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A Fluorescent Probe for the 70S-Ribosomal GTPase-Associated Center

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Resistance against commonly prescribed antibiotics constitutes a constant challenge for human healthcare.[1] The need for cures effective against multiply resistant pathogens has prompted a search for novel antibiotic targets and lead structures.[2] One validated target as yet unused for human therapy is the so-called GTPase-associated center of the bacterial ribosome.[3] For this target, high-affinity ligands of the thiopeptide antibiotic class are available from nature,[4] among them thiostrepton (**1**, Scheme 1), nosiheptide (**2**), and micrococcin (**3**). Thiostrepton (**1**) has been shown to bind with high affinity between the ribosomal protein L11 and the 23S rRNA,[4,5,6] thereby blocking peptide translocation at the 70S ribosome.[7–10] Beyond its ensuing strong antibacterial activity on Gram-positive organisms,[4,7] **1** has also been reported to display potency against the malaria parasite[11] and selected tumor cell lines,[12,13] and to have immunosuppressant properties.[14] Here we describe a fluorescent high-affinity probe derived from **1** that allows the interaction of thiopeptides with their target to be quantified.

Promising sites for the requisite semisynthetic modification[15] of **1** could be the dehydroalanine Michael acceptors.[16] We found that cropping of the dehydroamino acid tail[17,18] of **1** was crucial to allow clean transformations. With Et_2NH in $CHCl_3$ the compounds **4** or **5** could be obtained on preparative scale after simple flash chromatography (Scheme 1). To generate a highly fluorescent and water-soluble probe by sulfa-Michael addition, fluorescein isothiocyanate (FITC) was now appended with an oligoethylene glycol spacer terminated with mercaptopropionic acid (see the Supporting Information). A protic solvent and slightly basic conditions were essential for a clean sulfa-Michael addition of this thiol to **4**; trifluoroethanol (TFE) gave the best results. The attachment occurred selectively on the terminal dehydroalanine residue to deliver probe **6** (32% after prep. HPLC, Scheme 2), which was freely soluble in aqueous buffer.[19] Furthermore, the optimized reaction conditions also allowed access to a variety of thiostrepton derivatives (**7**–**11**) modified at the dehydroalanine terminus, through the use of readily available thiols. Diasteromeric mixtures were obtained after HPLC purification (26–56% yields, Table 1) in all cases, with the exception of the octyl derivatives **8a** and **b**, for which separation could be achieved.

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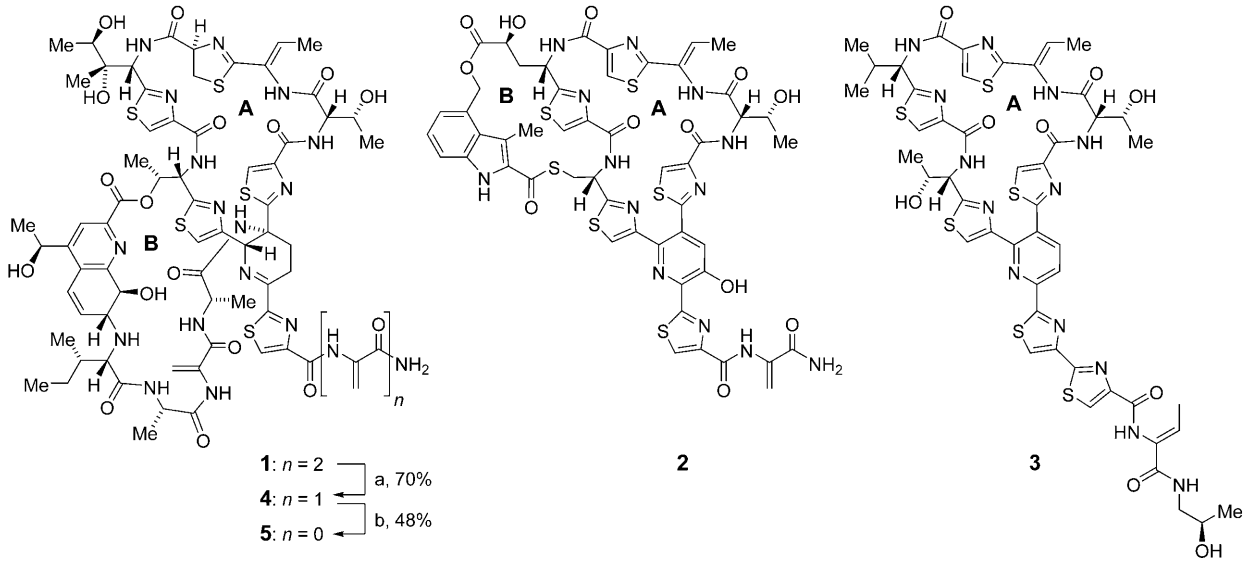
The binding of the fluorescent probe **6** to the L11/23S rRNA target[5] was then evaluated by fluorescence anisotropy measurements (Figure 1, Table 1).[20] In contrast with earlier reports on thiostrepton (**1**),[7b,21,22] any significant binding of the probe **6** to the individual components[5] of the L11/RNA complex could be ruled out (Scheme 3, paths A and B), because probe anisotropy remained unaffected by L11 protein or matching RNA alone (each $K_D > 10\ \mu M$). Strong binding was evident, however, in the presence of the composite L11/RNA complex (Figure 1).

