Microwave-assisted slipping synthesis of fluorescent squaraine rotaxane probe for bacterial imaging†

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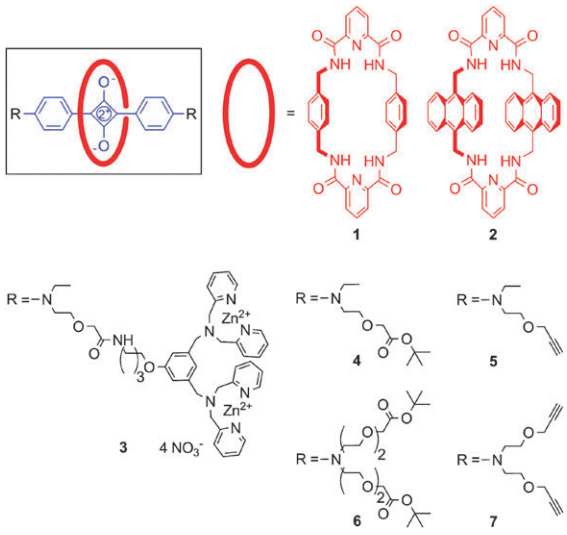
Microwave heating accelerates quantitative squaraine rotaxane formation by slipping and facilitates production of a bacterial imaging probe with zinc dipicolylamine targeting ligands.

We are developing squaraine rotaxanes as high performance fluorescence imaging probes.[1] Our first generation probes employ the phenylene tetralactam **1** to encapsulate various squaraine dyes (Scheme 1). The resulting rotaxanes exhibit almost identical absorption and emission wavelengths as the popular red-emitting cyanine dye Cy-5, however, the squaraine rotaxanes are brighter and much more photostable. One example is the probe **1⊃3** (abs.: 653 nm, em.: 669 nm) with two appended zinc dipicolylamine (Zn-DPA) affinity ligands that selectively target the surface of bacterial cells.[2] This probe is extremely resistant to photobleaching and allows real-time fluorescent movies to be generated showing bacterial cell division over long periods of time. The synthesis of **1⊃3** involves several low yielding reactions and requires chromatography in the later steps. This limits the amount of material that can be generated in a single batch and makes the probe relatively expensive to produce, which can be problematic for *in vivo* imaging studies that require repeated dosing of large numbers of animals. This drawback has motivated us to devise a related next generation squaraine rotaxane probe that can be assembled efficiently in high yield and large scale.



Scheme 1

We designed the rotaxane **2⊃3** which maintains the same squaraine thread component **3** with two Zn-DPA targeting ligands, but has the anthracene-containing macrocycle **2**. Based on previous work, we expected that **2⊃3** would exhibit the same excellent stability as **1⊃3**, and similar bacterial affinity, but that its absorption–emission wavelengths would be red-shifted.[3] The challenge was to develop a suitable high yielding and large scale synthetic process. The key step is the rotaxane forming reaction that makes the intermediate bis(ester) **2⊃4**. We first tried a standard clipping reaction; that is, a templated macrocyclization of 9,10-bis(aminomethyl)-anthracene and 2,6-pyridine dicarbonyl dichloride in the presence of **4** which produced **2⊃4** in 20% yield after chromatography (Scheme 2). In addition to the low yield, the clipping reaction required pseudo dilution conditions which limited the batch size. We next considered if rotaxane yield could be improved by a slipping approach.[4] Unlike macrocycle **1**, macrocycle **2** is quite soluble in weakly polar organic solvents, and previous studies have shown that it will spontaneously encapsulate squaraine dyes with high affinity.[3] For example, a 1 : 1 mixture of **2** and **5** in chloroform forms **2⊃5** in quantitative yield, but the assembly process takes several weeks to reach completion at room temperature, and two days when the sample is heated at 120 °C. Therefore, we investigated microwave heating, a technique that has proven to be quite effective at shortening reaction times,[5] but to the best of our knowledge has not been reported as a method to facilitate rotaxane slipping.‡ We discovered that microwave heating of a 1 : 1 mixture of **2** and **5** in dry chloroform at a temperature of 160 °C produced a quantitative yield of rotaxane **2⊃5** in 5 minutes. Furthermore, the same microwave procedure with a 1 : 1 mixture of **2** and **4** formed the desired bis(ester) rotaxane **2⊃4** in quantitative yield. Not only is the assembly time extremely short and high yielding, it is easily conducted on a large scale. Not surprisingly, there is a steric limitation on the types of squaraine rotaxanes that can be assembled by this microwave-assisted slipping process. Exploratory studies showed that no rotaxane was formed when

Scheme 2

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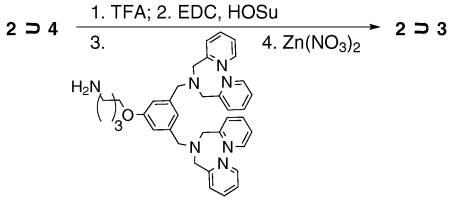
analogous reactions were conducted with squaraines **6** and **7** that have sterically more demanding terminal stopper groups.

