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Bioorganic & Medicinal Chemistry Letters 16 (2006) 794–797
Bioorganic & Medicinal Chemistry Letters

Design, synthesis, and biological evaluation of BODIPY®–erythromycin probes for bacterial ribosomes

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Received 18 August 2005; revised 8 November 2005; accepted 8 November 2005
Available online 23 November 2005

Abstract—BODIPY®–erythromycin probes of bacterial ribosomes were designed and synthesized by attaching a BODIPY® fluorophore to the 4″- and 9-positions of the erythromycin structure. The probes exhibited excellent binding affinity to bacterial ribosomes and competed with erythromycin and other drugs whose binding sites are in the same vicinity of the 50S subunit. The synthetic fluorescent probe **5** was successfully adapted in our ultra high-throughput screening (uHTS) to identify novel ribosome inhibitors.

Bacterial resistance to antibiotics has become a serious public health issue. Novel classes of antibacterial agents that have distinct chemical structure and no cross-resistance with currently used agents are urgently needed. Interruption of bacterial ribosome function represents a proven way to kill bacteria. There are naturally occurring antibiotics and synthetic antibacterial agents that target bacterial ribosomes. A prominent group of ribosome inhibitors is the macrolide family, which comprises a large number of naturally occurring and semi-synthetic antibiotic agents, such as erythromycin, clarithromycin, and azithromycin.

Macrolides bind to the large subunit (50S) of bacterial ribosomes and prevent the elongation step of protein synthesis. The structures of 50S ribosomal subunits co-crystallized with seven separate macrolides have been reported.[1–3] These crystal structures revealed that all macrolides bind to the entrance of the peptide exit tunnel located in the peptidyl transferase reaction center of the 50S subunit. The macrolide binding site is defined by a series of contacts between residues of the domain V region of the 23S ribosomal RNA and the antibiotic molecule. Macrolides bound at this site effectively block the peptide exit tunnel, and erythromycin class macrolides do so without directly interfering with the catalytic activity of the peptidyl transferase reaction center. The erythromycin co-crystal structure indicates that there are no productive interactions between the ribosome and the 4″-, 6-, 9-, and 11-positions of the macrolide molecule. This is consistent with the available structure–activity relationships as all four positions tolerate the introduction of large substituent groups without significantly reducing the binding affinity.[4]

Fluorescence polarization is a powerful technique applicable to the probing of ribosome–ligand interactions. A fluorescent probe bound to a ribosome when excited with polarized light of appropriate wavelength emits light with enhanced polarization relative to that of an unbound probe. Hence, when a bound probe is competitively or allosterically displaced by a ligand the polarized light signal diminishes. The ligand thus identified is likely to interact with the ribosome in a similar fashion at or near the same binding site as the probe. Since the binding site is well validated, the ligand therefore serves as an attractive start-point for further optimization. An advantage of this screening methodology is that the approximate binding site of the identified novel ribosome ligand is immediately apparent, which can be used for structure-guided drug design and

Keywords: Antibacterial agent; Ribosome inhibitor; Macrolides; Erythromycin; Fluorescent polarization probes; BODIPY; Synthesis.
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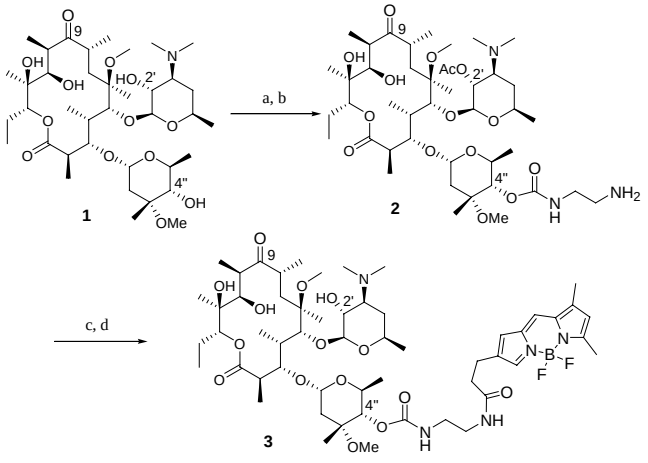
doi:10.1016/j.bmcl.2005.11.028

chemical optimization. Furthermore, the simple mixing of inexpensive reagents required for fluorescence polarization displacement assays means that they are readily adaptable for ultra high-throughput screening, unlike alternative methods that may require physical separation steps and/or expensive reagents.