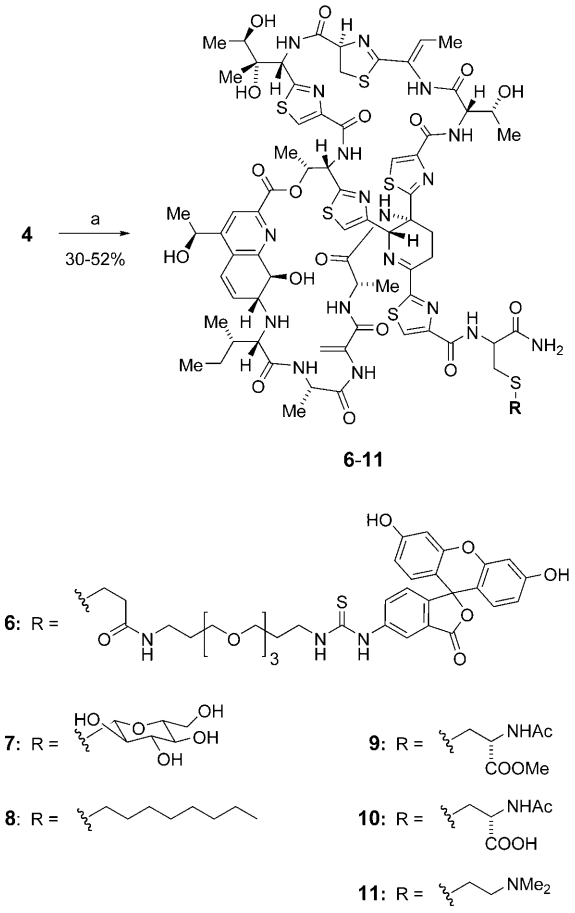
It could be shown that the arising scenario can be described analytically (see the Supporting Information) and could then be evaluated by least-squares fitting. This procedure allowed us directly to determine dissociation constants in the high-affinity regime ($K_D < 1$ nM) for protein and ligand (Figure 1A). All measurements were adjusted to 384-well plates and conducted in quintuplicate. The protein dissociation constant (K_{D1}) obtained in this way was validated independently by gel-shift experiments (17.7 ± 2.5 nM; see the Supporting Information) and was found to be identical with the anisotropy-derived result within experimental error. For probe **6** we determined a dissociation constant of $K_{D2} = 140 \pm 80$ pM, which is an extraordinarily tight binding event for an RNA ligand.

We then used the fluorescent probe **6** to assess binding of other thiopeptides by displacement titrations,[23] among them thiostrepton (**1**), nosiheptide (**2**), micrococcin (**3**), and the synthetic thiostrepton derivatives **4**–**11** (Table 1 and Figure 1B).[24] The K_D determined for thiostrepton (**1**) under homogenous equilibrium conditions with *T. thermophilus* L11 refines earlier nM estimates.[7,21,22] Nosiheptide (**2**) was found to bind twice as tightly as thiostrepton (**1**), whereas micrococcin (**3**) is an order of magnitude weaker.[4] Interestingly, modifications of the dehydroaminoacid tail of **1**—including truncation (**4**, **5**) and/or attachment of hydrophilic/moderately polar (**6**, **7**, **9**), anionic (**10**), or cationic substituents (**11**)—were found to be broadly tolerated. A lipophilic chain (**8a** and **b**), however, seems to be detrimental, as evidenced by an approximately 20-fold affinity loss. The stereochemistry of the addition product does not seem to have major influences on ligand binding (**8a** and **8b**). These data corroborate the supposition that the dehydro-Ala tail is not crucially involved in binding interactions with the target, consistent with recent studies by proximity-induced covalent capture (PICC)[5] and X-ray crystallography.[6]

Growth inhibition studies were then conducted with methicillin-susceptible and methicillin-resistant *S. aureus* (MSSA and MRSA). Considerable potency was observed overall, and both strains were equally challenged (Table 1). The activity in cell culture deviated from the biophysical binding data (*T. thermophilus*), but the trend in the natural products **1**–**3** was reflected well. The remarkable potency of nosiheptide[25] (**2**) against MSRA in cell culture is noteworthy and correlates with its su-



Scheme 1. Chemical structures of thiostrepton (**1**), nosiheptide (**2**), and micrococcin (**3**), and selective chemical truncation of the C-terminal dehydroalanine amino acids. Similar A-rings and divergent B-rings are marked. a) 10% $HNEt_2$ in $CHCl_3$, 3 h; b) 10–20% $HNEt_2$ in $CHCl_3$, 48 h.



