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# Tetracyanoresorcin[4]arene as a pH dependent artificial acetylcholine receptor

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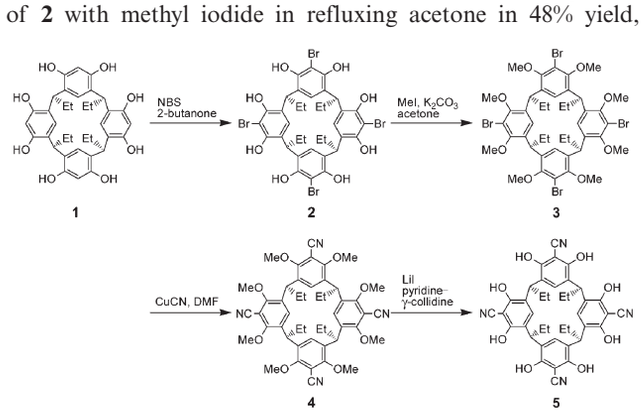
**This communication describes the facile synthesis of tetracyanoresorcin[4]arene and its high and pH dependent affinities toward biologically important acetylcholine in the physiological pH region.**

The biological importance of acetylcholine (neurotransmitter) spurs various efforts to construct its synthetic receptors,[1] aiming at developing acetylcholine sensor devices. In these aspects, resorcin[4]arenes,[2] readily available from the acid-catalyzed condensation of resorcinol with aldehydes, serve actively as promising building block. Due to their concave aromatic structures, electron-rich resorcin[4]arenes are especially attractive as very strong synthetic receptors[2,3] for choline type quaternary ammonium cations with association constants which partially exceed the corresponding constants of biological systems.[3b] Some elegant artificial acetylcholine receptors[1c,e,g] based on resorcin[4]arenes have been reported up to now, and their affinities toward acetylcholine were elucidated by various spectroscopic methods.

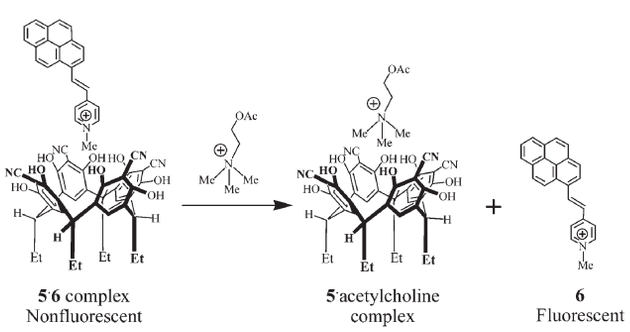
As part of our projects to develop sophisticated acetylcholine sensor devices which are active under physiological conditions, a resorcin[4]arene derivative, tetracyanoresorcin[4]arene **5** (Scheme 1), carrying strongly electron-withdrawing cyano groups at the 2-position of each resorcinol unit, was designed. The electron-withdrawing groups enable the phenolic hydroxyl groups of resorcin[4]arenes to dissociate in neutral aqueous media to afford negatively charged resorcin[4]arenes which can interact more strongly with the quaternary ammonium moiety of acetylcholine. Herein we describe the facile synthesis of **5** and its high and pH dependent affinities toward acetylcholine in the physiological pH region.[4] This compound, as recently described by us,[5] possesses p$K_a$'s in the physiological pH region, and thus can recognize acetylcholine with the aid of enhanced cation–π and electrostatic interactions.

