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Tetrabiphenylporphyrin-Based Receptors for Protein Surfaces Show Sub-Nanomolar Affinity and Enhance Unfolding

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Abstract—A family of tetrabiphenylporphyrin-based receptors has been synthesized. Receptor **7** showed sub-nanomolar affinity ($K_d = 0.67$ nM) in binding to the surface of cytochrome *c*. In addition, a stoichiometric amount of the receptor **7** caused a lowering in the T_m of cytochrome *c* from 85 to 35 °C.

The design of synthetic agents that modulate protein function remains an important area of study in modern bioorganic chemistry. While the development of inhibitors that bind small, well-defined cavities in enzymes is common,[1] synthetic receptors that bind to the exterior surface of proteins have been largely unexplored.[2] We have previously reported that synthetic receptors containing multiple peptide loops on the periphery of a calix[4]arene scaffold can bind to the exterior of certain proteins.[3] More recently, we have shown that the tetraphenylporphyrin scaffold provides an excellent platform for protein surface recognition and have described receptor **1** which binds to cytochrome *c* with a K_d of 20 nM.[4] The best of these early protein surface receptor designs bind to their targets with affinities in the 10^{-7}–10^{-8} M range. However, many protein–protein interactions involve large interfacial surface area contacts (>1000 Å^2) and very high affinities (K_d values of 10^{-9}–10^{-12} M).[5] In order to disrupt these strong protein–protein complexes it is essential to develop synthetic receptors that bind their target with sub-nanomolar affinity. In this paper, we describe a new family of protein binding agents that contain a large hydrophobic core surrounded by a periphery of negatively charged substituents and the best of which bind to the positively charged protein target, cytochrome *c*, with 10^{-9}–10^{-10} M affinity.

Cytochrome *c* is a well characterized 12.5 kDa protein with a pI of 10.4[6] that plays key roles in mitochondrial electron transfer and apoptosis. The region on cytochrome *c* that predominantly interacts with its natural redox partner proteins contains a hydrophobic patch near the edge of the heme surrounded by several invariant Lys and Arg residues.[7] The complementary domains on the partner proteins, such as cytochrome *c* peroxidase or cytochrome *c* oxidase, have a hydrophobic area and invariant Asp and Glu residues. This simple matching of hydrophobic and charged domains encouraged us to design artificial receptors for cytochrome *c* in which a hydrophobic core was surrounded by negatively charged substituents (Fig. 1). Our early results with receptor **1** suggested that improved recognition might be achieved by increasing the hydrophobic surface area and the negative charge density that surrounds it. This can be accomplished by expanding the hydrophobic core scaffold from tetraphenylporphyrin, with a diameter of 15.5 Å, to tetrabiphenylporphyrin which measures 24.0 Å across (Fig. 2).

The *meso*-tetrakis-(4-carboxybiphenyl)porphyrin **2** and *meso*-tetrakis-(3,5-dicarboxy-4,4′-biphenyl)porphyrin **5** were prepared as hydrochloride salts by a modification of Adler's method[8] [acetic acid and $Zn(OAc)_2$] from the corresponding aldehydes **10a** and **10b**, respectively, followed by hydrolysis (LiOH). The aldehydes **10a** and **10b** were synthesized by Suzuki coupling between the arylbromides and arylboronic acids using $Pd(PPh_3)_4$ as catalyst (Scheme 1). The protected forms of the receptors were prepared from the corresponding acids **2** or **5** by initial conversion to acid chlorides [$(COCl)_2$/DMF] followed by coupling reactions with hydrochloride salts of appropriately protected aspartic acid derivatives in the

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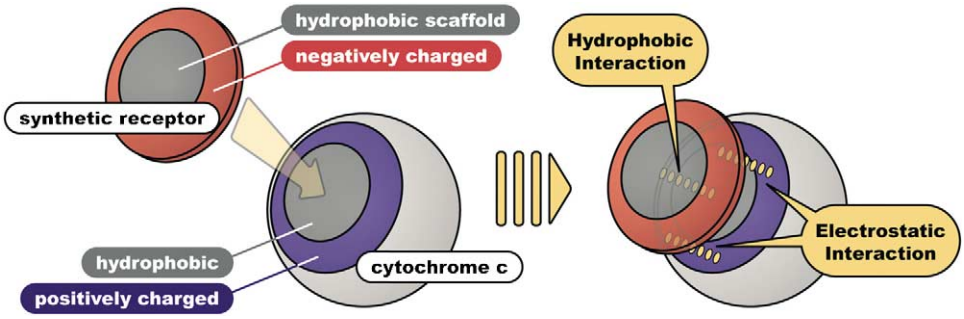


Figure 1. Schematic representation of cytochrome *c* binding by synthetic receptors.