Scheme 2. Preparation of the PEG-fluoresceine-tagged probe **6** and semisynthetic thiostrepton derivatives **7**–**11** through sulfa-Michael additions. a) HSR, NEt_3, TFE/H_2O, pH 9, 2–48 h.

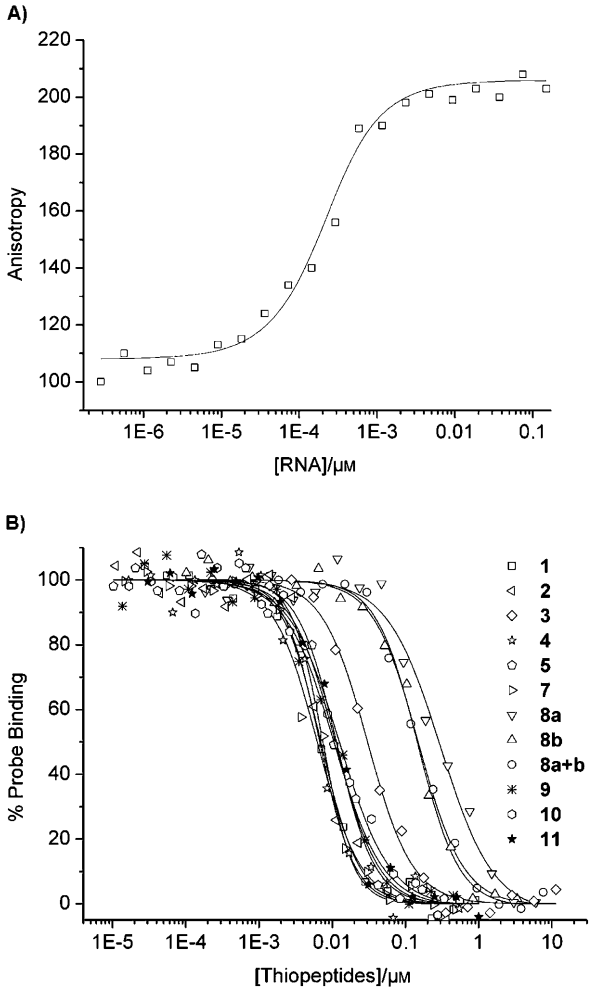
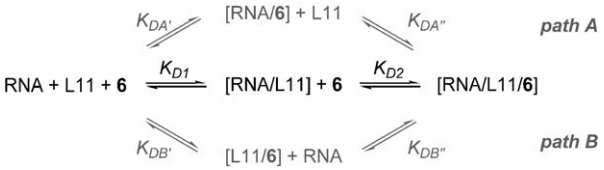


Figure 1. A) Representative fluorescence anisotropy titration curve for probe **6** (5 nM) and L11 protein (0.6 μM) fitted with the analytical solution for the coupled equilibrium (see the Supporting Information). B) Displacement titrations of probe **6** (5 nM) from the RNA–protein complex (5 nM RNA, 0.6 μM L11) with ligands **1**–**5** and **7**–**12**, fitted with the Hill equation.

Table 1. Chemical derivatization yields, affinities to the 23S rRNA (res. 1051–1109 *E. coli*)/L11 complex (*T. thermophilus*), and growth inhibition data for *S. aureus* strains.

Compound	Yield[a] [%]	K_{D2}[b] [nM]	MIC[c] [μM]	MIC[d] [μM]
probe **6**	32	0.14 ± 0.08	–	–
thiostrepton (**1**)	–	0.20 ± 0.05	0.0065	0.013
nosiheptide (**2**)	–	0.11 ± 0.02	0.00063	0.00063
micrococcin (**3**)	–	1.73 ± 0.90	0.34	0.42
4	70	0.19 ± 0.01	0.063	0.063
5	48	0.32 ± 0.05	0.013	0.013
7	56	0.22 ± 0.06	2.0	2.0
8a	6[e]	3.59 ± 1.20	0.30	0.30
8b	6[e]	5.41 ± 2.03	3.2	3.2
9	32	0.32 ± 0.08	0.67	0.41
10	42	0.32 ± 0.04	3.2	1.6
11	26	0.44 ± 0.11	0.37	0.37

[a] From **4**. [b] Data determined by displacement of probe **6** are apparent values. [c] MSSA strain (ATCC 25923). [d] MRSA strain (ATCC 43300). [e] After stringent separation by multiple prep. HPLC, yield of **8a**/**8b** mixture: 32%.



