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Bis-Catechol-Substituted Redox-Reactive Analogues of Hexamethonium and Decamethonium: Stimulated Affinity-Dependent Reactivity through Iron Peroxide Catalysis[†]

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Symmetrically bis-catechol-substituted analogues (**1** and **2**, respectively) of hexamethonium and decamethonium were synthesized and investigated as redox-activated affinity reagents toward the neurotoxin-binding sites of the nicotinic acetylcholine receptor (nAcChR), purified from *Torpedo californica* electroplax. These reagents bound to nAcChR with $K_d = 1.8 \times 10^{-8}$ and 2.3×10^{-7} M for **1** and **2**, respectively. In the presence of a metal, Fe(II)/Fe(III), and peroxide, both reagents produced a rapid and efficient half-of-sites inactivation of neurotoxin-binding sites in the nAcChR in a concentration-dependent manner, which paralleled the extent of receptor binding of the reagents. In the absence of Fe(II)/Fe(III) peroxide, redox-dependent inactivation occurred for both **1** and **2** more slowly and only at concentrations much higher (10^3-10^4 times) than those necessary to produce significant binding to nAcChR. However, receptor inactivation in the absence of added metal peroxide was still more efficient for **1** and **2** than observed previously for [(trimethylammonio)methyl]catechol (**3**), the prototypic redox-dependent affinity reagent after which **1** and **2** were patterned. Thus, the new reagents reported are expected to provide more efficient and selective conditions for redox-dependent inactivation at nAcChR and other macromolecular sites to which such reagents may be directed.

Introduction

Selective cholinergic neuronal inactivation may be used to dissect neurobiologically complex systems and is further useful in the study of the mechanisms underlying consciousness and cognition. Such selective cholinotoxins may assist in the development of animal models for disorders associated with cholinergic deficit, such as Alzheimer's disease.[1,2] Two affinity-directed approaches have been developed to meet this need, one case utilizing nitrogen mustard reactivity and a second employing affinity-directed redox-reactive reagents.[3,4]

In regard to the latter reagent type, simple, redox-reactive affinity reagents directed to choline and acetylcholine macromolecular binding sites have been constructed.[5,6] Their chemical mechanisms of action and biological specificities have been investigated.[4−8] Nickoloff et al.[6] demonstrated that 3-[(trimethylammonio)methyl]catechol (TMC, **3**) cleanly inactivated half of all available neurotoxin-binding sites of the nicotinic acetylcholine receptor (nAcChR), suggesting a novel redox-dependent affinity-directed inactivation mechanism which has been supported by subsequent mechanistic studies.[8] Affinity-dependent redox reactions occurring with these simple reagents lead to covalent modification of the macromolecular sites to which they were directed.[6]

Affinity reagents emphasizing the catechol redox-dependent theme, where two catechol rings were integrated into the reagent, offered unique possibilities for expanding both (1) their biochemical scope of application and (2) their modes of redox-initiated inactivation of macromolecular sites. Simple monosubstituted reagents such as **3** were believed to proceed through the early hydroxylated oxidation products facilitating subsequent direct redox-dependent affinity inactivation of sites. However, reagents which were disubstituted with catechols, where the catechols were strategically placed with respect to the affinity-directing functionality, could provide inactivation through a metal complexation site and a potentially more aggressive redox-inactivating reaction guided by a metal-bound reduced oxygen species, e.g., peroxide.

We have synthesized and examined as model reagents the symmetrical bis[[(peralkylammonio)methyl]catechol] analogues of hexamethonium and decamethonium[9] (**1** and **2**, Figure 1). Both agents demonstrated behavior similar to **3**; however, with both **1** and **2** addition of Fe(II)/Fe(III) salts and peroxide strongly facilitated inactivation of the nAcChR. These observations extended the manipulability and sensitivity of the catechol redox-affinity reagents in site-directed inactivation and, in addition, suggested a metal peroxide redox-catalyzed inactivation process *not* observed with simpler monofunctionalized reagents such as **3**. Here, we have described the synthesis of these hexamethonium and decamethonium analogues, their equilibrium constants for binding to the nAcChR, and their redox-initiated site-directed inactivation of neurotoxin sites of the nAcChR in the presence and absence of Fe(II)/Fe(III) and peroxides.

