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SHORT COMMUNICATION

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Improved Synthesis of the Triazacryptand (TAC) and its Application in the Construction of a Fluorescent TAC-BODIPY Conjugate for K^+ Sensing in Live Cells

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In this report, a vastly improved method for the synthesis of [2.2.3]-triazacryptand (TAC), an excellent K^+-selective cryptand, is disclosed, which provides a shorter synthetic route with > fivefold higher overall yield. On the basis of the improved synthesis, a TAC-BODIPY conjugate was developed for K^+ sensing. TAC-BODIPY is a fluorescence turn-on K^+ sensor exhibiting high selectivity toward K^+ over other biologically relevant metal cations. Cell imaging studies are presented and demonstrate that TAC-BODIPY is suitable for dynamic K^+ sensing in live cells.

As the most abundant intracellular metal cation, potassium plays indispensable roles in a wide range of biological processes, such as nerve transmission, cardiac excitability, epithelial fluid transport, muscle contraction, cell proliferation, and cellular ionic homeostasis.[1] On the other hand, abnormal potassium levels are early symptoms for certain diseases, including hypertension, heart disease, seizures, stroke, bulimia, anorexia, diabetes, alcoholism, renal disease, myasthenia, cancer, and AIDS.[2] Therefore, a delicate balance of potassium is essential for human health. Due to the biological significance of potassium, continuous efforts have been devoted to intracellular and extracellular potassium detection. Relative to other techniques, fluorescence approaches have attracted more and more interest because of their distinct advantages including non-invasiveness, high sensitivity, and convenience. Among various proposed fluorescent potassium sensors, PBFI (potassium-binding benzofuran isophthalate), which incorporates a diaza-18-crown-6 ether as the recognition unit and a benzofuran derivative as the fluorophore, is the most popular and currently the only one commercially available.[3] However, PBFI suffers insufficient potassium binding strength and poor selectivity for K^+ over Na^+.[4] To overcome the problem of poor K^+/Na^+ selectivity, He et al. developed a [2.2.3]-triazacryptand (TAC) platform as a K^+-selective receptor unit, which exhibits very high selectivity for detecting K^+ over the interfering Na^+.[5] Subsequently, fluorescent potassium sensors employing TAC as the K^+-recognition moiety have been synthesized and applied to biological imaging.[6]

However, the synthesis of TAC involves multistep reactions, which indirectly limits its wide application. Herein, we describe an optimized synthesis of TAC with a shorter synthetic route and a higher overall yield (Scheme 1) and its application in the construction of TAC-BODIPY, a BODIPY (4,4-difluoro-4-bora-3a,4a-diza-s-indacene) based fluorescent K^+-selective sensor.

The synthetic precursor compound **3** was obtained from compounds **1** and **2** (see Supporting Information). In the literature, this reaction was performed in refluxing MeCN with KI as catalyst and K_2CO_3 as base. However, after refluxing for 16 h, only a monosubstituted aniline derivative was produced as the major product and the desired bis-substituted aniline product was formed in trace amount. Repeated addition of **1** every 20 h (three times) resulted in a modest yield (ca. 50%) of compound **3**. DMF was also employed as the reaction medium rather than MeCN, and the reaction temperature was raised to 120 °C, but similar results were obtained. Finally, a totally different reaction system was adopted to perform this reaction, by using a mixture of 1,4-dioxane and water (2:3) as the reaction medium and $CaCO_3$ as the base (reaction details in Supporting Information). As a result, **3** was obtained in significantly higher yield (91%).

In the next step, a hydrogenation reaction was used as in literature methods for the reduction of the nitro groups of compound **3** to amino groups in a DMF solution of **3** at 2.2 atm, with Pd/C (palladium on activated carbon) as cata-