The synthetic route to tetracyanoresorcin[4]arene **5**[6] is outlined in Scheme 1. Bromination of resorcin[4]arene **1**[7] with *N*-bromosuccinimide (NBS) in 2-butanone gave **2** in 65% yield, according to the reported protocols.[8] After *O*-methylation of **2** with methyl iodide in refluxing acetone in 48% yield, Rosenmund–von Braun reaction of octamethylated compound **3** with CuCN in refluxing DMF gave *O*-octamethylated cyano derivative **4** in 38% yield. The structure of **4** was confirmed by ESI MS and NMR. It gave an ESI MS peak at *m*/*z* 830.5, corresponding to $[M + H_2O]^+$. Compared with the corresponding ones in **3**, the aromatic protons in **4** were shifted to downfield in the $^1$H NMR spectrum, compatible with the introduction of more strongly electron-withdrawing cyano groups. A newly appeared peak at 114 ppm in the $^{13}$C NMR spectrum was assignable to the resonance of cyano groups. *O*-Demethylation of **4** was achieved by using lithium iodide in refluxing 1 : 1 pyridine–γ-collidine to afford **5** in 49% yield. The structure of **5** was confirmed by ESI MS and NMR. A negative ESI MS spectrum showed the corresponding molecular ion peak at *m*/*z* 699.3 ($[M - H]^-$). The NMR signals of the aromatic methoxy groups disappeared, revealing the complete cleavage of the methyl ether bonds. Only one set of resorcin and alkyl parts in the NMR spectra guaranteed the formation of the symmetric all-axial conformer.




**Scheme 1** Synthetic route to tetracyanoresorcin[4]arene **5**.

The pH dependent signaling recognition of acetylcholine by **5** was assessed by use of pyrene modified pyridinium cation **6** as a fluorescent probe in physiological pH region (pH 5.0–8.5), since the recognition behavior related to neurotransmitters proceeds under physiological conditions. The sensing process, which is shown in Scheme 2, involves fluorescence quenching of **6** by **5** and then fluorescence regeneration of **6** by the competitive binding of acetylcholine.[1c,d] For comparison, the binding of acetylcholine to **1** was also investigated in a similar way.




**Scheme 2** Sensing mechanism of acetylcholine by **5** in the physiological pH region.

Fig. 1 shows the plots of the relative fluorescence intensity ($I/I_0$) of **6** *vs.* the concentrations of **1** and **5** at pH 8.0. It can be seen that **5** exhibited saturation behaviour at a lower [host]/[guest] ratio than **1** under the same measuring conditions, revealing that **5** recognizes **6** more strongly than **1**. Analyses of the relationship between $I/I_0$ and the host concentrations by nonlinear curve fitting methods[9] afford the binding constants of **6** with **5** ($K_{a1}$) and **1** ($K_{a2}$) (Table 1).

It can be found from Table 1 that both **1** and **5** show very high affinities toward **6** at any pH, and compound **5** recognizes **6** much more strongly than compound **1** at any pH. Secondly, the association constants between **5** and **6** increase with the increase

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**Table 1** Association constants ($M^{-1}$) of **6** with **1** ($K_{a2}$) and **5** ($K_{a1}$) at various pH's

| pH | $K_{a1}$ | $K_{a2}$ | $K_{a1}/K_{a2}$ |
|---|---|---|---|
| 5.0 | $(7.00 \pm 2.51) \times 10^6$ | $(3.41 \pm 0.71) \times 10^6$ | 2.1 |
| 5.5 | $(7.65 \pm 1.85) \times 10^6$ | $(3.73 \pm 0.20) \times 10^6$ | 2.1 |
| 6.0 | $(1.40 \pm 0.79) \times 10^7$ | $(3.77 \pm 0.23) \times 10^6$ | 3.7 |
| 6.5 | $(3.92 \pm 1.13) \times 10^7$ | $(3.79 \pm 0.50) \times 10^6$ | 10.3 |
| 7.0 | $(4.98 \pm 2.41) \times 10^7$ | $(3.83 \pm 0.46) \times 10^6$ | 13.0 |
| 7.5 | $(6.73 \pm 3.59) \times 10^7$ | $(4.00 \pm 0.57) \times 10^6$ | 16.8 |
| 8.0 | $(1.00 \pm 0.60) \times 10^8$ | $(4.26 \pm 1.56) \times 10^6$ | 23.5 |
| 8.5 | $(1.27 \pm 0.10) \times 10^8$ | $(4.31 \pm 0.50) \times 10^6$ | 29.5 |

