluorescence spectroscopy. The binding affinity in the order of TCPC > BCPC ≫ TPC reflects a clear dependence on the number of carboxyl ligating groups attached onto a receptor and suggests the electrostatic force may be the predominant factor driving the complexing process. The stable 1:1 complexes of TCPC and BCPC with cyt *c* were evidenced with the binding constants of 3.15×10^6 and 5.85×10^5 L mol^{-1}, respectively. Due to a large overlap between the emission spectrum of TCPC and the absorption spectrum of cyt *c*, and a short interaction distance (estimated to be 5.6 nm) between them, the fluorescence quenching of TCPC upon complexation with cyt *c* is attributed to an efficient energy transfer.

1. Introduction

There has been a growing interest in the development of synthetic protein binding agents based on macrocyclic molecules to identify, inhibit, separate and/or functionally modify specific proteins [1–3]. In recent years, Hamilton et al. [2,4] have developed a series of artificial protein binders, including those built by a central calix[4]arene scaffold with peripheral groups of cyclic peptides, acting as inhibitors to specific proteins. They also created a pattern-based detection approach for different proteins by using an array of functionalized porphyrins as fluorescent protein receptors, which can be used for high-throughput proteomics, medical diagnostics, and bioterrorism applications in which multiple types of proteins need to be rapidly detected [5]. On the other hand, many synthetic receptors, particularly calixarene-based ones, have been found to possess excellent capabilities to solubilize, extract and separate specific proteins, as well as to improve the efficiency or modify the function of enzymes in organic media or ionic liquids. Shimojo et al. [6] reported that dicyclohexano-18-crown-6 enables transfer of cyt *c* into ionic liquids via supramolecular complexation, and the solubilized cyt *c* was stable and showed peroxidase activity. Recently, they found that denatured cyt *c* can be extracted from an urea solution into an organic solution containing calixarene derivatives and can successfully regain its native structure through back-extraction into a denaturant-free aqueous solution [7]. Oshima et al. [8–10] have extracted and separated cyt *c* from water into organic solvents via calixarene derivatives, and utilized one of them as mobile carriers to continuous separation and transport of cyt *c* through a liquid membrane.

Calix[*n*]arenes, consisting of phenol rings bridged by methylenes, are one of the major classes of macrocyclic host compounds in supramolecular chemistry [11,12]. Calix[*n*]arenes possess a unique basketlike cavity that can be controlled by changing the number of phenol units. To date, calix[4]arene has been extensively used as a platform to construct synthetic receptors because of its tunable and unique three-dimensional structure together with the ease of functionalization [12–15]. However, there are few examples taking advantage of an extended preorganized rigid platform of calix[4]arene for construction of artificial receptors [16]. Besides the merit of improving the encapsulation and recognition properties [17], the extended calix[4]arene skeleton with π-conjugated units could act as a chromophore or fluorophore. Based on a change in fluorescence properties upon binding with a specific guest, chemical sensors are developed and are considered particularly attractive because they offer promise for high sensitivity at low analyte concentration [18,19]. In present work, calix[4]arene carboxylphenyl derivatives

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were designed and constructed by a calix[4]arene scaffold stabilized in the cone conformation with tetra- or di-carboxylphenyl groups born at the upper rim. Cytochrome *c* (cyt *c*) is one of the most thoroughly physicochemically characterized metalloproteins with a stable protein backbone and plays key roles in electron transfer and apoptosis [20]. As a highly basic protein (pI 9.79), it possesses abundant positively charged residues on its exterior surface. In addition, it has been shown that the unpaired electrons in the ferro moiety could quench fluorescence upon binding to a fluorogenic receptor [21]. So cyt *c* represents an excellent model protein for an investigation of the interactions of proteins with artificial protein binding agents designed with the electrostatic complementary principle via fluorescence spectroscopy. Various techniques [8,22–24], including circular dichroism, dynamic light scattering and Langmuir film balance have been used to probe the binding of calixarenes to proteins. However, there are only few reports on this topic by using fluorescence method [5,25,26].

We have recently reported a preliminary findings on a carboxylphenyl-substituted calix[4]arene derivative binding with cyt *c* and revealed the interaction mechanism by molecular modeling studies [27]. Continuing on the thorough investigation of the binding properties of carboxylphenyl-substituted calix[4]arene derivatives with cyto *c*, we herein report a complete study including (i) the design rationale, detailed synthesis and full characterization of three calix[4]arene-based fluorescent receptors, *tetrakis*-carboxylphenylcalix[4]arene (TCPC), 1,3-*bis*-carboxylphenylcalix[4]arene (BCPC), and a corresponding analogue *tetrakis*-phenylcalix[4]arene (TPC) (Fig. 1); (ii) the comparison of fluorescence behavior and binding affinity of these calix[4]arene derivatives binding upon cyt *c* in dimethylformamide (DMF); (iii) the mechanism of the fluorescence quenching upon binding; and (iv) the investigation of visible CD spectra of cyt *c*–TCPC complex in DMF. The results of the present work suggest that TCPC exhibits a higher binding affinity towards cyt *c* in DMF, which highlights its potential application in chemoenzymatic synthesis to stabilize proteins in organic media [28,29]. In addition, because of its high fluorescent nature and its efficient fluorescence quenching property upon binding with specific proteins, this artificial protein binder is anticipated to have widespread applications as a fluorescent detection tool e.g. fluorescent sensor for various proteins with cation-rich surface.