Scheme 3. Complex formation between the bacterial 23S rRNA (res. 1051–1109 *E. coli*, **RNA**), *T. thermophilus* L11 protein (**L11**), and probe **6** under equilibrium conditions. Paths A and B were experimentally not observed.

preme affinity to the molecular target. Removing the dehydroamino acids from **1** preserved the activity of the compound (**4** and **5**). For the derivatives **7**–**11** the activity on cells was found to be somewhat reduced. It should be noted, however, that these data (0.7–5 mg L^{-1}) still compare well with the activities of common antibiotics (e.g., cefoxitine or linezolide: 1–8 mg L^{-1}) against these strains. The increased solubility of **7** (>100 times higher than **1**) did not lead to improved cellular activity, whereas the lower target affinity of **8** did not reduce it further. However, stereochemistry was found to be influential in cells (**8a** versus **8b**). A cationic (**11**) appendage seems somewhat beneficial, but the influence is not strong (cf. compounds **9** and **10**). Overall, we reason that the attachments affect uptake or metabolism in the bacterial cell in a complex fashion, and might therefore be used to tailor specific compound profiles in the future.

In summary, we have quantified ligand binding at the 70S-ribosomal GTPase-associated center with the aid of a fluorescent small molecule probe (**6**) derived from commercially available thiostrepton (**1**). In the quest for novel antiinfectives, such a probe would be expected to enable focused screening setups,[26] which we have explored here with novel thiostrepton derivatives. Initial studies on their antibiotic activities against *S. aureus* cell cultures indicate that potent and soluble thiopeptides can be obtained. Design parameters for in vivo activity of semisynthetic thiostrepton antibiotics will be subject to further investigation.

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Keywords: antibiotics · antitumor agents · drug design · ribosome · RNA binders · thiopeptides