With considerable amounts of **2⊃4** in hand, we were able to produce the desired probe **2⊃3** using the straightforward synthetic sequence shown in Scheme 3.§ Compound **2⊃3** exhibited excellent water solubility, and as shown in Fig. 1a, absorption–emission wavelengths (abs.: 663 nm, em.: 690 nm) that match the common cyanine dye Cy-5.5. The ability of **2⊃3** to act as a bacterial imaging probe was evaluated using fluorescence microscopy and whole body optical imaging. As expected, the probe readily stains Gram-positive and Gram-negative bacteria.[2] Shown in Fig. 1b is a typical fluorescence image of *Salmonella typhimurium* AM3 that has been incubated with **2⊃3** for fifteen minutes. The image shows strong localization of the probe in the bacterial envelope which agrees with previous studies indicating that the Zn-DPA affinity ligands associate with the anionic phospholipids and amphiphiles that are embedded in the bacterial membrane and peptidoglycan layer. In the case of Gram-negative bacteria like *S. typhimurium* the likely Zn-DPA probe targets are the phosphate esters within the lipid A component of the lipopolysaccharides in the outer monolayer of the exterior bacterial membrane.[6]

Most importantly, the red-shifted wavelength of **2⊃3** allows it to be distinguished spectrally from **1⊃3** using appropriate microscopy filter sets or spectral unmixing algorithms that are available on modern imaging stations. This is demonstrated in Fig. 2, which shows whole body optical images of a living mouse after it was injected with two separate populations of *S. typhimurium*. The population in the left rear leg was prelabeled with **1⊃3** and the population in the right rear leg was prelabeled with **2⊃3**. Whole body fluorescence images were acquired using a 750 ± 18 nm emission filter while scanning the excitation from 550–690 nm. Spectral unmixing of the emission data separated the signals of the two probes, and produced the three images in Fig. 2. The images in panels A and B show the locations of probes **1⊃3** (green) and **2⊃3** (red), respectively, relative to a planar X-ray image. Panel C



Scheme 3

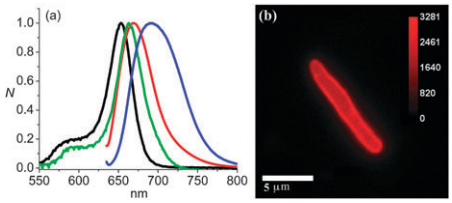


Fig. 1 (a) Normalized absorption and fluorescence emission spectra for probes **1⊃3** (abs.: black, em.: red) and **2⊃3** (abs.: green, em.: blue) in water (5.0 μM); (b) fluorescence micrograph of *S. typhimurium* AM3 after treatment with **2⊃3**.

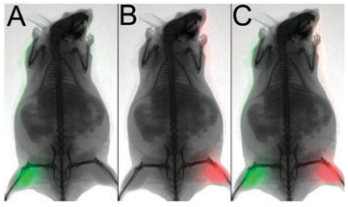


Fig. 2 X-Ray and fluorescence emission overlay of a living mouse with two separate prelabeled populations of *S. typhimurium* AM3 injected into: (A) left rear leg (**1⊃3**, green); (B) right rear leg (**2⊃3**, red). Panel C is a combination of A and B.

shows a combination of panels A and B, and the lack of yellow color indicates complete unmixing of the two probe signals. The ability to spectrally separate the fluorescence emission signals from these two probes demonstrates how the parent squaraine rotaxanes can be employed as a complementary pair of fluorophores for simultaneous tracking of two imaging targets in the same biological sample.

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Notes and references

‡ Successful rotaxane assembly by microwave-assisted slipping requires the slipping equilibrium to remain highly favored at high temperature. Rotaxane synthesis has been promoted by grinding methods, see for example ref. 7. However, attempts to promote slipping by grinding dry solid samples of **2** and **4** did not produce any squaraine rotaxane.

§ As discussed in the ESI†, there is no rotaxane unthreading during the synthetic sequence shown in Scheme 3, in agreement with the conclusion of ref. 1*c* that squaraine rotaxane integrity is maintained in weakly polar solvents with small stopper groups.

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