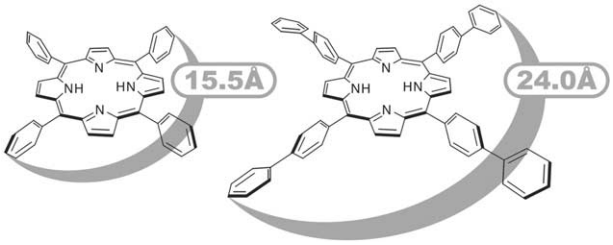
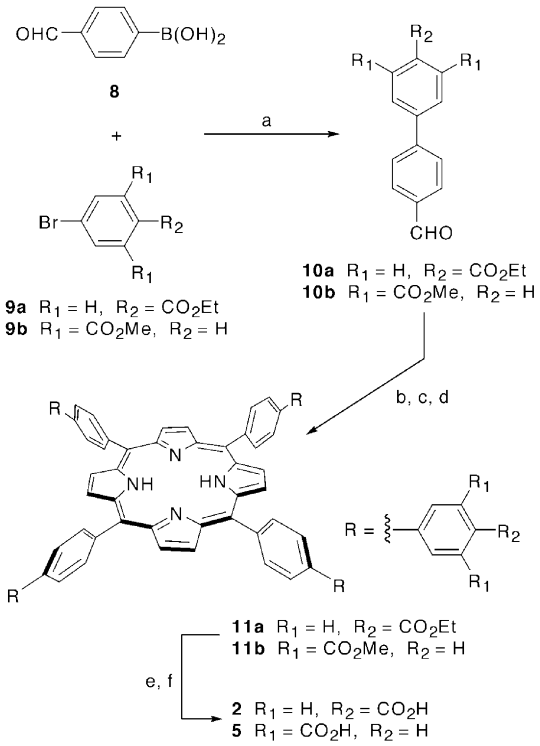
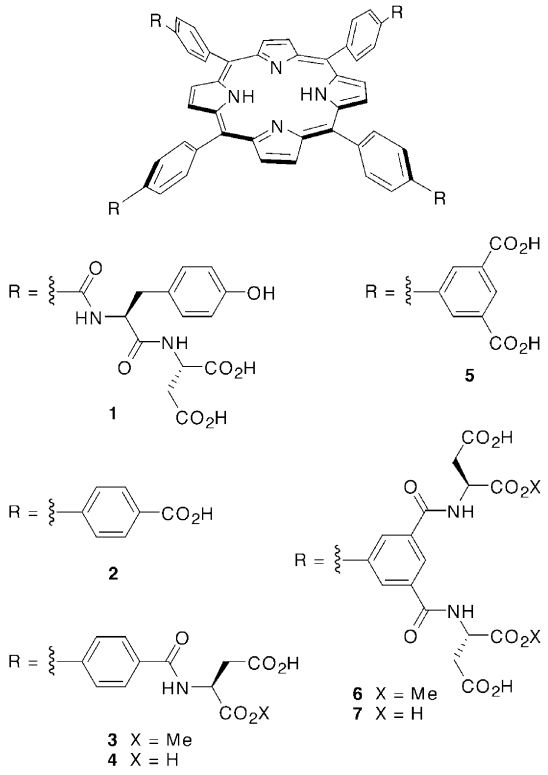


Figure 2. Comparison of tetraphenylporphyrin and tetra-biphenylphorphyrin scaffolds.

