JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Selective Covalent Labeling of miRNA and siRNA Duplexes Using HEN1 Methyltransferase

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Supporting Information

ABSTRACT: MicroRNAs regulate gene expression in numerous biological pathways and are typically methylated at their 3′-termini in plants but not in animals. Here we show that the HEN1 RNA 2′-O-methyltransferase from *Arabidopsis thaliana* catalyzes the transfer of extended propargylic moieties from synthetic AdoMet cofactor analogs to duplex miRNAs or siRNAs. The presented approach permits selective and efficient covalent labeling of small RNA duplexes with a variety of functional or reporter groups for their enrichment and analysis.

MicroRNAs (miRNAs) are short (21−25 nt) noncoding RNAs found in organisms ranging from plants and insects to mammals.[1] miRNAs modulate the expression of over 30% of mammalian genes during development, apoptosis, metabolism, or environmental response.[2−4] Individual miRNAs exhibit characteristic expression patterns in normal and disease cells depending on the tissue type, stage, and treatment,[5,6] and therefore analysis of miRNA profiles proved valuable for diagnostics and development of novel therapies.[7] Current methods for small RNA discovery largely rely on size-dependent cloning of nucleic acids strands followed by their massive parallel sequencing.[8] However, such approach lacks the specificity required to discriminate against other similarly sized types of cellular RNA and DNA molecules and their degradation fragments.[9] *In situ* hybridization, DNA microarray, and qPCR analyses are extensively employed for quantitation of miRNAs, but these methods are limited to analyzing known biological species.[10] Therefore, despite a growing need for efficient, reliable, and convenient miRNA identification and profiling techniques, most existing methods fall short to address the challenges presented by the structural peculiarities of the target molecules.

A distinctive feature of microRNA biogenesis in plants, not commonly found in mammals, is the methylation of the 3′-terminal nucleotides. HEN1 is a methyltransferase from *Arabidopsis thaliana* that transfers methyl groups from the cofactor *S*-adenosyl-L-methionine (AdoMet) onto the 2′-OH group of the 3′-terminal nucleotides of double-stranded RNA molecules, like miRNA/miRNA* and siRNA/siRNA* (Figure 1A).[11−13] Here we explore the properties of HEN1 in the context of developing molecular tools for selective tagging of mammalian miRNAs and siRNAs for their subsequent detection and analysis. Our strategy is based on the methyltransferase-directed Transfer of Activated Groups (mTAG) approach (Figure 1B,C) which combines the cognate specificity of a methyltransferase and a synthetic AdoMet analogue carrying a sulfonium-bound extended side chain replacing the methyl group.[14] The mTAG approach was successfully implemented for sequence-specific covalent labeling of DNA[15−17] and RNA[18,19] or targeted labeling of methylation sites in tRNAs[18,19] and histone proteins.[20,21] To examine the potential of HEN1 for small RNAs modification, we first used synthetic cofactors with extended propargyllic side chains carrying a terminal amine or alkyne group (cofactors **2**−**4**). The 2′-O-alkylation of the 3′-terminal ribose in an RNA oligonucleotide was monitored by PAGE experiments in which alkylated RNA strands appear longer due to the attached moiety.[22] We found that the extended side chains with reactive functional groups are efficiently transferred from the synthetic cofactors to both strands of the miR173/miR173* duplex which is a natural substrate of HEN1 in *A. thaliana* (Figure 2a, left). To test the transalkylation reactions with human small RNAs, we examined the hypoxia-related potential cancer biomarker miR-210.[22] Complete alkylation of both strands of the miR-210/miR-210* duplex (Figure 2a, center) suggests that the HEN1 transalkylation activity is not limited to plant microRNAs. HPLC/ESI-MS analysis of the alkylation products confirmed that a complete side chain is transferred from the synthetic cofactors onto the ribose moiety of a 3′-terminal G, A, or C nucleotide (Figures 2b,c and S1,S2 and Table S1). Moreover, as shown in Figure 2a (right), the methyltransferase successfully modified a representative of siRNA, which differs from the previous examples by its perfect base pair complementarity in the double-stranded part. Kinetic analysis of the HEN1-directed alkylation of miR-210/miR-210* or miR173/miR173* with synthetic cofactors performed under single turnover conditions showed that the modification rates (Figure S3) were lower (0.03−10 min^{-1} at 100 μM cofactor) as compared to the methyl group transfer from the natural cofactor AdoMet (4−35 min^{-1} at 100 μM AdoMet)[13] but were sufficiently high to permit a complete or nearly complete modification within 5−60 min. Thus, it can be concluded that the HEN1-directed mTAG alkylation reaction is well-suited for practical laboratory use.

