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COMMUNICATION

Rationally-designed fluorescent lysine riboswitch probes†

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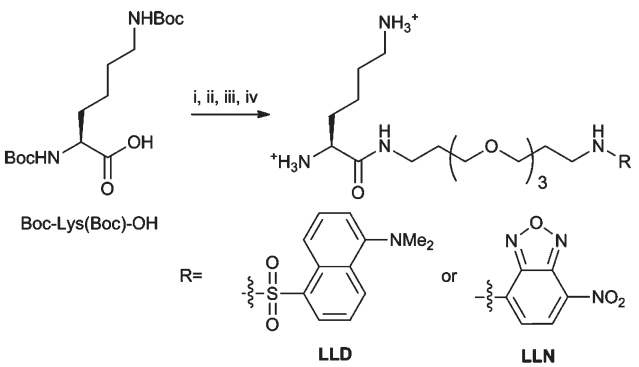
Two fluorescent lysine amide analogs, in which the carboxyl end of lysine was covalently attached to dansyl or NBD groups through an ethylene glycol-based linker, were rationally designed and synthesized. Both probes showed high binding affinity to the lysine riboswitch *in vitro* and their fluorescence intensities decreased by riboswitch binding.

Typically found in the 5′ untranslated regions (5′-UTRs) of certain bacterial mRNAs, riboswitches are the structured RNA molecules that can specifically bind to cellular metabolites and regulate the genes associated with the biosynthesis, transport, or degradation of the metabolites.[1] To date, more than 20 distinct classes of riboswitches have been found for many essential cellular metabolites including coenzymes, amino acids, nucleobases, sugars, and metal ions.[2] These recently characterized RNA molecules have attracted attention as potential antibiotic targets.[3] Lysine riboswitches, one of two amino acid-specific riboswitch classes, detect lysine within cells and repress the expression of the lysine biosynthesis and transport genes, which are essential for the cell wall biosynthesis and spore formation in bacteria. A few antibacterial lysine analogs that target lysine riboswitches have been reported in the literature.[4]

For the characterization of riboswitch–ligand interactions, fluorescence-based assays offer many advantages including highly sensitive and rapid measurements without radioactive labeling, the ability to measure multiple fluorescence properties (quenching, anisotropy, and resonance energy transfer), and parallel detection capability in microarray platforms.[5] Previously, the fluorescence quenching that occurs when a riboswitch binds to its intrinsically fluorescent ligand (*e.g.* riboflavin, 2-aminopurine) was monitored to study the riboswitch–ligand interactions.[6,7] However, only a few riboswitch ligands are intrinsically fluorescent and they often do not have the desired fluorescence properties. Therefore, fluorescent riboswitch binding probes were prepared by linking riboswitch ligands to various fluorescent dyes with the desired characteristics.

In order to design new fluorescent lysine riboswitch-binding probes, we examined the crystal structures of the *Thermotoga maritima* lysine riboswitch, which were independently reported by two research groups.[8,9] The structures of the lysine-bound riboswitch revealed two openings from the carboxylate and ε-amino groups of lysine in the binding pocket, which potentially could be modified without significantly reducing the riboswitch–ligand interactions.[8,9] In fact, L-homoarginine and N^6-1-iminoethyl-L-lysine, two structural analogs of lysine in which the ε-amino group is substituted by a slightly larger but still positively charged guanidinium or acetamidine group, respectively, also selectively bind to the lysine riboswitch *in vitro*.[4] Whereas no K^+ ion was found in one structure of the *T. maritima* lysine riboswitch, another crystal structure showed a K^+ ion bound to a carboxyl oxygen of lysine in the lysine-binding pocket. Nevertheless, the carboxyl group has been suggested as a potential site for modification, possibly by replacing the K^+ ion.[8] Indeed, it has been recently reported that carboxyl-modified lysine analogs including lysine amide, lysine hydroxamate and lysine ethyl ester are more effective than lysine for the *lysC* riboswitch-mediated transcription termination.[10]