Chemistry

The symmetrical bis-catechol analogues (**1**, **2**) of hexamethonium and decamethonium were prepared through the condensation of (2,3-dimethoxybenzyl)-

† Abbreviations: AcCh, acetylcholine; AcChE, acetylcholine esterase; nAcChR, nicotinic acetylcholine receptor; TMC, [(trimethylammonio)methyl]catechol; 4-HTMC, 4-hydroxy-3-[(trimethylammonio)methyl]catechol; 5-HTMC, 5-hydroxy-3-[(trimethylammonio)methyl]catechol; [^{125}I]-α-Btx, [^{125}I]-α-bungarotoxin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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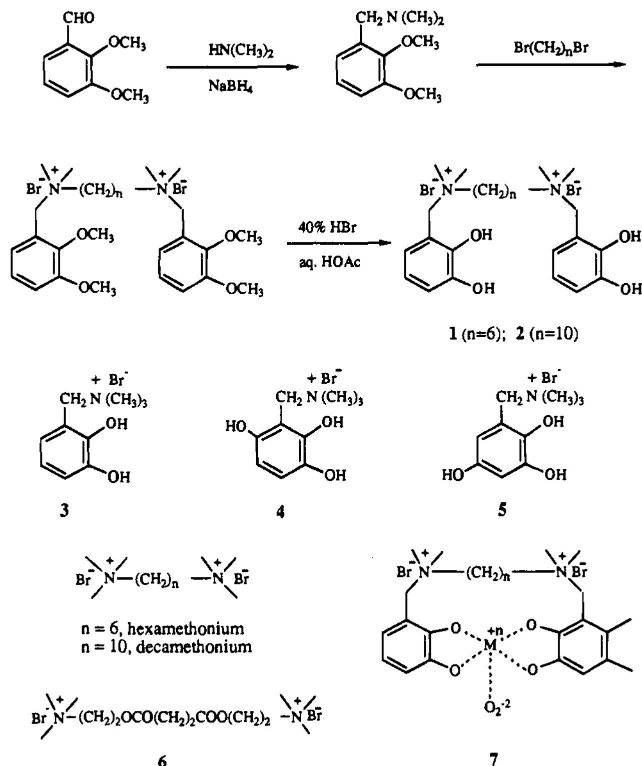

Figure 1. General synthesis of symmetrical bis-catechol hexamethonium and decamethonium analogues N,N'-(2,3-dihydroxybenzyl)-$N,N,N'N'$-tetramethyl-1,6-hexanediamine bromide (1) and N,N'-(2,3-dihydroxylbenzyl)-$N,N,N'N'$-tetramethyl-1,10-decanediamine dibromide (**2**). Structures of [(trimethylammonio)methyl]catechol bromide (TMC, **3**), 4-hydroxy-3-[(trimethylammonio)methyl]catechol (4-HTMC, **4**), 5-hydroxy-3-[(trimethylammonio)methyl]catechol (5-HTMC, **5**), hexamethonium, decamethonium, suxamethonium (**6**), and hypothetical metal peroxy complexes of 1 and **2**.

dimethylamine as noted in Figure 1. The latter was prepared from the commercially available 2,3-dimethoxybenzaldehyde. This appeared to represent an efficient general scheme for the preparation of homologous bis-methonium analogues with symmetrically substituted catechol rings. The products were unambiguously identified by chemical analysis and NMR spectroscopy.

Biological Results

The binding constants for association of both **1** and **2** with nAcChR were determined at concentrations well below those necessary to observe reagent-induced inactivation of the nAcChR. Both the hexamethonium and decamethonium bis-catechol analogues bound as well or more tightly to the AcChR as did the corresponding hexamethonium and decamethonium. Hexamethonium has $K_d = 1.8 \times 10^{-6}$ M, whereas decamethonium binds about 100 times more tightly. The binding curves together with the Hill plots (Figure 2 inserts) from which estimated binding constants were determined are shown in Figure 2 [K_d(hexamethonium analogue) = 1.8×10^{-8} M and K_d(decamethonium analogue) = 2.3×10^{-7} M]. The increased binding affinities of the bis-catechol analogues, particularly for the hexamethonium analogue, suggested that, when appropriately placed, one or both catechol substituents made constructive contributions to the binding of the analogues to the receptor. Thus, the catechol rings themselves in the hexamethonium analogue may bind in close productive association with receptor residues.