Received: July 21, 2014
Published: August 29, 2014

dx.doi.org/10.1021/ja507390s | *J. Am. Chem. Soc.* 2014, 136, 13550−13553

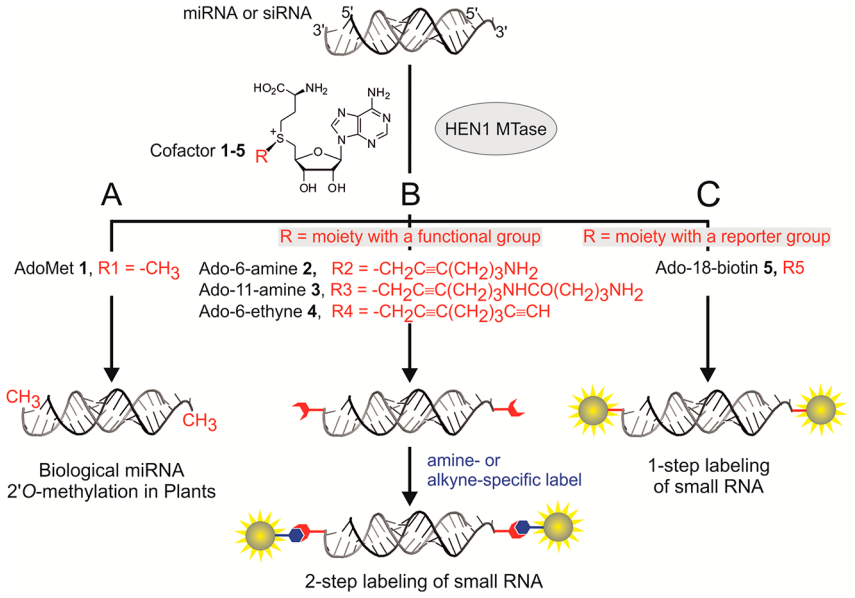

Figure 1. Strategies of HEN1-directed labeling of small RNAs. (A) Biological HEN1-directed reaction: the methyl group transfer from the *S*-adenosyl-L-methionine cofactor to the 2′-O position of a 3′-terminal nucleotide of a target miRNA duplex. (B) A two-step RNA labeling strategy, whereby a functional group (primary amine, thiol, alkyne, azide, etc.) embedded in the side chain R of a synthetic cofactor is first transferred to the 3′-terminal nucleotide. In the second step, a desired reporter (fluorophore, biotin, etc.) is attached to the functional group. (C) An alternative strategy depicts one-step labeling by direct HEN1-dependent transfer of a reporter group embedded in the extended side chain R.