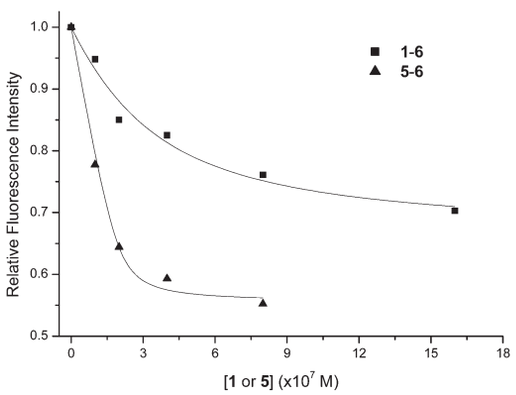


**Fig. 1** Relative fluorescence intensity of **6** ($2.0 \times 10^{-7}$ M) plotted against the concentrations of **1** and **5** in pH 8.0 aqueous solution buffered with 10 mM phosphate at room temperature, excitation 430 nm, emission 570 nm.

of pH, while the binding of **6** to **1** is insensitive to pH. These features, together with the efficient fluorescence quenching by **5**, enable compound **6** to act as a probe for the pH dependent binding of acetylcholine to **5**.

The properties of **5** as an acetylcholine receptor were examined by the fluorescence regeneration of **6** induced by the competitive binding of acetylcholine. Fig. 2 shows the plots of the relative fluorescence intensity ($I/I_0$) of **6** against the concentration of acetylcholine added at pH 8.0. It can be seen that the fluorescence intensities of **6** increase on the addition of acetylcholine and recover to their initial values after the addition of excess acetylcholine,[10] indicating that acetylcholine can substitute **6** bound to the cavity of **5**. Though the binding constant of **6** with **5** was 23.5 times greater than with **1** at pH 8.0, the addition of acetylcholine of identical concentrations induced comparable fluorescence regeneration (Fig. 2), indicating that acetylcholine binds to **5** much more strongly than to **1**. Analyses of $I/I_0$ as a function of the added acetylcholine concentrations by nonlinear curve fitting methods[9] afford the binding constants of acetylcholine with **5** ($K_{a3}$'s) and **1** ($K_{a4}$'s) (Table 2). The plots of $K_{a3}$ and $K_{a4}$ against pH are illustrated in Fig. 3.

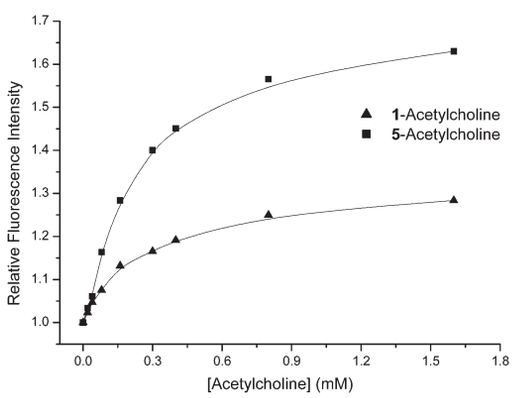


**Fig. 2** Fluorescence regeneration of **6** ($2.0 \times 10^{-7}$ M) caused by the competitive binding of acetylcholine to **1** ($8.0 \times 10^{-7}$ M) and **5** ($8.0 \times 10^{-7}$ M) in pH 8.0 aqueous solution buffered with 10 mM phosphate at room temperature, excitation 430 nm, emission 570 nm.