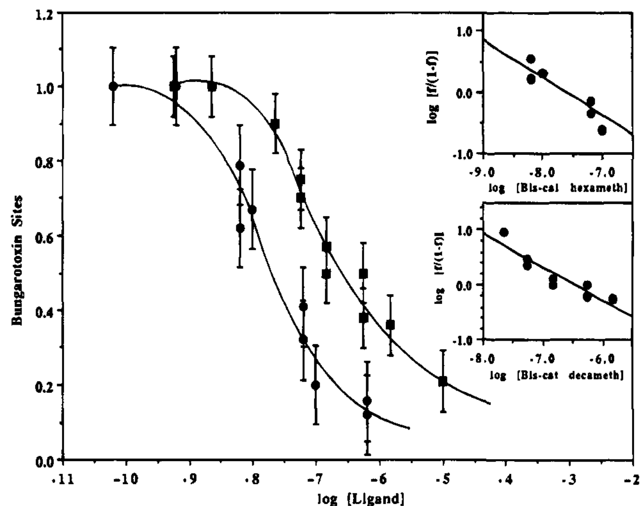

Figure 2. Relative binding of symmetrical bis-catechol hexamethonium and decamethonium analogues N,N'-(2,3-dihydroxylbenzyl)-$N,N,N'N'$-tetramethyl-1,6-hexanediamine dibromide (1; ●) and N,N'-(2,3-dihydroxylbenzyl)-$N,N,N'N'$-tetramethyl-1,10-decanediamine dibromide (**2**; ■) to purified n-AcChR from *T. californica*. Insert (top) and insert (bottom) are Hill plots based on the data plotted in the main figure. Error bars represent standard error of the data.

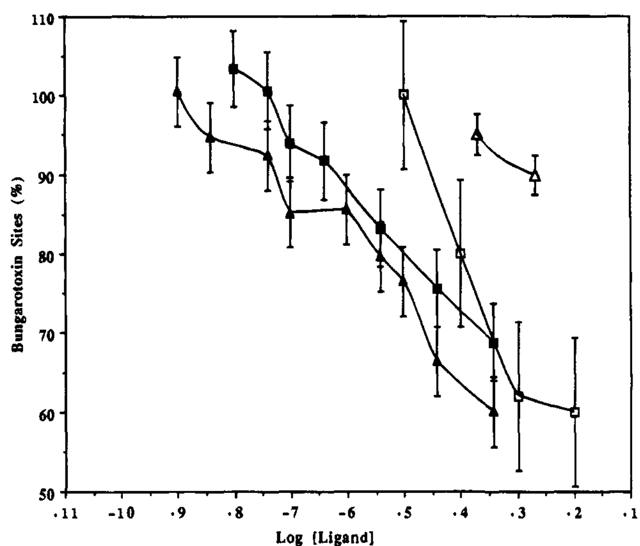

Figure 3. Inactivation of bungarotoxin sites in nAcChR in the presence of various concentrations of symmetrical bis-catechol hexamethonium and decamethonium analogues N,N'-(2,3-dihydroxylbenzyl)-$N,N,N'N'$-tetramethyl-1,6-hexanediamine dibromide (1) and N,N'-(2,3-dihydroxylbenzyl)-$N,N,N'N'$-tetramethyl-1,10-decanediamine dibromide (**2**) in the presence (1, ▲; **2**, ■) or absence (**1**, □; **2**, △) of Fe(II)/Fe(III) peroxide mixtures, respectively. Error bars represent standard error of the data.

The Fe(II)/Fe(III) peroxide-initiated inactivation of the receptor in the presence of **1** and **2** was examined using iron peroxide conditions, which did not, in themselves, inactivate the receptor (Figure 3). Both the hexamethonium and decamethonium analogues produced nearly complete half-of-sites inactivation in the high-concentration ranges of the reagents. Further, the extent of inactivation paralleled the levels of binding of the analogues to the receptor as both decreased with concentration.