Previous reports used fluorescent probes that covalently link fluorophores to ribosome inhibitors to probe inhibitor–ribosome interactions and to estimate the location of the inhibitor binding sites.[5–9] Some of those probes were also successfully used in uHTS to identify small molecules that interact with ribosomes. In an ongoing ribosome program, we desired to synthesize ribosome probes suitable for our ultra high-throughput screening needs. We settled on 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza,s-indacene (BODIPY-FL) as our fluorophore for two reasons. First, the relatively small size of BODIPY-FL should minimize the impact on binding affinity of the resulting macrolide-BODIPY probes to the ribosome. Second, BODIPY-FL has excitation and emission wavelengths (504 nm excitation and 513 nm emission in methanol, respectively) that are distinct from most compounds in our screening library. The distinct spectral properties should facilitate fluorescence anisotropy-based high-throughput screening. We report herein the synthesis and biological evaluation of three fluorescent probes (**3**, **5**, and **7**) which have BODIPY fluorophore tethered to the 4″- or 9-position of the erythromycin structure through different linker groups.

The preparation of probe **3** commenced with 6-*O*-methyl -erythromycin (clarithromycin) **1** (Scheme 1).[10] The 2′-hydroxy group was selectively protected by an acetate group. The 4″-hydroxy group was selectively reacted with 1,1′-carbonyldiimidazole (CDI), followed by addition of ethylenediamine in situ to generate intermediate **2**. Compound **2** was then coupled with BODIPY-FL propionic acid in the presence of succinimidyl ester followed by the deprotection of the acetate group to give the desired BODIPY-fluorescent probe **3**.[11]



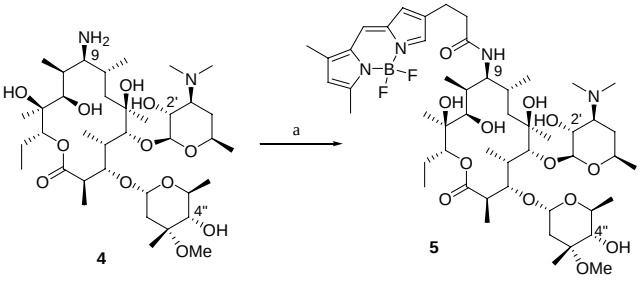
Scheme 1. Preparation of probe **3**. Reagents and conditions: (a) 1.0 equiv Ac_2O, 3.0 equiv Et_3N, CH_2Cl_2, rt, 16 h; (b) 1.0 equiv CDI, THF, 35 °C, 12 h, then added 10 equiv $NH_2CH_2CH_2NH_2$, 45 °C, 1 h; (c) BODIPY-FL propionic acid, succinimidyl ester, DMF, rt, 1 h, 34% from compound **1**; (d) MeOH, 60 °C, 1 h, 33%.

Fluorescent probe **5** was prepared by tethering BODIPY fluorophore to the 9-position of 9-aminoerythromycin **4** (Scheme 2), which in turn was prepared from erythromycin A by a known process described in the literature.[12] Treatment of **4** with BODIPY-FL propionic acid in the presence of succinimidyl ester produced the desired probe **5**.

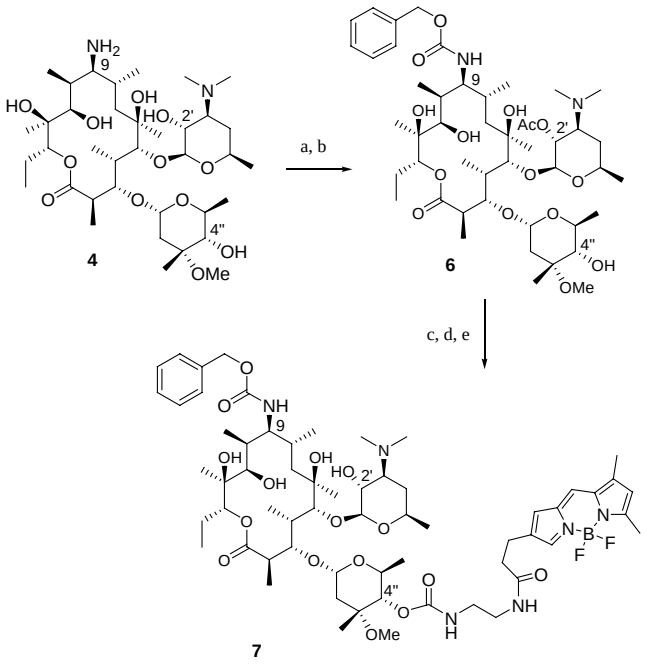
BODIPY probe **7** is a version of probe **3** (Scheme 3) optimized to be more useful in screening due to an increase in the ribosomal off rate as described below. The synthesis involved protecting the amine group of compound **4** with benzyl carbamate and otherwise followed the similar synthesis described for the preparation of fluorescent probe **3**.