This paper reports the synthesis of the rationally-designed fluorescent lysine riboswitch probes **LLD** and **LLN**, in which the lysine's carboxyl end is attached to a dansyl or an NBD group, respectively, through *O*,*O*′-bis(3-aminopropyl)diethylene glycol (BAPDG), an 18 Å long ethylene glycol-based linker containing three oxygen atoms (Scheme 1). This linker was specifically selected because it would be long enough to fit in the solvent-exposed narrow channel (about 14 Å long) next to the lysine



Scheme 1 (i) TSTU, iPr_2NEt, DMF; (ii) 4,7,10-trioxa-1,13-tridecanediamine, Et_3N, DMF, 69% in two steps; (iii) dansyl chloride, Et_3N, CH_2Cl_2, 50% or NBD-Cl, Et_3N, CH_2Cl_2, 30%; (iv) HCl, MeOH, 82% for **LLD** or 91% for **LLN**.

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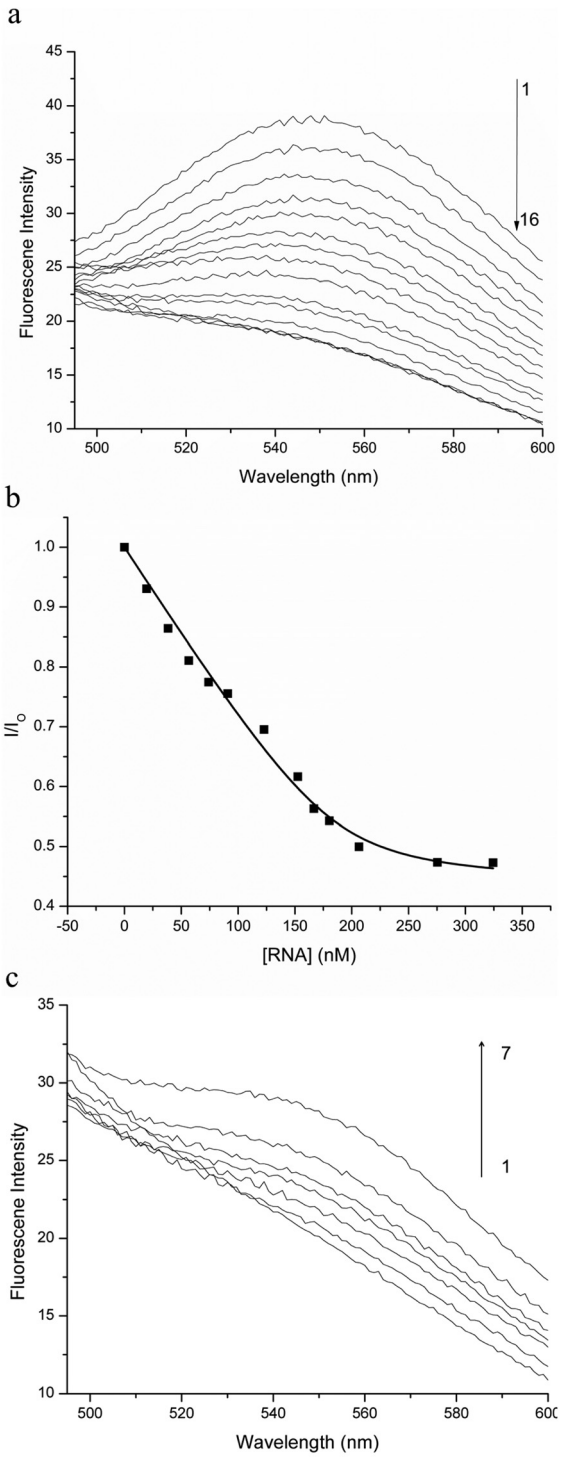

Fig. 1 (a) Fluorescence intensity of 200 nM **LLD** alone (1) and with 20 nM (2), 38 nM (3), 57 nM (4), 74 nM (5), 107 nM (6), 123 nM (7), 138 nM (8), 153 nM (9), 167 nM (10), 180 nM (11), 194 nM (12), 206 nM (13), 219 nM (14), 275 nM (15), and 324 nM (16) of the *lysC* ribowitch RNA. (b) Relative fluorescence intensity of 200 nM **LLD** as a function of increasing concentrations of the *lysC* ribowitch RNA. (c) Fluorescence intensity of 200 nM **LLD** in the presence of 500 nM *lysC* riboswitch RNA (1) and with 571 nM (2), 721 nM (3), 888 nM (4), 1.0 μM (5), 1.1 μM (6), and 1.2 μM (7) of lysine.