[1] a) A. Coates, Y. Hu, R. Bax, C. Page, *Nat. Rev. Drug Discovery* **2002**, *1*, 895–910; b) D. M. Livermore, *Clin. Microbiol. Infect.* **2004**, *10*, Suppl. 4, 1–9.
[2] a) E. D. Brown, G. D. Wright, *Chem. Rev.* **2005**, *105*, 759–774; b) F. von Nussbaum, M. Brands, B. Hinzen, S. Weigand, D. Häbich, *Angew. Chem.* **2006**, *118*, 5194–5254; *Angew. Chem. Int. Ed.* **2006**, *45*, 5072–5129.
[3] D. N. Wilson, K. H. Nierhaus, *Crit. Rev. Biochem. Mol. Biol.* **2005**, *40*, 243–267.
[4] a) M. C. Bagley, J. W. Dale, E. A. Merritt, X. Xiong, *Chem. Rev.* **2005**, *105*, 685–714; b) R. A. Hughes, C. J. Moody, *Angew. Chem.* **2007**, *119*, 8076–8101; *Angew. Chem. Int. Ed.* **2007**, *46*, 7930–7954.
[5] S. Baumann, S. Schoof, S. D. Harkal, H.-D. Arndt, *J. Am. Chem. Soc.* **2008**, *130*, 5664–5666.
[6] J. M. Harms, D. N. Wilson, F. Schluenzen, S. R. Connell, T. Stachelhaus, Z. Zaborowska, C. M. T. Spahn, P. Fucini, *Mol. Cell* **2008**, *30*, 26–38.
[7] a) E. Cundliffe, *Biochem. Biophys. Res. Commun.* **1971**, *44*, 912–917; b) E. Cundliffe in *The Ribosome: Structure, Function and Evolution* (Eds.: W. E. Hill, A. Dahlberg, R. A. Garrett, et al.), American Society for Microbiology, Washington, DC, **1990**, pp. 479–490.
[8] Y. Xing, D. E. Draper, *Biochemistry* **1996**, *35*, 1581–1588.
[9] M. V. Rodnina, A. Savelsbergh, V. I. Katunin, W. Wintermeyer, *Nature* **1997**, *385*, 37–41.
[10] a) B. T. Porse, I. Leviev, A. S. Mankin, R. A. Garrett, *J. Mol. Biol.* **1998**, *276*, 391–404; b) M. V. Rodnina, A. Savelsbergh, N. B. Matassova, V. I. Katunin, Y. P. Semenkov, W. Wintermeyer, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9586–9590.
[11] a) G. A. McConkey, M. J. Rogers, T. F. McCutchan, *J. Biol. Chem.* **1997**, *272*, 2046–2049; b) S. Chaubey, A. Kumar, D. Singh, S. Habib, *Mol. Microbiol.* **2005**, *56*, 81–89.
[12] a) K. Jounghee, WO2002066046, **2002**; b) B. D. Bowling, N. Doudican, P. Manga, S. J. Orlow, *Canc. Chemother. Pharmacol.* **2008**, *63*, 37–43; c) U. G. Bhat, P. A. Zipfel, D. S. Tyler, A. L. Gartel, *Cell Cycle* **2008**, *7*, 1851–1855.
[13] K. C. Nicolaou, M. Zak, S. Rahimipour, A. A. Estrada, S. H. Lee, A. O'Brate, P. Giannakakou, M. R. Ghadiri, *J. Am. Chem. Soc.* **2005**, *127*, 15042–15044.
[14] M. Ueno, S. Furukawa, F. Abe, M. Ushioda, K. Fujine, S. Johki, H. Hatori, J. Ueda, *J. Antibiot.* **2004**, *57*, 590–596.
[15] For selected semisynthesis on other thiopeptides see: a) P. Hrnciar, Y. Ueda, S. Huang, J. E. Leet, J. J. Bronson, *J. Org. Chem.* **2002**, *67*, 8789–8793; b) B. N. Naidu, M. E. Sorenson, J. J. Bronson, M. J. Pucci, J. M. Clark, Y. Ueda, *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2069–2072.
[16] a) U. Schmidt, E. Öhler, *Angew. Chem.* **1976**, *88*, 54; *Angew. Chem. Int. Ed. Engl.* **1976**, *15*, 42; b) Y. Zhu, W. A. van der Donk, *Org. Lett.* **2001**, *3*, 1189–1192; c) D. P. Galonic, W. A. van der Donk, D. Y. Gin, *Chem. Eur. J.* **2003**, *9*, 5997–6006; d) A review on asymmetric *sulfa*-Michael reactions: D. Enders, K. Lüttgen, A. A. Narine, *Synthesis* **2007**, 959–980.
[17] A. Regueiro-Ren, Y. Ueda, *J. Org. Chem.* **2002**, *67*, 8699–8702.
[18] K. C. Nicolaou, M. Zak, B. S. Safina, A. A. Estrada, S. H. Lee, M. Nevalainen, *J. Am. Chem. Soc.* **2005**, *127*, 11176–11183.

[19] For **1** we determined solubilities of 3 μM in standard PBS buffer and 5 μM in H_2O. This agrees with ref. [22].
[20] a) W. J. Checovich, R. E. Bolger, T. Burke, *Nature* **1995**, *375*, 254–256; b) A. J. Pope, U. M. Haupts, K. J. Moore, *Drug Discovery Today* **1999**, *4*, 350–362; c) M. H. A. Roehrl, J. Y. Wang, G. Wagner, *Biochemistry* **2004**, *43*, 16056–16066.
[21] a) P. C. Ryan, M. Lu, D. E. Draper, *J. Mol. Biol.* **1991**, *221*, 1257–1268; b) D. E. Draper, Y. Xing, L. Laing, *J. Mol. Biol.* **1995**, *249*, 231–238.
[22] S. L. Bausch, E. Poliakova, D. E. Draper, *J. Biol. Chem.* **2005**, *280*, 29956–29963.
[23] Other examples: a) A. L. Rodriguez, A. Tamrazi, M. L. Collins, J. A. Katzenellenbogen, *J. Med. Chem.* **2004**, *47*, 600–611; b) F. Stricher, L. Martin, P. Barthe, V. Pogenberg, A. Mechulam, A. Menez, C. Roumestand, F. Veas, C. Royer, C. Vita, *Biochem. J.* **2005**, *390*, 29–39.
[24] H.-D. Arndt, S. Schoof, S. Baumann, B. Ellinger, DE 10 2008 014 485.1, **2008**.
[25] F. Benazet, M. Cartier, J. Florent, C. Godard, G. Jung, J. Lunel, D. Mancy, C. Pascal, J. Renault, P. Tarridec, J. Theilleux, R. Tissier, M. Dubost, L. Ninet, *Experientia* **1980**, *26*, 414–416.
[26] H.-D. Arndt, S. Schoof, S. Baumann, DE 10 2008 014 487.8, **2008**.

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