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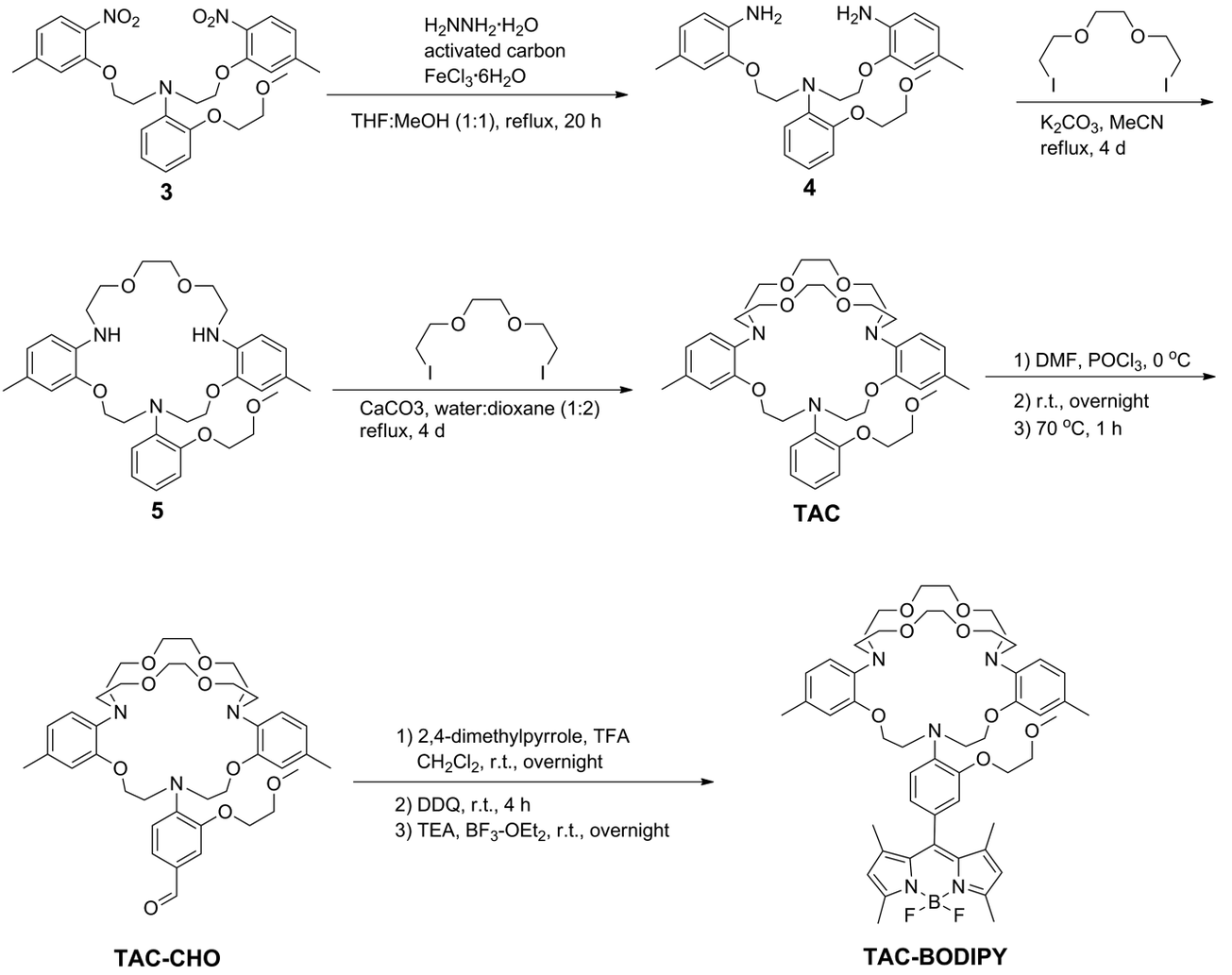

Scheme 1. Synthetic route of TAC-BODIPY.

lyst. In this way, amine **4** was prepared in 97% yield. To avoid the use of hydrogen gas, hydrazine monohydrate was employed as the reducing reagent. A comparable yield (97%) was obtained by this method relative to that under Pd/C catalytic conditions. Furthermore, a more economic catalyst $FeCl_3$/C (a mixture of $FeCl_3$ and activated carbon) also provided compound **4** in a similarly high yield (96%).

The following two 21-membered macrocyclization reactions are the two key steps for the synthesis of TAC. In the literature methods, both macrocycles were achieved by an amide synthesis (steps Sd and Sf in Scheme S1). Two amide groups were synchronously formed through reactions between the amines (compound **4** for step Sd and compound **5** for step Sf) and 3,6-dioxaoctanedioic acid dichloride (DODC), which was obtained from reaction of 3,6-dioxaoctanedioic acid with oxalyl chloride. Following each of these two steps, a reduction reaction takes place, which reduces the newly formed amides (compounds **6** and **7** in Scheme S1) to amines (compound **5** and TAC), respectively. Here we attempted to synthesize macrocycle **5** directly from **4** and TAC directly from **5**, as displayed in Scheme 1, which eliminates the synthesis of compounds **6** and **7**.