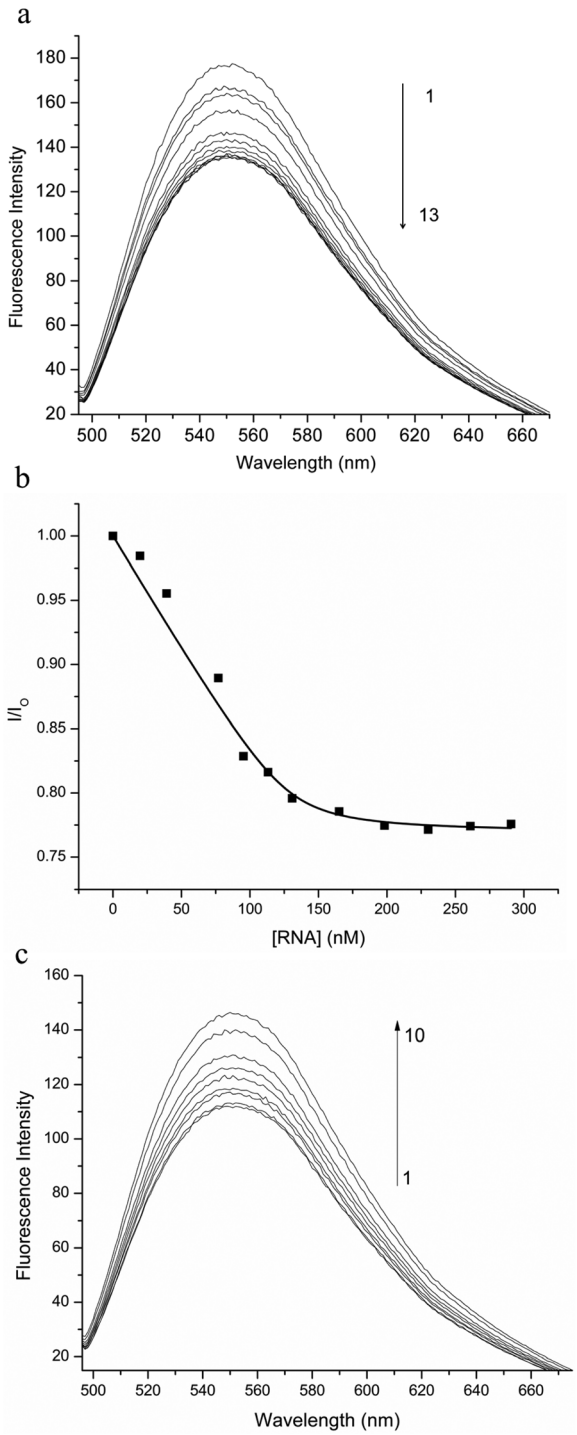

Fig. 2 (a) Fluorescence intensity of 200 nM **LLN** alone (1) and with 20 nM (2), 39 nM (3), 58 nM (4), 77 nM (5), 95 nM (6), 113 nM (7), 131 nM (8), 165 nM (9), 198 nM (10), 230 nM (11), 261 nM (12), and 291 nM (13) of the *lysC* riboswitch RNA. (b) Relative fluorescence intensity of 200 nM **LLN** as a function of increasing concentrations of the *lysC* ribowitch RNA. (c) Fluorescence intensity of 200 nM **LLN** in the presence of 500 nM *lysC* riboswitch RNA (1) and with 95 nM (2), 182 nM (3), 333 nM (4), 571 nM (5), 750 nM (6), 889 nM (7), 1.0 μM lysine (8), 1.1 μM (9), and 1.2 μM (10) of lysine.

carboxyl group and could also mimic three water molecules found within the presumably hydrophilic channel.[8]