**Table 2** Association constants ($M^{-1}$) of **1** ($K_{a4}$) and **5** ($K_{a3}$) with acetylcholine at various pH's

| pH | $K_{a3}$ | $K_{a4}$ | $K_{a3}/K_{a4}$ |
|---|---|---|---|
| 5.0 | $(6.94 \pm 2.95) \times 10^4$ | $(2.45 \pm 0.47) \times 10^4$ | 2.8 |
| 5.5 | $(7.46 \pm 2.06) \times 10^4$ | $(2.51 \pm 0.43) \times 10^4$ | 3.0 |
| 6.0 | $(3.08 \pm 2.77) \times 10^5$ | $(2.43 \pm 0.37) \times 10^4$ | 12.7 |
| 6.5 | $(4.38 \pm 1.42) \times 10^5$ | $(2.72 \pm 0.57) \times 10^4$ | 16.1 |
| 7.0 | $(6.22 \pm 1.97) \times 10^5$ | $(2.64 \pm 0.35) \times 10^4$ | 23.6 |
| 7.5 | $(9.20 \pm 3.49) \times 10^5$ | $(3.88 \pm 1.30) \times 10^4$ | 23.7 |
| 8.0 | $(1.01 \pm 0.30) \times 10^6$ | $(4.22 \pm 1.22) \times 10^4$ | 23.9 |
| 8.5 | $(1.13 \pm 0.40) \times 10^6$ | $(4.92 \pm 1.96) \times 10^4$ | 23.0 |

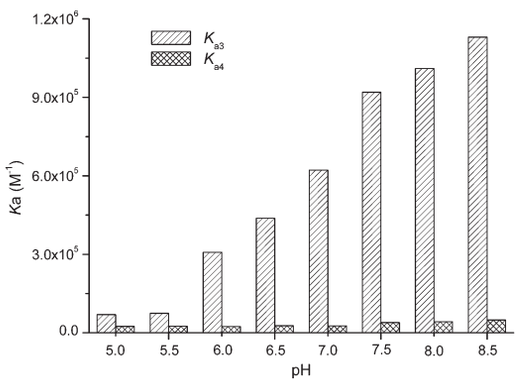


**Fig. 3** Plot of the association constants of **1** ($K_{a4}$) and **5** ($K_{a3}$) with acetylcholine against pH in the physiological pH region.

Some interesting observations can be extracted from Table 2 and Fig. 3. The first observation is that the association of acetylcholine with **5** shows a strong pH dependence, while that with **1** is scarcely affected by pH. The association constant of acetylcholine with **5** increases by over 16 times, from $(6.94 \pm 2.95) \times 10^4$ $M^{-1}$ to $(1.13 \pm 0.40) \times 10^6$ $M^{-1}$, when the pH rises from 5.0 to 8.5, while these values with **1** only double in size. Secondly, compound **5** shows much greater affinities toward acetylcholine than **1** under the same conditions, and this difference becomes greater with the increase of pH, *e.g.* the ratio of $K_{a3}$ to $K_{a4}$ is 2.8 at pH 5.0, while it increases to 23 at pH 8.5.

The foregoing results can be rationalized if the structural characteristics inherent in **5** are taken in account. The introduction of cyano groups makes **5** possess some unique features: firstly, the strongly electron-withdrawing cyano groups enable compound **5**, as recently described by us,[5] to have $pK_{a1}$, $pK_{a2}$ and $pK_{a3}$ values in the physiological pH region. Thus, in the pH range of 5.0–8.5 compound **5** changes from neutral to trianionic species. The increased dissociation of **5** with pH causes the increase of electron density at the aromatic part in **5**. In contrast, compound **1** has $pK_{a1}$ of 7.9 in aqueous media (1 : 4 water–methanol)[11] and thus remains undissociated in the physiologically important pH regions examined here. Therefore, the binding of acetylcholine (and also **6**) to **5** exhibits a pH dependence, while the association of acetylcholine with **1** is scarcely affected by pH. Secondly, the linearly aligned cyano groups deepen the concave electron-rich cavity and expand the conjugated aromatic π-systems of resorcin[4]arene, and thus **5** can offer larger contact areas with the bound guest. These factors may contribute to the stronger molecular recognition of acetylcholine (and also **6**) by **5** than by **1**, through enhanced electrostatic and cation–π interactions.

