DOI: 10.1002/cbic.201000177

A Modular Approach to Synthetic RNA Binders of the Hepatitis C Virus Internal Ribosome Entry Site

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Natural products that target the RNA components of bacterial ribosomes and thereby act as antibiotics that shut down microbial protein synthesis, have provided a rich source of inspiration for the design and synthesis of small-molecule ligands directed at RNA targets.[1,2] A prominent example of a privileged scaffold for RNA recognition occurring in natural aminoglycoside antibiotics is 2-deoxystreptamine (2-DOS),[3] which contains a rigid framework of hydrogen-bond donors among which the rigid *cis*-1,3 arrangement of amino groups is responsible for selective interaction with structural motifs in RNA targets (Figure 1).[4] In an approach to reduce the complexity of chemical library synthesis that involves the highly functionalized 2-DOS scaffold, we have recently developed the 3,5-diaminopiperidine heterocycle (DAP) as a structural mimetic of the RNA-recognizing pharmacophore of the 2-DOS scaffold (Figure 1). Structure-guided design had been applied to discover a series of antibacterial DAP-triazine derivatives that act on the same ribosomal RNA target as the natural aminoglycoside antibiotics, which initially served as the inspiration for the conception of the DAP compounds.[5,6]

Here, we describe the synthesis of a novel class of modular ligands (**1**) that contain the DAP scaffold as the key moiety for RNA recognition in nonribosomal targets (Figure 1).[7] Screening of modular DAP ligands against the subdomain IIa, an RNA target in the internal ribosome entry site (IRES) of hepatitis C virus (HCV) (Figure 1), revealed a set of N-amido substituted α-amino acid conjugates of DAP (**2**) as micromolar binders of this RNA. We had previously shown that ligand-induced conformational change in the subdomain IIa RNA disrupts the function of the IRES and blocks viral protein synthesis; this ultimately leads to inhibition of HCV in infected cells.[8]

The common building block **4** used in the synthesis of the modular DAP compounds **2** was obtained from 2-chloro-3,5-dinitro-pyridine (**3**) following an established route,[9] which required, in the last step, high-pressure hydrogenation to reduce the pyridine (Scheme 1). As the yield in this transformation critically depends on the hydrogen pressure (>1000 psi), we sought to employ an alternative reduction procedure. Among the explored methods, neither transformation of the pyridine

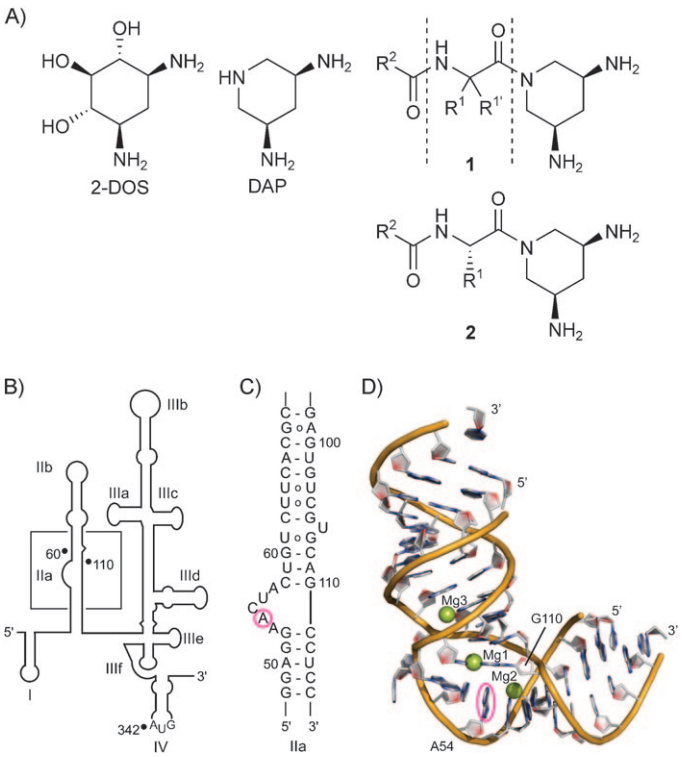
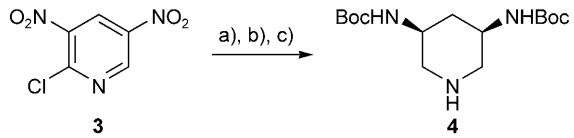