2. Experimental

2.1. Apparatus

Fluorescence spectra were measured at $20 \pm 1\,^{\circ}C$ in standard quartz cells of 1 cm path length on a Hitachi F-4500 spectrofluorimeter equipped with a xenon lamp source and a thermostat bath. Both of the excitation and emission bandwidths were set at 5 nm. Circular Dichroism (CD) measurements were carried out on a Jasco J-810 spectropolarimeter. ^{1}H NMR and ^{13}C NMR spectra were recorded by a JEOL JHM-EX270 FT NMR or Varian INOVA-400 FT NMR spectrometer. Mass spectroscopy measurements were carried out by using fast atom bombardment (FAB) on the API ASTAR Pulsar I Hybrid Mass Spectrometer or matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) technique.

2.2. Reagents

Cytochrome *c* from bovine heart (C_{2037}) was purchased from Sigma and employed without further purification. Quinine sulfate was of biochemical reagent grade and purchased from Shanghai second reagent factory of China. All other reagents were of reagent grade for synthesis or analytical reagent grade for spectroscopic measurements and were used as received.

2.3. Preparation of TCPC and BCPC

The synthetic route is shown in Scheme 1. The corresponding analogue 5,11,17,23-*tetrakis*(4-phenyl)-25,26,27,28-tetrapropoxycalix[4]arene (TPC) was synthesized by the literature procedure [30]. All characterization data of ^{1}H NMR, ^{13}C NMR and MS for TCPC and BCPC are shown in Fig. S1–S5.

2.3.1. 5,11,17,23-Tetrakis(4-formylphenyl)-25,26,27,28-tetrapropoxycalix[4]arene (**2**)

To a 100-mL flask containing compound **1** (0.23 g, 0.25 mmol) and 4-formylphenylboronic acid (0.23 g, 1.53 mmol) in 50 mL of toluene was added $Pd(OAc)_2$ (11 mg, 5 mol%), P(*o*-tol)$_3$ (30 mg, 10 mol%), 8 mL of methanol and 3 mL of 2 M K_2CO_3. The mixture was stirred at 75 °C under N_2 overnight. After cooling to room temperature, 20 mL of water was added. The reaction mixture was acidified to pH 3–4 using 6 M HCl and then extracted by ethyl acetate (20×3 mL). The combined organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue obtained was purified by silica gel flash column chromatography using gradient elution method with petroleum ether and ethyl acetate as eluent affording a white solid (0.16 g, 64% yield). ^{1}H NMR (400 MHz, $CDCl_3$, δ) 9.85 (s, 4H), 7.56 (d, J = 8 Hz, 8H), 7.22 (d, J = 8 Hz, 8H), 6.98 (s, 8H), 4.60 (d, J = 13.2 Hz, 4H), 3.97 (t, J = 7.2 Hz, 8H), 3.33 (d, J = 13.2 Hz, 4H), 1.96–2.04 (m, 8H), 1.06 (t, J = 7.2 Hz, 12H). ^{13}C NMR (100 MHz, $CDCl_3$, δ) 191.7, 157.3, 146.7, 135.5, 134.4, 133.5, 129.9, 127.2, 126.7, 77.1, 31.3, 23.3, 10.3. MS (FAB) m/z 1008.1 [M$^+$].

2.3.2. 5,11,17,23-Tetrakis(4-carboxylphenyl)-25,26,27,28-tetrapropoxycalix[4]arene (TCPC)

To a 100 mL flask containing **2** (0.2 g, 0.20 mmol) in 10 mL $CHCl_3$ and 30 mL acetone was added 1 mL aqueous sulphamic acid (0.3 g, 3.1 mmol) and 1 mL aqueous sodium chlorite (0.28 g, 3.1 mmol). The mixture was stirred at room temperature for 1 h and then evapo-

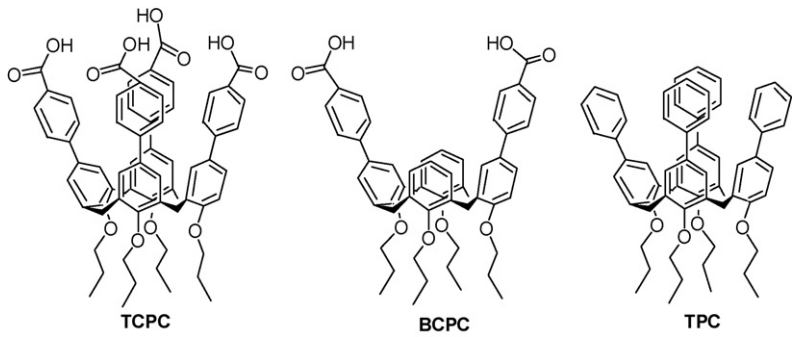

Fig. 1. The molecular structures of TCPC, BCPC and TPC.