Enlightened by the results obtained in the synthesis of **3**, in which the MeCN/K_2CO_3 system worked well in preparing mono-substituted anilines but poorly in producing bis-substituted anilines, we employed those conditions to prepare macrocycle **5**. Different conditions were evaluated to achieve the optimized reaction conditions, which included reactants, bases, and solvents. As shown in Table S1, the **A**/K_2CO_3/MeCN system (entry 1) turned out to be the most efficient one among the systems examined. Macrocyle **5** was generated by reaction of **4** and 1,2-bis(2-iodoethoxy)-ethane with K_2CO_3 as base in MeCN under reflux. The yield (40%) of this reaction was moderate but comparable to the overall yield (42%) of the literature two-step methods. Similarly, TAC was produced in 69% yield, much higher than the overall yield (32%) of the literature two-step method by refluxing a mixture of **5**, 1,2-bis(2-iodoethoxy)ethane, and $CaCO_3$ in 1,4-dioxane/water (2:1).

TAC-CHO was obtained by following a literature procedure.[5] Subsequently, a TAC-BODIPY conjugate was

synthesized for K^+ sensing through a trifluoroacetic acid catalyzed condensation reaction of TAC-CHO with 2,4-dimethylpyrrole. The fluorescence response of TAC-BODIPY to K^+ ions was investigated in 5 mM HEPES buffer (pH 7.2)/MeCN (4:1, v/v). Figure 1 shows the fluorescence emission spectra of TAC-BODIPY in the presence of K^+ and other biologically relevant metal cations at their physiological concentrations. As expected, TAC-BODIPY exhibits very high selectivity for K^+ over the other metal cations. In the absence of cations, free TAC-BODIPY emits a very weak fluorescence (quantum yield: 0.03) at 512 nm. Upon addition of K^+ ions, the fluorescence intensity dramatically increases while the addition of Na^+ and other biologically important metal cations induces no obvious change to the fluorescence spectrum of TAC-BODIPY.

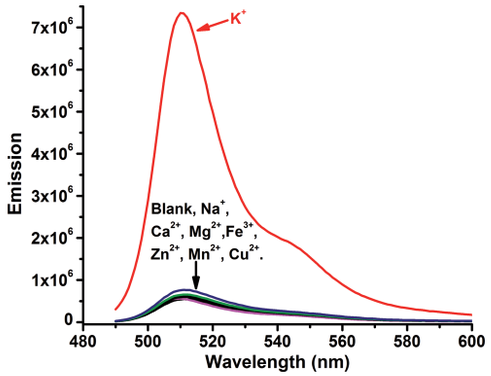

Figure 1. Fluorescence emission spectra of TAC-BODIPY (10 μM) in 5 mM HEPES buffer (pH 7.2)/MeCN (4:1, v/v) upon addition of various biologically relevant metal cations at their physiological concentrations: Na^+ (150 mM), K^+ (150 mM), Ca^{2+} (0.5 mM), Mg^{2+} (2.5 mM), Fe^{3+} (18 mM), Zn^{2+} (45 mM), Mn^{2+} (0.9 mM), Cu^{2+} (16 mM).

To further examine the efficacy of TAC-BODIPY towards sensing K^+ ions, fluorescence emission titration experiments were carried out in 5 mM HEPES buffer (pH 7.2)/MeCN (4:1, v/v). Solutions were balanced with NaCl to maintain a constant ionic strength of 150 mM. As illustrated in Figure 2, a gradual enhancement of the fluorescence intensity of TAC-BODIPY was observed upon progressive addition of K^+ ions. The dissociation constant (K_d) of the sensor was determined to be 65 mM (see Supporting Information), according to a literature method.[6e] The linear fitting of the plot (Figure S1) evidenced a 1:1 binding ratio between TAC-BODIPY and K^+.[6e] The fluorescence intensity increased 14-fold in the presence of 150 mM K^+ (quantum yield: 0.43). Higher concentrations of K^+ ions were not able to further increase the fluorescence intensity of TAC-BODIPY. Moreover, TAC-BODIPY was capable of detecting 1 mM K^+ with the co-existence of a high concentration of Na^+. The detection limit of TAC-BODIPY for K^+ was determined to be 1.5×10^{-6} M. Since the extracellular and intracellular concentrations of potassium are of the order of 5 mM and 150 mM,[7] respectively, the TAC-BODIPY probe is potentially suitable for determining physiological K^+ levels.