Biological evaluation of all three BODIPY probes **3**, **5**, and **7** included determination of their binding affinity to 70S bacterial ribosomes isolated from *Escherichia coli* using a method reported by Turconi et al.[8] As shown in Table 1, binding affinities (K_d) to the ribosome vary from less than 0.5 nM for probe **3** to 30 nM for probe **5**. The excellent binding affinity of probe **3** may be attributable to the BODIPY-fluorophore attachment point. The 4″-position of erythromycin seems to tolerate large groups without detrimental effect. This finding was reinforced by probe **7** where position 9 was modified while maintaining the large BODIPY fluorophore linked to the 4″-position, yet the affinity of probe **7** was only slightly affected when compared to probe **3**. However, attaching BODIPY fluorophore to the 9-position led to probe **5** with weaker (but still potent) binding affinity for the ribosome. Only probes with appropriate binding affinities and kinetics are useful in a FP displacement assay. Higher affinity probes allow greater resolution of ligand potency and require less assay reagents,[13] but they may also result in slower displacement binding kinetics or in low signal as probe levels should ideally not be significantly above the probe K_d.

Inhibition of ribosome function mediated by erythromycin and the synthesized probes **3**, **5**, and **7** was evaluated in a coupled transcription and translation assay using *E. coli* S30 extracts by measuring inhibition of light production based on expression of a luciferase



Scheme 2. Preparation of probe **5**. Reagents and conditions: (a) 1.0 equiv BODIPY-FL propionic acid, 1.0 equiv succinimidyl ester, DMF, rt, 1 h, 62%.



Scheme 3. Preparation of probe **7**. Reagents and conditions: (a) 1.0 equiv *N*-(benzyloxycarbonyloxy)succinamide, DMF; (b) 1.2 equiv Ac_2O, 4.0 equiv Et_3N, CH_2Cl_2, rt, 16 h; (c) 2.0 equiv CDI, THF, 35 °C, 12 h, then added 10 equiv $NH_2CH_2CH_2NH_2$, 45 °C, 1 h; (d) 1.0 equiv BODIPY-FL propionic acid, 1.0 equiv succinimidyl ester, DMF, rt, 1 h, 48% from 1; (e) MeOH, 60 °C, 1 h, 42%.

Table 1. Binding constants and inhibition IC_{50}s of fluorescent probes

Compound	Ribosome binding K_d (nM)	Transcription and translation IC_{50} (μM)
Erythromycin	—	0.08
3	<0.5	0.16
5	30	1.5
7	1.5	0.55

reporter.[14] As shown in Table 1, probe **3** with tight binding affinity exhibited a roughly equivalent inhibition when compared to erythromycin. Similarly, the lower affinity probes **5** and **7** showed reduced inhibition accordingly.

We undertook competitive binding studies to demonstrate that the synthesized probes bind to the bacterial ribosome in a biologically relevant manner, using methods similar to those previously described.[8] Erythromycin competed off all three probes with potent IC_{50}s. However, in fluorescence polarization competition assays, the potency of an inhibitor that binds to ribosomes more tightly than the assay probe itself cannot be determined but rather is limited by the affinity of the probe.[13] For example, the observed IC_{50} of erythromycin using probe **5** was 30 nM, corresponding to the probe affinity. The binding site for lincosamides such as clindamycin overlaps with the macrolide binding site.[1] Displacement of probe **5** or **7** by clindamycin yielded identical IC_{50} values of 6 μM (corresponding to a $K_i = 2$ μM), in reasonable agreement with the published literature.[15] Finally, in high-throughput screening with probe **5**, a variety of macrolides and ribosome inhibitors with adjacent binding sites that were present in the screening library were identified as hits, while known inhibitors that bind to more distal ribosome sites were silent. These data further validate the nature and utility of these BODIPY-FP probes as tools in the discovery of novel ligands for bacterial ribosomes.

As indicated above, the binding affinity and kinetics are important for a fluorescent probe. Although probe **3** demonstrated the best binding affinity, the competitive ligand exchange rate was slow and required up to two days incubation. Fluorescent probe **7**, with an additional group at the 9-position, also showed strong binding affinity and slow kinetics requiring an overnight incubation for full displacement under the conditions in which it was employed in high-throughput screening. However, fluorescent probe **5**, with a moderate binding affinity and shorter ligand exchange time (about 1 h), was found to be ideal for use in high-throughput screening. Probe **5** was successfully used to identify a series of novel ribosome ligands that competitively or allosterically displace the fluorescent probe from the bacterial ribosome. This result will be reported in elsewhere (Roche et al., manuscript in preparation).

In summary, macrolides with BODIPY-FP fluorophore groups attached at either the 4″- or 9-positions were designed, synthesized, and evaluated. The binding specificity of the prepared BODIPY-FP probes to the product exit channel of the peptidyl transferase reaction center of the ribosome was verified through competitive binding studies that employed erythromycin and other antibiotics known to bind in the same location of the 50S subunit. Finally, erythromycin-BODIPY-FL probe **5** was found to be ideal for use in an ultra high-throughput screen to identify novel small molecules that bind to the bacterial ribosome. These small molecules are being evaluated as potential start-points for chemistry optimization efforts toward the development of a novel antibacterial drug.

References and notes

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