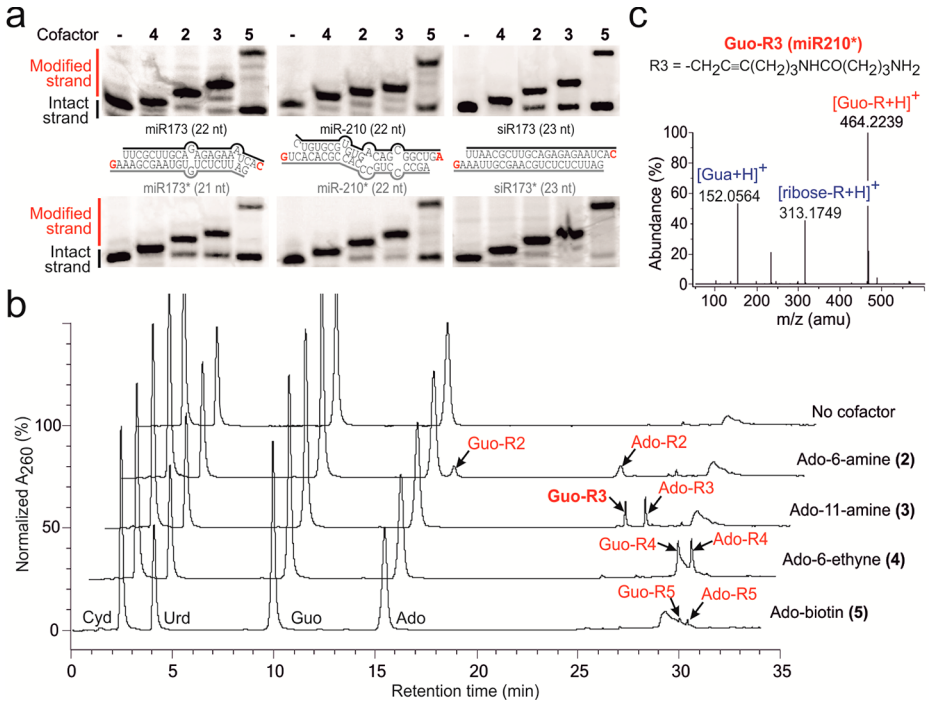

Figure 2. HEN1-directed transfer of extended moieties to various types of small RNAs. (a) Alkylation of the guide strand (top row) or the star strand (bottom row) of plant miR173/miR173*, animal miR-210/miR-210*, or siR173/siR173* in the presence of a synthetic cofactor: Ado-6-ethyne (**4**), Ado-6-amine (**2**), Ado-11-amine (**3**), Ado-18-biotin (**5**), or none [−]. Target adenosine, cytidine, and guanosine nucleotides in RNA strands are marked in red. (b) Reversed-phase HPLC analysis of nucleosides derived from miR-210/miR-210* incubated with HEN1 in the presence of the above synthetic cofactors (100 μM, if any). (c) A representative MS analysis of a modified nucleoside, Guo-R3. The transalkylation reactions lead to modified Ado and Guo derivatives (shown in red) carrying ribose-bound chemical moieties matching the sulfonium-bound side chains of the donor AdoMet analogs.

To demonstrate actual labeling of small RNAs, the miRNA duplexes were first modified in the presence of HEN1 and the synthetic Ado-6-amine (**2**) or Ado-6-ethyne (**4**) cofactors followed by attachment of a desired fluorophore employing conventional amino-NHS ester or alkyne−azide click chemistry, respectively. As shown in Figure S4, the Cy5 or eterneon labeled miRNAs are detectable using appropriate laser illumination but remained invisible in control reactions

dx.doi.org/10.1021/ja507390s | J. Am. Chem. Soc. 2014, 136, 13550−13553

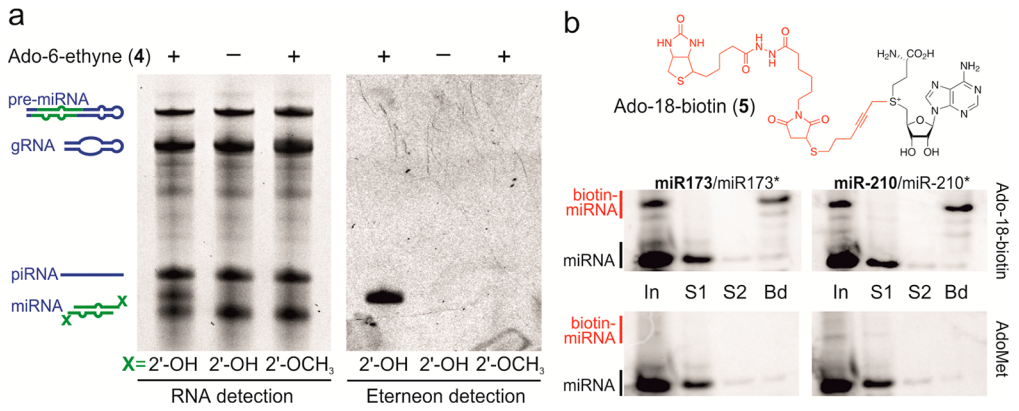

Figure 3. HEN1-directed selective labeling of miRNA using two-step and one-step approaches. (a) Selective two-step labeling of unmodified (X = OH) or premethylated (X = OCH_3, control) mature miR173/miR173* (21/22 nt) in a mixture of plant miR173 stem-loop precursor (pre-miRNA, 102 nt), partially self-complementary archaeal guide RNA sR47-A31 (gRNA, 64 nt), and single-stranded animal piR3 (piRNA, 28 nt) (common elements in miRNA and pre-miRNA are shown in green). The RNAs were incubated with HEN1 and the Ado-6-ethyne cofactor, treated with eterneon-480/635-azide and copper(I) catalyst, and then analyzed on a denaturing 12% PAA gel. Eterneon fluorescence was detected using a 473 nm laser (right), and bulk RNA was visualized after staining with ethidium bromide (left). (b) One-step biotin labeling of miRNA using Ado-18-biotin cofactor (transferable moiety shown in red). ^{33}P-miR173/miR173* or ^{33}P-miR-210/miR-210* was treated with HEN1 and either Ado-18-biotin or AdoMet cofactor (control). The initial reactions (In) were loaded on streptavidin beads, and supernatant (S1) was collected. Following a buffer wash (S2 fraction), streptavidin beads were resuspended in water to release bound RNA (Bd). All fractions analyzed on a 12% PAA gel.

performed with the natural cofactor AdoMet. The HEN1-mediated alkyl transfer reaction followed by eterneon fluorophore treatment allows selective two-step labeling of miR173/miR173* in a mixture containing pre-miRNA,[11] C/D guide RNA,[23] and piRNA.[24] Notably, no eterneon labeling occurred when premethylated miR173^{CH_3}/miR173*^{CH_3} duplex was used in the reaction (Figure 3a), attesting that HEN1 retains its cognate specifity toward natural substrates. Similarly, efficient biotinylation of miR-210/miR-210* premodified with the alkylamino side chains was achieved by treatment with biotin-NHS ester (Figure S5a) as demonstrated by increasing mobility shifts of RNA strands modified in the presence of Ado-6-amine or Ado-11-amine cofactors and upon subsequent attachment of a biotin reporter. These findings indicate that the attached functional groups stably reside on the RNA and are well accessible for subsequent reactions. The reporter groups are fully functional as well since the labeled miRNAs can be successfully enriched using capture on streptavidin beads (Figure S5b).