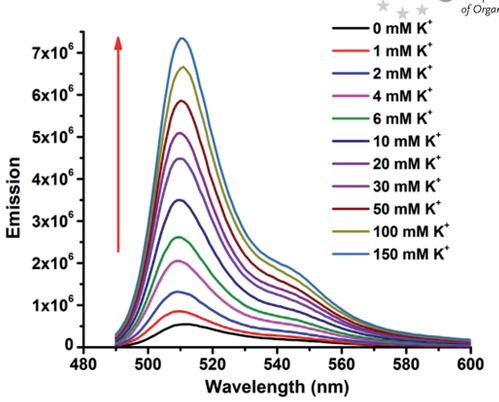

Figure 2. Enhancements in fluorescence emission spectra of TAC-BODIPY (10 μM) in 5 mM HEPES buffer (pH 7.2)/MeCN (4:1, v/v) upon continuous addition of 150 mM K^+ ions. Ionic strength was maintained at 150 mM by addition of NaCl.

Encouraged by these results, TAC-BODIPY was used to detect changes of intracellular potassium levels. Figure 3 shows images of cells taken at different times (0, 30, 60, 90, 120 min), and the average fluorescence emission intensity of each image is shown in Figure S2. U87MG cells were incubated with a 5 μM solution of TAC-BODIPY in complete MEM medium for 10 min. As expected, the cells were highly fluorescent as shown in Figure 3A and F, which is consistent with the forgoing observed fact that the intracellular K^+ level is high enough to trigger strong fluorescence of TAC-BODIPY. To examine the ability of TAC-BODIPY to respond to changes in K^+ levels in living cells, a mixture of nigericin (5 μM), ouabain octahydrate (10 μM), and bumetanide (10 μM) in complete MEM medium was pumped into the cell chambers and images of the living cells were taken at different times. It has been shown that the combination of nigericin, ouabain octahydrate, and bumetanide can effectively bring about K^+ efflux of cells.[6e,8] With the depletion of K^+ induced by nigericin, ouabain octahydrate, and bumetanide, the fluorescence intensity of the cells gradually decreases (from Figure 3F–J). For the control experiment, after the same time, the cells still appeared highly fluorescent (Figure 3B–E), which exhibit little visible change relative to their original state (Figure 3A). These results indicate that TAC-BODIPY is a

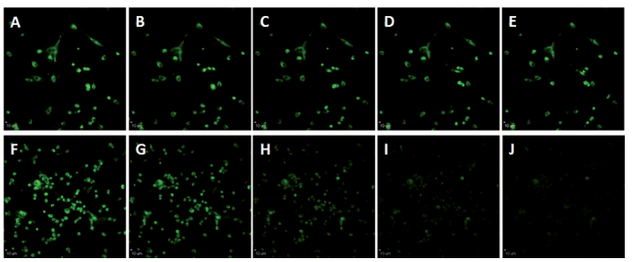

Figure 3. Images of live U87MG cells in MEM medium (A–E), and in an MEM medium solution of nigericin (5 μM), ouabain octahydrate (10 μM), and bumetanide (10 μM) (F–J), at 0 min (A, F), 30 min (B, G), 60 min (C, H), 90 min (D, I), and 120 min (E, J) after incubation with TAC-BODIPY (5 μM).

promising probe to monitor intracellular K^+ levels in living cells.

In summary, the synthesis of TAC was improved significantly. The synthetic route was shortened and the overall yield was increased fivefold from 4% to nearly 20%. A fluorescent K^+-selective sensor, TAC-BODIPY, was then synthesized and characterized. TAC-BODIPY exhibited excellent selectivity toward K^+ over other biologically important metal cations and high sensitivity for K^+ sensing. Fluorescence microscopy experiments demonstrated that the TAC-BODIPY probe is capable of sensing K^+ in living cells.

Supporting Information (see footnote on the first page of this article): Information on the procedures for the improved synthesis of compounds **1**, **3**–**5**, and TAC, synthesis of TAC-BODIPY, cell culture for imaging, fluorescence intensity of cell images, and copies of ^1H and ^{13}C spectra are presented.

Acknowledgments

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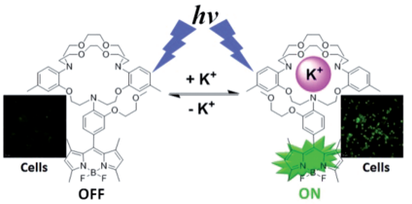
The dye, TAC-BODIPY, emits very weak fluorescence at 512 nm. Upon binding with K^+, it becomes highly fluorescent, emitting very strong fluorescence. TAC-BODIPY exhibits excellent selectivity toward K^+ over Na^+ and desirable sensitivity for K^+ sensing. This fluorescent K^+ sensor is capable of efficiently sensing the K^+ levels in living cells through cell imaging with fluorescence microscopy.



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