In summary, tetracyanoresorcin[4]arene with four axial ethyl chains has been successfully synthesized. This compound shows very high and pH dependent affinities toward biologically important acetylcholine in physiological pH region. This result ensures the development of tetracyanoresorcin[4]-arene-based tailored acetylcholine receptors whose affinities can be modulated by pH. The present results also indicate that simply modified resorcin[4]arenes can lead to some sophisticated receptors with strong affinities toward biologically related cationic species.

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## Notes and references


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4 For recent examples for the pH dependent behaviour of resorcin[4]arene derivatives toward ammonium cations, see: S. M. Biros, E. C. Ullrich, F. Hof, L. Trembleau and J. Rebek, Jr., *J. Am. Chem. Soc.*, 2004, **126**, 2870–2876.

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6 Synthetic procedures for compounds **2**: To a solution of **1** (2.51 g) in 2-butanone (35 mL), NBS (6.10 g) was added slowly keeping the temperature below 25 °C by ice cooling. The resulting solution was stirred at room temperature for 17 h with exclusion of light. The reaction mixture was concentrated. Then the mixture was filtered and the precipitates were washed with methanol several times and dried *in vacuo* to afford **2** (2.48 g, 65%); $\delta_H$(500 MHz; $CD_3OD$; $Me_4Si$) 0.93 (12 H, t, C$H_3$), 2.15 (8 H, m, C$H_2$CH$_3$), 4.35 (4 H, t, Ar$_2$C$H$), 7.14 (4 H, s, Ar$H$); $\delta_C$(125 MHz; DMSO-$d_6$; DMSO-$d_6$) 12.48 (CH$_2$C$H_3$), 26.42 (C$H_2$CH$_3$), 37.53 (Ar$_2$C$H$), 101.18, 123.77, 125.50, 148.70 (Ar); *m*/*z* (ESI) 915 ([M − 1]$^-$, 100%). **3**: MeI (15 mL) was added to a mixture of **2** (2.48 g) and $K_2CO_3$ (4.49 g) in acetone (50 mL). The mixture was refluxed for 36 h. After the solvents were evaporated, the solid residues obtained were partitioned between water and chloroform. The organic layer was separated, and the aqueous layer was extracted further with chloroform twice. The combined organic solutions were dried over anhydrous sodium sulfate and concentrated. Purification was accomplished by chromatography on a silica-gel column, eluted with chloroform, to give **3** (1.34 g, 48%); $\delta_H$(500 MHz; $CDCl_3$; $Me_4Si$) 0.98 (12 H, t, CH$_2$C$H_3$), 1.89 (8 H, m, C$H_2$CH$_3$), 3.66 (24 H, s, OC$H_3$), 4.39 (4 H, t, Ar$_2$C$H$), 6.58 (4 H, s, Ar$H$); $\delta_C$(125 MHz; $CDCl_3$; $CDCl_3$) 12.88 (CH$_2$C$H_3$), 28.17 (C$H_2$CH$_3$), 40.27 (Ar$_2$C$H$), 60.56 (ArOC$H_3$), 113.07, 125.47, 134.50, 154.52 (Ar); *m*/*z* (ESI) 1046.1 ([M + $H_2O$]$^+$, 100%). **4**: A mixture of **3** (1.34 g) and CuCN (1.88 g) in dry DMF (20 mL) was refluxed for 30 h. After being concentrated to *ca.* 5 mL and cooled to room temperature, $FeCl_3 \cdot 6H_2O$ (5.00 g) in water (10 mL) was added to the reaction mixture. After stirring for 30 min, concentrated HCl (5 mL) was added and the aqueous layer was extracted with chloroform thrice. The combined chloroform solutions were dried over anhydrous sodium sulfate and concentrated. The purification was accomplished by chromatography on a silica-gel column, eluted with chloroform, to afford **4** (0.40 g, 38%); $\delta_H$(500 MHz; $CDCl_3$; $Me_4Si$) 0.93 (12 H, t, C$H_3$), 2.15 (8 H, m, C$H_2$CH$_3$), 3.86 (24 H, s, C$H_3$), 4.37 (4 H, t, Ar$_2$C$H$), 6.77 (4 H, s, Ar$H$); $\delta_C$(125 MHz; $CDCl_3$; $CDCl_3$) 12.44 (CH$_2$C$H_3$), 27.66 (C$H_2$CH$_3$), 37.96 (Ar$_2$C$H$), 61.72 (ArOC$H_3$), 114.29 (C$N$), 99.79, 130.55, 132.78, 159.98 (Ar); *m*/*z* (ESI) 830.5 ([M + $H_2O$]$^+$, 100%). **5**: A solution of **4** (900 mg) and anhydrous LiI (2.70 g) in dry 1 : 1 pyridine–γ-collidine (20 mL) was refluxed for 30 h. After the solvents were evaporated under reduced pressure, the solid residues obtained were dissolved in water, acidified to *ca.* pH 3 with dilute HCl and filtered. The residues obtained were dried, and then dissolved in 1 : 10 methanol–chloroform and purified by chromatography on a silica-gel column, eluted with 1 : 5 methanol–chloroform, to afford **5** (430 mg, 49%); $\delta_H$(500 MHz; $CD_3OD$; $Me_4Si$) 0.93 (12 H, t, C$H_3$), 2.18 (8 H, m, C$H_2$CH$_3$), 4.29 (4 H, t, Ar$_2$C$H$), 7.29 (4 H, s, Ar$H$); $\delta_C$(125 MHz; DMSO-$d_6$; $Me_4Si$) 12.90 (CH$_2$C$H_3$), 27.00 (C$H_2$CH$_3$), 37.09 (Ar$_2$C$H$), 116.51 (C$N$), 92.57, 125.85, 129.44, 157.14 (Ar); *m*/*z* (ESI) 699.3 ([M − 1]$^-$, 100%).