Figure 3 shows that the inactivation of the receptor by the hexamethonium analogue (**1**) occurs at relatively high concentrations, when compared with inactivation

in the presence of metal and peroxide. Inactivation was initiated at concentrations as low as 0.1 mM **1**. These were 10–100-fold lower than observed with **3**;[6] however, this increased sensitivity was consistent when viewed in the context of the relative 1000× increase in the receptor-binding affinity of **1** (18 nM) cf. **3** (28 μM). On a comparative receptor-binding basis, in the absence of metals and peroxide, **1** and **2** were, in fact, about as active or a little less active than **3**. In the presence of metal and peroxide, both reagents were about as active as 4- and 5-HTMC (**4** and **5**, respectively, Figure 1). In the absence of metal and peroxide, the decamethonium analogue (**2**) was at least 10-fold less reactive than the hexamethonium analogue (**1**). Compound **2** did not appear to appreciably inactivate the receptor at concentrations as high as 2 mM and produced a relatively low level of inactivation even after a 70 h incubation in the presence of receptor. As this concentration was near the solubility limit for **2**, we have been restricted in investigating its activity at higher concentrations.

Discussion

Bis-quarternary ammoniums were originally prepared as simplistic models of *d*-tubocurarine.[9] The original efforts produced compounds which blocked neuromuscular activity, e.g., decamethonium.[10,11] Such compounds acted initially as agonists of neuromuscular contraction and subsequently produced extended relaxation. Decamethonium was used for a number of years to produce neuromuscular blockade during light anesthesia but was eventually replaced by suxamethonium (**6**, Figure 1) which may be regarded as a decamethonium analogue. The duration of the action of suxamethonium was more easily controlled due to its rapid hydrolysis to inactivated products by cholinesterase. Later, it was shown that hexamethonium primarily antagonized nerve–nerve terminals, while decamethonium primarily acted at the neuromuscular junction. While both of these bis-quaternary ammoniums were primarily of historical interest, they were initially useful in differentiating types of nicotinic cholinergic receptors.[12–15] Further, these bis-alkylammoniums have some still unresolved mechanistic significance in inhibitor studies on cholinesterases. Binding of different bis-quaternary ammoniums enhanced rates of cholinesterase acylation by neutral substrates and produced additional anomolous effects on acylation with anionic substrates.[16–19] These observations provided an early evaluation of distance relationships involving the primary and secondary "anionic" sites and the catalytic site on the enzyme as well as a speculative analysis on the hydrophilic–hydrophobic character of each of these sites.[20] This problem has become analyzable in greater depth on the basis of the recently solved crystal structure of the AcChE–decamethonium complex.[21,22]

The metal peroxide catalysis observed in the bis-catechol-substituted quaternary ammoniums is unique to the agents prepared here. No similar activation was observed with **3**. Addition of metal and peroxide to **3** did not increase rate or extent of inactivation of nAcChR. Receptor inactivation through these bis-catechol quaternary ammonium analogues may have been uniquely mediated through metal coordination complexes such as **7** (Figure 1). However, we have not directly measured the formation of such complexes, and it should be pointed out that additional mechanisms for metal-dependent inactivation may be considered. Nevertheless, the formation of a complex of a still-to-be defined structure between metal peroxide and these agents must occur to induce the affinity-directed inactivation of the receptor. Such a complex may explain the facilitation of inactivation apparently brought about by the presence of two potentially interacting catechol substituents within the same molecule.

In summary, the description of two redox-activatable bis-catechol-substituted bis-methonium analogues has provided two additional and potentially more selective inactivators of choline-binding macromolecules. Direct studies with these agents on more complex neurobiologic systems may provide a useful analysis of the contributions that multiple cholinergic receptors for these molecules make in the functioning of those systems.

Experimental Section

General. NMR spectra were obtained on a Bruker AC-F 300 MHz instrument. Chemical shifts are expressed in ppm downfield from internal tetramethylsilane. Coupling constants (J) are in hertz. Melting points are uncorrected.