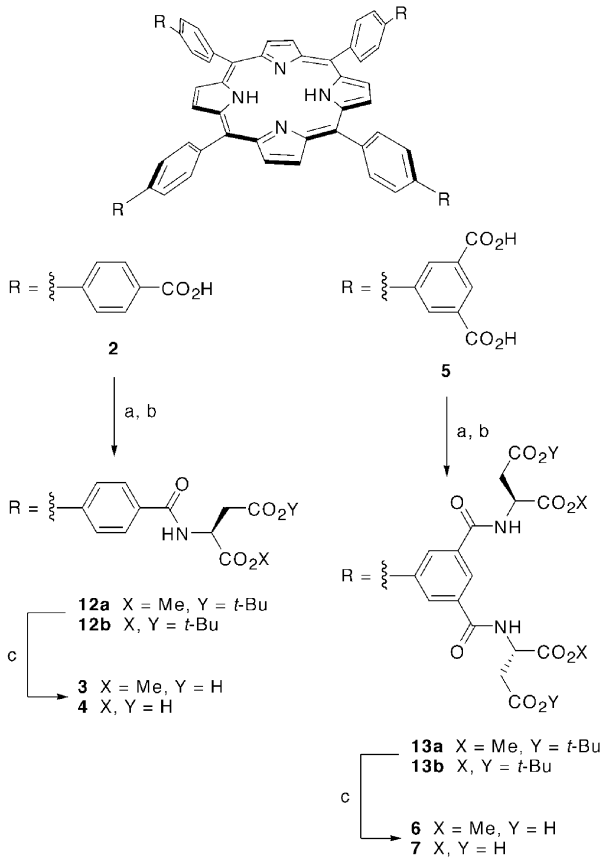


Scheme 1. Reagents and conditions: (a) $Pd(PPh_3)_4$, K_2CO_3, DMF (**10a**: 93%, **10b**: 62%); (b) AcOH, $Zn(OAc)_2$, Δ, air; (c) DDQ; (d) 18% HCl (**11a**: 11%, **11b**: 16%); (e) LiOH, dioxane, MeOH; (f) HCl (**2**: quant, **5**: 86%).

presence of base (diisopropylethylamine). Final deprotection by trifluoroacetic acid gave trifluoroacete salts of receptors **3**, **4**, **6** and **7** (Scheme 2). All the compounds were identified by ^{1}H NMR and mass spectroscopy, and the purity was confirmed by HPLC.



The binding affinities of receptors **1**–**7** for horse heart cytochrome *c* were investigated by fluorescence quenching measurements. To a solution of each receptor (10 nM), aliquots of cytochrome *c* solution were added and the fluorescence intensity at 650 nm was recorded (excitation at 420 nm) after each titration. An amphiphilic nonionic detergent, Tween20, was added (0.05 v/v%) to the aqueous 5 mM sodium phosphate buffer (pH 7.4) to minimize aggregation effects from the receptor.[9] In the best cases, a dramatic quenching of the fluorescence emission of the porphyrin was seen on addition of the protein (Fig. 3). The plots of fluorescence emission intensity vs. receptor concentration for **2**–**7** (as well as **1** in comparison) fitted well to a 1-to-1 binding isotherm (within 15% standard error except for the case of **7**) to give dissociation constants (Fig. 4, Table 1). The dissociation constants indicated a clear relationship between binding affinity and the number of anionic groups on the periphery. Receptors **2** and **3**, which bear four carboxyl groups, were found to bind to cytochrome *c* with a K_d of 12–17 μM. In contrast, receptors **4**, **5** and



Scheme 2. Reagents and conditions: (a) $(COCl)_2$, DMF; (b) H-Asp(O*t*-Bu)-OMe or H-Asp(O*t*-Bu)-O*t*-Bu, DIEA (**12a**: 54%, **12b**: 63%, **13a**: 68%, **13b**: quant.); (c) TFA (**3**: 59%, **4**: quant., **6**: 49%, **7**: quant.).

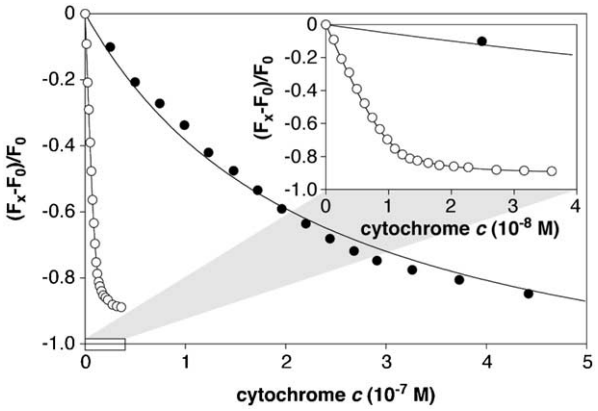


Figure 4. Fluorescence quenching profiles of **1** (●) and **7** (○) upon addition of cytochrome *c* ([**1**] = [**7**] = 10 nM). The titrations were carried out in 5 mM sodium phosphate buffer, pH 7.4, 0.05% Tween 20, rt.