LLD was easily prepared by the reaction of the *N*-hydroxylsuccinimidyl (NHS) ester of Boc-protected lysine with BAPDG and subsequent reaction with dansyl chloride followed by Boc-deprotection in methanolic HCl (Scheme 1). The 271-base *Escherichia coli lysC* riboswitch RNA was prepared by *in vitro* transcription of the corresponding DNA under control of the T7 promoter using T7 RNA polymerase.[11] The three dimensional structure of *E. coli lysC* riboswitch is not available. However, found in a broad range of bacterial species, the *lysC* riboswitches, also known as the L-box riboswitches, have highly conserved sequence and secondary structure, suggesting that their tertiary structure may be conserved as well.[12]

In order to examine whether **LLD** binds to the *E. coli lysC* riboswitch *in vitro*, the fluorescence intensity changes of **LLD** were monitored in the presence of varying concentrations of the *lysC* riboswitch. The fluorescence emission intensity of **LLD** decreased upon addition of the riboswitch RNA, indicating complex formation between the riboswitch and **LLD** (Fig. 1a). As shown in Fig. 1b, the data points fit well a curve obtained from an equation derived for 1 : 1 complex formation with a dissociation constant K_d = 7.8 ± 6 nM, a value at least 25-fold lower than the reported K_d for lysine (360 nM).[4] This result indicates an enhanced affinity of **LLD** to the lysine riboswitch compared to lysine. This increased affinity is probably due to the binding of the BAPDG linker to the solvent-exposed hydrophilic pocket at the carboxyl end of lysine.

In order to examine whether **LLD** competes with lysine for binding to the *lysC* riboswitch, the fluorescence intensity of 200 nM **LLD** in the presence of 500 nM *E. coli lysC* riboswitch was measured upon addition of lysine. As shown in Fig. 1c, the quenched fluorescence of **LLD** in the presence of the riboswitch RNA was recovered by increasing the concentration of lysine. The selectivity of **LLD** was also investigated by observing fluorescence intensity changes in the presence of increasing concentrations of the *E. coli thiM* riboswitch, which specifically recognizes thiamine pyrophosphate. As shown in Fig. S1,† little fluorescence intensity changes were observed for **LLD** even in the presence of 5 molar equivalents of the *thiM* riboswitch. These results clearly suggest that **LLD** does not non-specifically bind to the *thiM* riboswitch.

Due to the relatively low sensitivity of **LLD**, whose molar extinction coefficient at 328 nm was 200 M^{-1} cm^{-1}, **LLN** was also prepared by the same synthetic scheme as **LLD**. The molar extinction coefficient of **LLN** at 478 nm was 2000 M^{-1} cm^{-1}. Upon addition of the *lysC* riboswitch, the fluorescence intensity of **LLN** decreased as shown in Fig. 2a. Consistent with **LLD**, the data points fit well on the curve for a 1 : 1 binding stoichiometry with a K_d of 3.0 ± 2 nM (Fig. 2b). The quenched fluorescence of **LLN** in the presence of 500 nM *lysC* riboswitch RNA was recovered by increasing the concentration of lysine, clearly indicating that **LLN** also competes with lysine for binding to the *lysC* riboswitch (Fig. 2c). In the presence of 1.2 μM lysine, the fluorescence of **LLN** almost completely recovered to the level of the dye itself. When incubated with the *E. coli thiM* riboswitch, **LLN** also showed very little fluorescence intensity changes, indicating that **LLN** specifically binds to the *lysC* riboswitch (Fig. S2).†

In order to investigate the effect of **LLD** or **LLN** on the bacterial cell growth, the MG1655 *E. coli* cells were incubated on M9 minimal media plates containing 1 mM **LLD** or **LLN**. No inhibition of cell growth was observed, probably because these molecules were not transported into the cell. Evidently, the *E. coli* colonies on the media plates were not fluorescent whereas the media plate showed fluorescence from **LLD** or **LLN**. Therefore, *in vivo* studies with these molecules were not further pursued.

In summary, two fluorescent lysine riboswitch probes were rationally designed and synthesized. These probes will be useful for the high-throughput identification of new riboswitch modulators from small molecule libraries and for the analysis of lysine in complex chemical and biological samples *in vitro*.

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