Figure 1. A) Structures of RNA-binding scaffolds 2-deoxystreptamine (2-DOS) and 3,5-diaminopiperidine (DAP) as well as modular RNA binders, **1**[7] and **2** that contain the DAP moiety. B) Secondary structure of the internal ribosome entry site (IRES) of the hepatitis C virus (HCV).[19] The position of the subdomain IIa target is indicated by a box. C) Secondary structure of the IRES subdomain IIa. D) Three-dimensional structure of the subdomain IIa RNA as determined by X-ray crystallography.[13] Mg^{2+} ions are shown as spheres. For a ligand binding assay used here, the A54 residue has been replaced by a fluorescent 2-aminopurine.



Scheme 1. Reagents and conditions: a) Pd/C, H_2, MeOH, RT, 48 h, quant.; b) $(Boc)_2O$ (4 equiv), Et_3N (2 equiv), MeOH, 0 °C→RT, 12 h, 70%; c) AcOH (1 equiv), Rh/C (wet, 5 mol%), H_2 (1600 psi), MeOH, 110 °C, 24 h, 54%. Boc = *tert*-butoxycarbonyl.

to an N-oxide followed by ammonium formate treatment[10] nor reduction by lithium triethylborohydride[11] afforded the desired piperidine, which led us to pursue the original route to the building block **4**. Synthesis of the DAP compounds **2** commenced with coupling of **4** with N-Cbz and side-chain protected α-L-amino acids **5** (Scheme 2). Commercially available pro-

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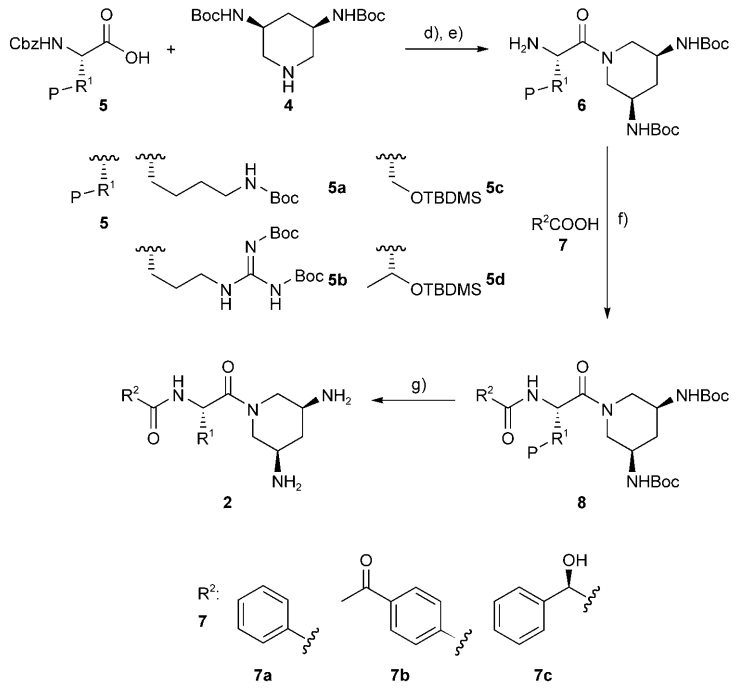
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201000177.



Scheme 2. Reagents and conditions: a) Et_3N (7 equiv), HATU (1.1 equiv), HOAT (1.2 equiv), CH_2Cl_2, 0 °C→RT, 5 h, 96%; b) Pd/C, H_2, MeOH, RT, 24 h; c) **7** (1 equiv), Et_3N (7 equiv), HATU (1.1 equiv), HOAT (1.2 equiv), CH_2Cl_2, 0 °C→RT, 3.5 h, 70% (2 steps); d) deprotection; for **5a** = N-Cbz-L-Lys(Boc) and **5b** = N-Cbz-L-Arg(Boc)$_2$: HCl/dioxane (4 M), MeOH (1:2 *v/v*), 0 °C→RT, 5 h, 50% after RP HPLC; for **5c** = N-Cbz-L-Ser(TBDMS) and **5d** = N-Cbz-L-Thr(TBDMS): TBAF (3 equiv), THF, 0 °C→RT, 4 h; then Boc deprotection as above. Cbz = carboxybenzyl; HATU = 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAT = 1-hydroxy-7-aza-benzotriazole; TBAF = tetra-n-butylammonium fluoride; TBDMS = *tert*-butyldimethylsilyl; THF = tetrahydrofuran. Spectroscopic characterization of the final products **2** is provided in the Supporting Information.

tected amino acids were used except for fully Boc-protected L-arginine, which was synthesized by alkylation of N-Cbz-L-ornithine with *N*,*N*′-Boc$_2$-(*S*)-methylisothiourea.[12] Selective deprotection of the coupling products gave DAP-amino acid conjugates **6**, which were further treated with carboxylic acids **7** to furnish the final products **2** after deprotection of the intermediates **8** and HPLC purification.