A key advantage of the two-step labeling approach is the flexibility in manipulating the chemical parameters of the labeling reaction (linker length, conjugation chemistry, reporter group) by simply combining different cofactors and chemoselective reporter compounds. Alternatively, the mTAG reaction could in principle be used for single-step labeling by direct transfer of a side chain carrying a desired reporter group, which would be beneficial in situations when minimal sample manipulations, simplicity, and speed are required. This entails added synthetic complexity to the cofactor analogue as reporter groups are typically larger and more complex than functional groups. Moreover, an increased steric bulk of the transferable side chain may also lead to a partial or complete impairment of the enzymatic transfer reaction.[17,19] As a proof of principle we went on to explore these challenges by designing a novel cofactor analogue that carries a biotin moiety in the transferable group. In the first step, a cofactor carrying a terminal thiol group was produced using trityl-protecting chemistry, which was then conjugated with maleimide-biotin to yield Ado-18-biotin cofactor **5** (Scheme S1). The mTAG reactions performed with the cofactor indicated that the HEN1 methyltransferase can append the extended side chain to both strands of plant and human miRNA/miRNA* or siRNA/siRNA* duplexes with high efficiency (Figure 2a,b and S1 and Table S1). It was remarkable that the wild-type HEN1 was able to accommodate the bulky side chain in the active site in light of poor acceptance of even shorter side chains by wild-type DNA C5-methyltransferases and, even more so, by C/D box snRNP 2′-O-RNA methyltransferases. We could identify no obvious structural features of the catalytic pocket attributable to its higher steric tolerance by visual inspection of available crystal structures.[25] From the point of view of substrate structure, the methylation target of HEN1 is located in a terminal extension of an RNA duplex, as opposed to the previously studied DNA and RNA methyltransferases, which all modify an inner nucleotide in a prolonged DNA or tRNA/rRNA substrate.[15,17,19] Since terminal nucleotides are generally more "embraceable" than centrally located ones, such substrates should impose fewer steric constrains on the catalytic site (lower density/crowding of critical residues) resulting in a higher structural plasticity during catalysis.

The exceptionally high intrinsic tolerance of HEN1 to bulky moieties is an extremely valuable feature for developing new tagging approaches not only *in vitro* but also for *in vivo* studies. To further illustrate practical utility of the one-step approach, the biotin-labeled miR173 and miR-210 RNAs were quantitatively bound to magnetic streptavidin beads (Figure 3b), while the unmodified fraction and control methylated RNA remained in solution. Together, these results for the first time demonstrate a facile incorporation of a functionally active biotin moiety permitting efficient capture and enrichment of target RNAs. It should be noted that miRNA/miRNA* duplexes are rapidly processed in mammalian cells such that one of the two strands is selectively bound in a RNA-induced silencing complex (RISC) and is thus predominantly detected in cellular RNA samples.[26] One way to analyze specific single miRNA strands would be their *in situ* hybridization with non-modifiable complementary oligonucleotide probes to produce HEN1-modifiable miRNA-like duplexes. Another possible

dx.doi.org/10.1021/ja507390s | J. Am. Chem. Soc. 2014, 136, 13550−13553

approach could be a forced accumulation of cellular double-stranded miRNA/miRNA* by blocking the formation of RISC complexes. That could potentially be achieved by coexpression of suppressor proteins that bind double-stranded miRNAs or by Argonaute silencing using siRNA knock-down or small molecule inhibitors.

In summary, we demonstrate that the HEN1 methyltransferase can be employed for attaching desired functional and reporter groups at the 3′-ends of small RNA duplexes in a simple two-step (Figure 1B) or even one-step procedure (Figure 1C). This is the first reported technique permitting selective labeling of a whole class of cellular RNAs. The new approach offers unprecedented control in covalent manipulation of miRNAs and siRNAs paving the way to develop numerous novel techniques ranging from affinity purification, high-throughput profiling, and specific single-molecule analysis using nanopore sensors.[27]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting figures and table and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): A.P., G.V., and S.K. are inventors on a related patent application.

■ ACKNOWLEDGMENTS

The authors thank Miglė Tomkuvienė for a gift of sR47-A31 RNA, Zita Liutkevičiūtė and Audronė Rukšėnaitė for valuable help with MS analysis, and Darius Kavaliauskas for experimental assistance. This work was funded by the European Social Fund under National Integrated Programme Biotechnology & Biopharmacy (grant VP1-3.1-SMM-08-K01-005).

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