General Procedure for the Synthesis of *N,N'*-(2,3-Dihydroxylbenzyl)-*N,N,N'N'*-tetramethyl-1,6-hexanediamine Dibromide (1) and *N,N'*-(2,3-dihydroxylbenzyl)-*N,N,N'N'*-tetramethyl-1,10-decanediamine Dibromide (2). (2,3-Dimethoxybenzyl)dimethylamine was prepared following the previously reported procedures.[6] Dibromohexane or dibromodecane (0.2 mmol) was refluxed with (2,3-dimethoxybenzyl)dimethylamine (0.8 g, 0.41 mmol) in acetonitrile (30 mL) for 16 h. The acetonitrile was evaporated and the residue partitioned in 50 mL of water and 50 mL of ether. The water layer was separated and washed with ether (2 × 40 mL). The aqueous layer was evaporated to dryness and gave the desired compounds. *N,N'*-(2,3-Dimethoxybenzyl)-*N,N,N'N'*-tetramethyl-1,6-hexanediamine bromide: 1.10 g, 88% yield; ^{1}H-NMR (D_2O) δ 7.1 (m, 4H), 6.8 (m, 2H), 4.3 (s, 4H), 3.7 (s, 6H), 3.6 (s, 6H), 3.1 (m, 4H), 2.8 (s, 12H), 1.7 (br s, 4H), 1.2 (br s, 4H). *N,N'*-(2,3-Dimethoxybenzyl)-*N,N,N'N'*-tetramethyl-1,10-decanediamine dibromide: 1.3 g, 95% yield; ^{1}H-NMR (D_2O) δ 7.0 (m, 4H), 6.9 (m, 2H), 4.2 (s, 4H), 3.7 (s, 6H), 3.6 (s, 6H), 3.0 (m, 4H), 2.7 (s, 12H), 1.7 (br s, 4H), 1.1 (br s, 12 H). Each of these intermediates (1 g) were refluxed in 20 mL of 1:1 HOAc:40% HBr for 16 h. Evaporation *in vacuo* in each case gave white solids.

In the case of *N,N'*-(2,3-dihydroxylbenzyl)-*N,N,N'N'*-tetramethyl-1,6-hexanediamine dibromide (**1**), the solid was recrystallized from MeOH and acetone with a yield of 68% (0.62 g): UV (water) λ_{max} 285 nm; ^{1}H-NMR (D_2O) δ 7.0–6.7 (m, 6H), 4.3 (s, 4H), 3.2 (m, 4H), 2.9 (s, 12H), 1.7 (br s, 4H), 1.3 (br s, 4H); ^{13}C-NMR 147.1 (C), 146.3 (C), 127.0 (CH), 129.1 (CH), 119.5 (CH), 116.7 (C), 65.8 (CH_2), 63.8 (CH_2), 51.2 (CH_3), 26.8 (CH_2), 23.6 (CH_2). Anal. Calcd for $C_{24}H_{38}N_2O_4Br_2$: C, 49.82; H, 6.57; N, 4.84. Found: C, 49.39; H, 6.86; N, 4.77.

N,N'-(2,3-Dihydroxybenzyl)-*N,N,N'N'*-tetramethyl-1,10-decanediamine dihydrobromide (**2**) was recrystallized from water: 0.88 g, 96% yield; UV (water) λ_{max} 286 nm; ^{1}H-NMR (D_2O) δ 6.8 (m, 2H), 6.7 (m, 4H), 4.3 (s, 4H), 3.1 (m, 4H), 2.8 (s, 12H), 1.7 (br s, 4H), 1.2 (br s, 12H); ^{13}C-NMR 146.4 (C), 145.7 (C), 126.2 (CH), 121.2 (CH), 118.8 (CH), 115.8 (CH), 65.3 (CH_2), 63.5 (CH_2), 50.5 (CH_3), 29.1 (CH_2), 26.4 (CH_2), 22.9 (CH_2). Anal. Calcd for $C_{28}H_{46}N_2O_4Br_2 \cdot 2H_2O$: C, 50.14; H, 7.46; N, 4.18. Found: C, 49.70; H, 7.72; N, 4.08.