RNA binding of the modular DAP compounds **2** was tested in two different fluorescence assays that we had previously developed for the subdomain IIa target in the HCV IRES. In the first assay, an oligonucleotide construct was used in which an adenine residue at position 54 in the internal loop of IIa (Figure 1) is replaced by the fluorescent base analogue 2-aminopurine (2AP) as a sensitive probe of the RNA folding state.[13] An increase of the fluorescence intensity upon addition of a ligand to IIa-2AP54 indicates unfolding, or destabilization, of the internal loop, while a signal decrease suggests further compaction of the RNA fold or, more likely, additional shielding of the 2AP label by a bound ligand. The second assay, which is based on fluorescence resonance energy transfer (FRET) between cyanine dyes attached at the termini of a IIa oligonucleotide construct,[8] was used to measure the impact of ligand binding on the interhelical angle between the base paired stems as a probe of the RNA conformation at the internal loop. A decrease in FRET intensity signals a larger distance between the stem termini, which follows from an increase in the interhelical angle in IIa. We have recently used this assay to demonstrate that a benzimidazole inhibitor of HCV IRES-driven translation acts by such conformational induction at the IIa target.[8]

Titrations of 2AP54 labeled IIa RNA with modular DAP compounds resulted in fluorescence quenching at low micromolar concentrations for three ligands (**2ab**, **2bb**, and **2cb**), all of which contained a 4-acetylbenzoic acid moiety (Table 1). None of the other tested compounds showed an appreciable change of 2AP fluorescence. The dose-dependent decrease of 2AP fluorescence observed for the 4-acetylbenzoic acid DAP derivatives (Figure 2) suggested that these compounds bind to the IIa RNA at the internal loop region, where 2AP54 is located, and thereby shield the fluorescent label from solvent exposure. The fact that a residual signal was observed even at the highest concentrations tested suggested that the 2AP labeled target remained in solution and that the fluorescence decrease was not due to nonspecific RNA aggregation. Binding was in competition with salt; this indicates electrostatic contributions to the ligand-RNA interaction (Figure 2). All titrations were performed in a background of 100 μM Mg^{2+} to ensure that the IIa RNA was properly folded. Our previous studies on the three-dimensional structure and metal binding of the IIa target revealed two tightly bound Mg^{2+} ions in close proximity of 2AP54; these ions are essential for the stabilization of the L-shaped RNA fold (Figure 1). For the DAP ligands that showed binding to the IIa target, the affinity was substantially reduced in the presence of 1 mM Mg^{2+} (Table 1); this suggests direct competition with one, or both, of the structural Mg^{2+} ions. Frame-

Table 1. Structure–activity relationships for modular DAP compounds.

Compound	Component		2AP assay[a] EC_{50} [μM] [μM]		FRET assay[b] I_{FRET}	
2	**5**	**7**	100 μM Mg^{2+}	1 mM Mg^{2+}	100 μM	1 mM
2aa	**5a**	**7a**	n.a.	n.d.	n.a.	n.a.
2ab	**5a**	**7b**	6.3 ± 0.6	270 ± 100	n.a.	1.3
2ac	**5a**	**7c**	n.a.	n.d.	n.a.	n.a.
2ba	**5b**	**7a**	n.a.	n.d.	n.a.	n.a.
2bb	**5b**	**7b**	7.2 ± 1.2	92 ± 30	n.a.	1.1
2cb	**5c**	**7b**	68 ± 8	n.a.	n.a.	n.a.
2cc	**5c**	**7c**	n.a.	n.d.	n.a.	n.a.
2dc	**5d**	**7c**	n.a.	n.d.	n.a.	n.a.