Table 1. Dissociation constants[a] and charge of synthetic receptors **1**–**7**

Receptors	K_d (nM)	Charge
1	220 (±17), 20 (±5)[b]	−8
2	17,000 (±840)	−4
3	12,000 (±950)	−4
4	1300 (±130)	−8
5	1500 (±170)	−8
6	1700 (±97)	−8
7	0.67 (±0.34)	−16

[a]Determined in 5 mM sodium phosphate buffer, pH 7.4, 0.05% Tween 20, rt. Standard error is given in parentheses.
[b]Determined in 5 mM sodium phosphate buffer, pH 7.4, rt see ref. 4.

6, which bear eight carboxyl groups, showed stronger affinity to cytochrome *c* (K_d = 1.3–1.7 μM). The binding of receptor **1** (K_d = 220 nM), with essentially the same composition of hydrophobic and anionic groups, to cytochrome *c* was shown to be about 6-fold stronger, indicating that flexible binding groups might better fit the recognition features on the protein surface. Most interestingly, the fluorescence quenching profile for receptor **7** carrying 16 carboxyl groups revealed significantly stronger affinity to cytochrome *c* (K_d = 0.67 nM). To our knowledge, this is the strongest binding affinity of all synthetic protein surface receptors reported.

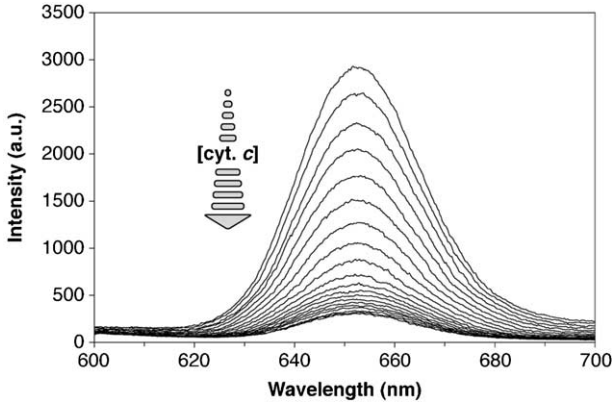


Figure 3. Fluorescence quenching of **7** upon addition of cytochrome *c* ([**7**] = 10 nM, [cyt. *c*] = 0–36 nM). The titrations were carried out in 5 mM sodium phosphate buffer, pH 7.4, 0.05% Tween 20, rt.

The selectivity of receptor **7** for cytochrome *c* relative to other proteins was investigated by fluorescence quenching at the same receptor concentration of 10 nM. Titrations were carried out (Fig. 5) with closely related cytochrome c_{551} (from Pseudomonas aeruginosa, 9.3 kDa, pI = 4.7[6]) and ferredoxin (from *Clostridium pasteurianum*, 6.2 kDa, pI = 2.75[10]) and the affinity of **7** was found to be, respectively, 270- and 25,000-fold weaker (K_d = 180 and 17,000 nM) than cytochrome *c*, suggesting that charge and size matching are required for strong binding.

To further investigate the influence of the receptors on cytochrome *c*, circular dichroism (CD) spectroscopy was performed to trace the thermal denaturation of the protein in the presence and absence of the receptors (5 mM sodium phosphate buffer, pH 7.4). The denaturation profile of horse heart cytochrome *c* at 222 nm showed a clear melting temperature (T_m) of 85 °C (10 μM cytochrome *c*). Earlier work had shown that a slight excess of the receptor **1** (12 μM) lowers the T_m of the protein to about 64 °C.[11] However, in the presence of receptor **7** the T_m decreased to 35 °C (Fig. 6). Titration of receptor **7** into cytochrome *c* solution (2 μM) at 65 °C showed a saturation for the effect at one equiv, suggesting