Acetylcholine Receptor (AcChR): Purification and [^{125}I]Bungarotoxin Assay. AcChR was purified from the electric organ of *Torpedo califonica* (Pacific Biomarine, Venice, CA) by cobratoxin-affinity chromatography of the Triton X-100-solubilized electroplax using procedures given earlier.[6,8,23] The neurotoxin was purified by the method of Karlsson et al.[24] from the lyophilized venom of the Thailand cobra, *Naja naja siamensis* (obtained from the Miami Serpentarium, Miami, FL). Purification of nAcChR was followed by observed increases in [^{125}I]-α-bungarotoxin ([^{125}I]-α-Btx) binding sites per

milligram of protein,[25] by SDS–PAGE on 7% acrylamide gels, and by separation from acetylcholinesterase, which was followed by the procedure of Ellman.[26] [^{125}I]-α-Btx was purchased from New England Nuclear. Protein was determined by the method of Lowry et al.[27]

Binding Constants of *N,N'*-(2,3-Dihydroxylbenzyl)-*N,N,N'N'*-tetramethyl-1,6-hexanediamine Dibromide (1) and *N,N'*-(2,3-Dihydroxylbenzyl)-*N,N,N'N'*-tetramethyl-1,10-decanediamine Dibromide (2) to nAcChR. Dilute solutions of nAcChR previously titrated with known concentrations of [^{125}I]-α-Btx were adjusted to contain 3–4 pmol of toxin sites. Various concentrations of samples prepared in buffer (0.1% Triton X-100, 10 mM sodium phosphate, pH 7.4) were added to the receptor solutions. Solutions of **1** and **2** were prepared fresh in concentrated form in pH 2.2 at room temperature and aliquoted to give appropriate concentrations in receptor buffer. The total reduction of toxin-binding sites in these receptor solutions was determined by the [^{125}I]-α-Btx procedure of Schmidt and Raftery.[28] Prior to counting, the disks were washed with buffer. To minimize the tendency of toxin to stick to plastic and glass, all toxin containers were coated with 1 mg/mL BSA (bovine serum albumin).

Extent of Inactivation of Bungarotoxin Sites of the Acetylcholine Receptor by *N,N'*-(2,3-Dihydroxylbenzyl)-*N,N,N'N'*-tetramethyl-1,6-hexanediamine Dibromide (1) and *N,N'*-(2,3-Dihydroxylbenzyl)-*N,N,N'N'*-tetramethyl-1,10-decanediamine Dibromide (2). Various concentrations of samples were incubated at 4 °C with dilute solutions of purified nAcChR previously titrated with known concentrations of [^{125}I]-α-Btx. Final concentrations of the samples varied from 0.1 to 10 mM. The nAcChR solutions were incubated alone or in the presence of samples in the buffer. The toxin-binding sites were determined by the [^{125}I]-α-Btx procedure of Schmidt and Raftery[25] after 2 h dialysis against buffer to remove excess reagent. During dialysis buffer solutions were changed twice. Control dialyses of samples oxidized in the absence of AcChR were carried out to assure removal of toxin binding inhibiting polymers that may have been formed during the oxidation of samples, that is, after dialysis addition of receptor and [^{125}I]-α-Btx under the usual conditions, no inhibition of toxin binding to the receptor was observed.

Effect of Fe(II) or Fe(III) on the Covalent Binding of *N,N'*-(2,3-Dihydroxylbenzyl)-*N,N,N'N'*-tetramethyl-1,6-hexanediamine Dibromide (1) and *N,N'*-(2,3-Dihydroxylbenzyl)-*N,N,N'N'*-tetramethyl-1,10-decanediamine Dibromide (2) to nAcChR. *N,N'*-(2,3-Dihydroxylbenzyl)-*N,N,N'N'*-tetramethyl-1,6-hexanediamine dibromide (**1**) or *N,N'*-(2,3-dihydroxybenzyl)-*N,N,N'N'*-tetramethyl-1,10-decanediamine dibromide (**2**) (1 mM) in the presence of dilute solutions of nAcChR and Na_2O_2 were incubated with equimolar concentrations of either $FeCl_2$ or $FeCl_3$ at 4 °C. All the samples were prepared in buffer (0.1% Triton X-100, 10 mM sodium phosphate, pH 7.4). Prepurified argon was briefly bubbled through buffer to remove saturating oxygen before use. In controls, receptor was incubated alone or with either $FeCl_2$ or $FeCl_3$ in the presence of Na_2O_2 but in the absence of **1** or **2**. The remaining toxin-binding sites were determined after 2 h dialysis against buffer. Controls were identical to those noted in the procedure given immediately above.

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