[a] 2AP EC_{50}, concentration required for 50% of the 2AP fluorescence decrease triggered by compound interaction with 2AP54 labeled IIa RNA, in the presence of 100 μM or 1 mM Mg^{2+}, in an assay that was described previously.[13] [b] FRET I_{FRET}, relative change of the FRET intensity of Cy3/Cy5 5′-labeled IIa RNA in the presence 100 or 1000 μM compound, measured in an assay that was described previously.[8] Compounds were tested for optical interference with the fluorescent labels for both the 2AP and FRET assays and no significant interference was found. n.a. = no activity; n.d. = not determined.

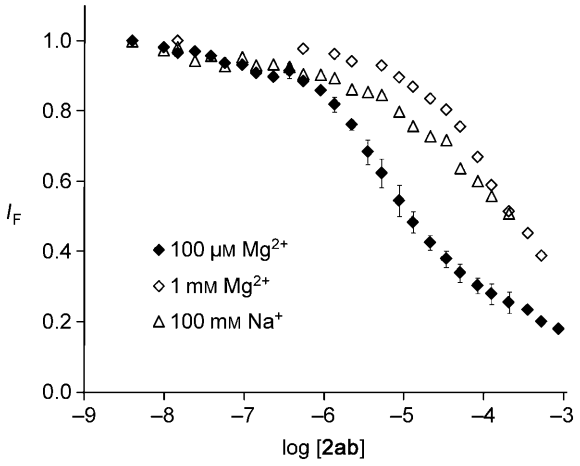


Figure 2. Titrations of 2AP54 labeled IIa RNA with DAP compound **2ab** in the absence and presence of salt. The assay was performed as previously described.[13] For the titration in 100 mM NaCl, 100 μM Mg^{2+} was present as well. Error bars represent $\pm 1\sigma$ of triplicate titrations. EC_{50} values for all tested DAP compounds are listed in Table 1. I_F = relative fluorescence intensity.

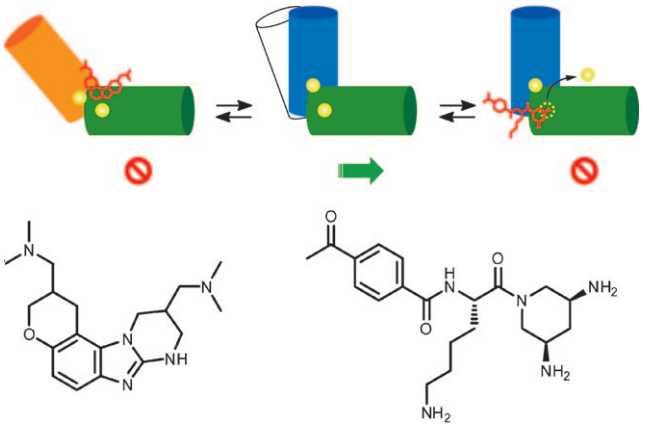


Figure 3. Model for the binding of small-molecule ligands to the HCV IRES IIa RNA. The subdomain IIa RNA adopts a magnesium-stabilized bent fold that plays a key role for the correct positioning of the IRES on the ribosome. Flexibility of the L-shaped IIa structure might be required for release of the ribosome after translation initiation (center). Benzimidazole inhibitors of IRES-driven translation (left) induce a widened angle in the IIa target, which leads to disruption of IRES function and inhibition of viral protein synthesis in infected cells.[8] Modular DAP compounds (right) bind in competition with structural Mg^{2+} ions and immobilize the L-shaped RNA, which might provide another mechanism for inhibition of IRES function. See text for discussion.

works of amino groups, which are positively charged under physiological conditions, play an important role for target recognition of aminoglycoside antibiotics that interact with RNA often by exploiting structural electrostatic complementarity with divalent metal-ion binding sites.[14] Similarly, binding data derived from the 2AP54 assay suggest that the DAP scaffold in the modular ligands described here participates in interactions with Mg^{2+} sites at the IIa target.