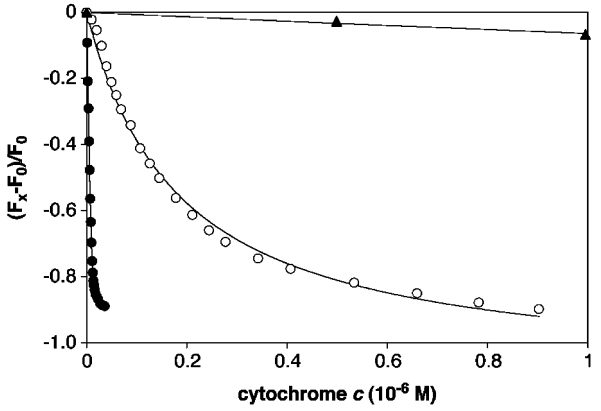


Figure 5. Fluorescence quenching profiles of **7** ([**7**] = 10 nM) upon addition of cytochrome *c* (●), cytochrome c_{551} (○) and ferredoxin (▲). The titrations were carried out in 5 mM sodium phosphate buffer, pH 7.4, 0.05% Tween 20, rt.

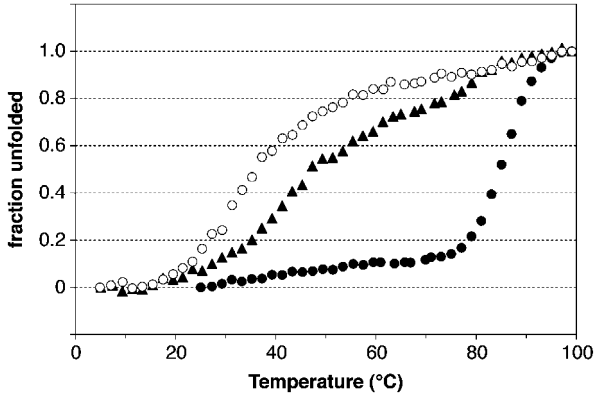


Figure 6. CD-monitored thermal denaturation profiles (θ at 222 nm) of cytochrome *c* in the absence and presence of **7**. ●: cyt. *c* (10 μM); ○: cyt. *c* + **7** (10 + 12 μM); ▲: cyt. *c* + **7** + NaCl (10 + 12 μM + 50 mM). All experiments were carried out in 5 mM sodium phosphate buffer, pH 7.4.

that the denaturation process involved a 1-to-1 stoichiometry (Fig. 7). The thermal denaturation profile at high salt concentration (additional 50 mM NaCl) showed a higher T_m, indicating that electrostatic interactions between the receptor and cytochrome *c* have a significant contribution to the more facile denaturation.[12]

In conclusion, we have designed and synthesized a family of protein surface receptors based on a tetrabiphenylporphyrin scaffold. Receptor **7** showed a remarkably strong affinity to cytochrome *c*. Stoichiometric amounts of receptor **7** lower the melting temperature of cytochrome *c* to 35 °C. These results may be extended to the development of other subnanomolar protein surface receptors. The findings may also provide an insight to the design of protein denaturants that works at stoichiometric concentration and physiological temperature.

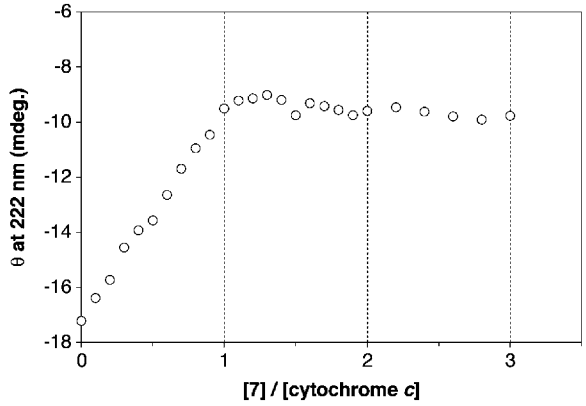


Figure 7. CD-monitored thermal denaturation profile (θ at 222 nm) of cytochrome *c* as a function of [**7**]/[cyt. *c*] at 65 °C. Titrations were carried out in 5 mM sodium phosphate buffer, pH 7.4.

Acknowledgements

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