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9 Association constants were derived from nonlinear curve fitting, using the equations $I/I_0 = 1 + ((I_\infty - I_0)/(2I_0[P]_0)) \times \{([H]_0 + [P]_0 + 1/K_{a1(or2)}) - (([H]_0 + [P]_0 + 1/K_{a1(or2)})^2 - 4[H]_0[P]_0)^{1/2}\}$ in the fluorescence quenching studies (Z. Xi, G. B. Jones, G. Qabaja, J. Wright, F. Johnson and I. H. Goldberg, *Org. Lett.*, 1999, **1**, 1375–1377; ) and $[G]_0 = \{K_{a1(or2)}(I_\infty - I)/(K_{a3(or4)}(I - I_0)) + 1\} \times \{[H]_0 - (I - I_0)/(K_{a1(or2)}\text{-}(I_\infty - I)) - [P]_0(I - I_0)/(I_\infty - I_0)\}$ in the fluorescence regeneration studies (K. Hamasaki and R. R. Rando, *Anal. Biochem.*, 1998, **261**, 183–190), wherein $[P]_0$, $[H]_0$ and $[G]_0$ are the initial concentrations of **6**, **5** (or **1**) and acetylcholine, respectively; $I$, $I_0$ and $I_\infty$ represent the fluorescence intensities of the sample, **6** alone and the intensity when **6** was totally bound, respectively.

10 For example, the fluorescence intensities of **6** alone ($2.0 \times 10^{-7}$ M), containing **5** ($8.0 \times 10^{-7}$ M) before and after the addition of exccess acetylcholine ($1.6 \times 10^{-3}$ M) were 42.1, 25.0 and 40.7, respectively, in pH 8.0 aqueous solution buffered with 10 mM phosphate at room temperature (excitation 430 nm, emission 570 nm).

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