The IIa-binding DAP compounds **2ab**, **2bb** and **2cb** were inactive in the FRET assay at micromolar concentrations in which binding occurred in the 2AP assay (Table 1); this indicates that these ligands interact with the RNA target without inducing a conformational change. For two of the ligands, **2ab** and **2bb**, both of which contain a basic amino acid (Lys or Arg), a small increase of the FRET signal was observed at millimolar concentrations. As optical interference of the DAP compounds with the fluorescent cyanine dyes was ruled out, the increased FRET intensity was likely due to immobilization of the IIa RNA target in the L-shaped fold upon ligand binding. Comparison of structural data from an NMR study of the IRES domain II[15] and crystal structure analysis[13] suggests that the bent subdomain IIa shows some flexibility, attested by an interhelical angle that averages at $>90^\circ$ in solution and that is fixed at 90° in the crystal. The DAP ligands are able to bind and arrest the IIa RNA in the 90° bent state in solution, thereby increasing the overall intensity of the FRET signal. This immobilizing action of the modular DAP ligands might provide a mechanistic basis for a novel class of HCV IRES inhibitors (Figure 3).[7]

Cryo-electron microscopy investigations of the HCV IRES bound to the 40S ribosomal subunit[16] and whole 80S ribosomes[17] revealed an overall extended shape of the RNA with the domain II located at one end, and adopting a hook-like structure that directs the highly conserved terminal hairpin IIb toward the ribosomal E site in proximity of the active site.[16] Interaction of domain II with the ribosome induces a conformational change in the 40S head[17] that leads to closure of the mRNA binding cleft.[16] Correct positioning of the IIb hairpin and the viral mRNA at the ribosome depends critically on the L-shaped fold of the domain II.[18] Intrinsic flexibility in the subdomain IIa might be required for release of the ribosome from the IRES during translation initiation. We have previously demonstrated that IRES-binding benzimidazole inhibitors of the HCV replicon act by conformational induction of a widened interhelical angle in the subdomain IIa (Figure 3), which facilitates the undocking of the hairpin IIb from the ribosome and ultimately leads to inhibition of IRES-driven translation in HCV-infected cells.[8] In a distinct mode of action, the IIa RNA-binding modular DAP ligands described here might represent a second class of novel IRES inhibitors that affect HCV translation by arresting subdomain IIa in a 90° bent state and thereby interfering with translation initiation (Figure 3).

Preliminary testing of one of the IIa RNA-binding DAP compounds in a cell based HCV replicon assay revealed that the ligand **2ab** induced dose-dependent reduction in HCV IRES-driven translation of a luciferase reporter (Figure 4). The inhibition of reporter expression in infected human cells was specific for the replicon construct and not due to cytotoxicity. Future work will be focused on the iterative optimization of the modular DAP compounds to improve RNA target binding and HCV replicon inhibition.

Acknowledgements

This work was supported in part by the National Institutes of Health, Grant Nos. AI72012 (T.H.), CA132753 (T.H.) and AI069989 (D.L.W.). Support of the NMR facility by the National Science Foundation is acknowledged (CRIF grant CHE-0741968). We thank Prof. Yitzhak Tor for providing access to a semipreparative HPLC instrument.

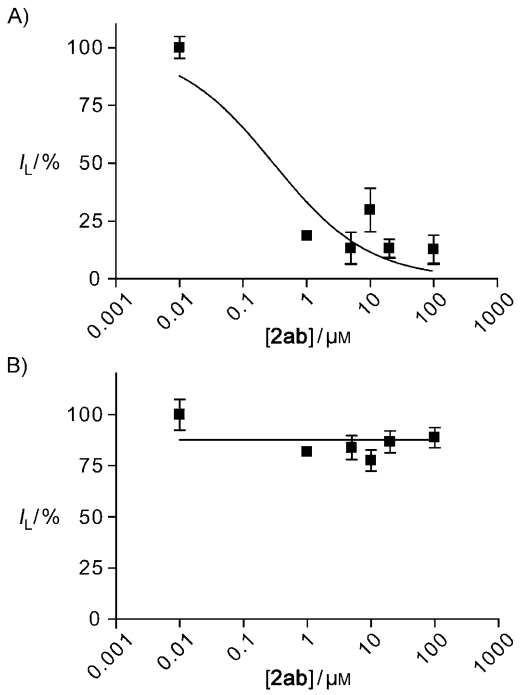


Figure 4. Biological activity of DAP compound **2ab** in human Huh-7.5 cells. A) Dose-dependent translation inhibition of luciferase reporter under HCV IRES control in the BM4-5 FEO HCV replicon.[20] B) Cytotoxicity as determined in a colorimetric viability assay. The luciferase reporter replicon assay and cytotoxicity testing were performed as described before.[8] I_L = relative luminescence intensity.

Keywords: antibiotics · antiviral agents · proteins · RNA recognition · viruses

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Received: March 17, 2010
Published online on June 16, 2010

