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Double metal-catalyzed synthesis of bisubstrate analogues for RNA methylation studies

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ABSTRACT: RNA methyltransferases (RNA MTases) are a family of enzymes that catalyze the methylation of RNA using S-adenosyl-L-methionine (SAM) as the cofactor. While RNA MTases are promising drug targets, new molecules are needed to fully understand their roles in disease and to develop safe and effective drugs that can modulate their activity. Since RNA MTases are suitable for bisubstrate binding, we report here an original strategy for the synthesis of a new family of m⁶A MTases bisubstrate analogues. Six compounds containing a SAM analogue unit covalently tethered by a triazole ring to the N-6 position of an adenosine, mimicking the RNA part were synthesized. A double metal-catalyzed reaction was used to introduce the α -amino acid motif mimicking the methionine chain of the cofactor SAM. First, an iCuAAC reaction afforded the 5-iodo-1,4-disubstituted-1,2,3-triazole and a second post-functionalization reaction based on palladium-mediated cross-couplings was used to connect the α -amino acid substituent. Docking studies of our molecules in the active site of a MTase show that i) the use of triazole as a linker provides additional interactions and ii) the presence of the α -amino acid chain stabilizes the bisubstrate. The synthetic methodology developed here enhances the structural diversity of bisubstrate analogues to explore the active site of RNA modification enzymes and to develop new inhibitors.

INTRODUCTION.

Bisubstrate approach to target methyltransferases (MTases) has been well documented and is considered as a promising strategy for the development of selective therapies against cancers, viral infections, and neurological diseases.¹ This strategy has been used for proteins, small molecules, DNA and RNA MTases studies and has been facilitated by the development of methodologies that enable the synthesis of bisubstrate molecules. One compound, the EPZ5676² was designed as a bisubstrate inhibitor of DOTL1, an histone methyltransferase, and entered phase II clinical trials for the treatment of acute leukemia. The bisubstrate approach is based on the design of molecules mimicking the transition state formed during the methylation process catalyzed by the MTases (Figure 1A and 1B). Since most of MTases use S-adenosyl-L-methionine (SAM) as methyl donor, the structure of bisubstrate analogues relies on the design of conjugates containing a SAM cofactor analogue linked to a mimic of the substrate (Figure 1C). In the literature, the use of bisubstrates to explore RNA MTases was described for N7-G MTases^{3,4} and for 2'O-RNA MTases.^{5,6} In these cases, the link between the two substrates is based on a sulfonamide function (Figure 1D). Optimization of the substituents on the sulfonamide led to powerful inhibitors of SARS-CoV N7-G MTases (nsp14) (IC₅₀ in submicromolar range).^{5,6} Recently, we reported the development of chemical strategies to access to bisubstrates for m⁶A MTases studies. Activation of the N6 position of an adenosine with isopropenyl carbamate allowed us to obtain SAM-adenosine conjugates containing an urea linker whereas the convertible nucleoside

strategy, starting from N6-hydroxybenzotriazole-inosine led to bisubstrates with alkyl linkers (with two and three carbons)⁷⁻⁹ (Figure 1D). Interestingly, the former strategy was successfully employed to access to oligonucleotides linked to SAM cofactor analogues (SAM-RNA conjugates).⁹ These compounds were then used in crystallization assays with RlmJ, a bacterial m⁶A rRNA MTase. Three crystal structures of RlmJ in complex with the bisubstrate conjugates were solved and revealed the RNA-specific recognition elements used by RlmJ to bind the RNA substrate in its active site. Moving forward to explore the impact of the linker of the bisubstrate, we next examined the copper(I) azide-alkyne cycloaddition reaction (CuAAC) to introduce 1,4 substituted-triazole ring as a linker (Figure 1D).¹⁰ However, these conjugates lack the α -amino acid motif.

These examples show that bisubstrates turn out to be potent molecular tools to explore the active site of RNA MTases.¹⁻⁹ Important features must be considered: i) α -amino acid motif of the cofactor SAM is essential to successfully co-crystallize bisubstrates in the presence of m⁶A RNA MTases and ii) the nature of the linker impacts the binding efficiency. In the present work, we report a synthetic route to incorporate the α -amino acid motif into on the triazole molecules. The introduction of such substituents requires the formation of a carbon-carbon bond between α -amino acid chain analogues and the triazole ring through the development of a double metal-catalyzed process.

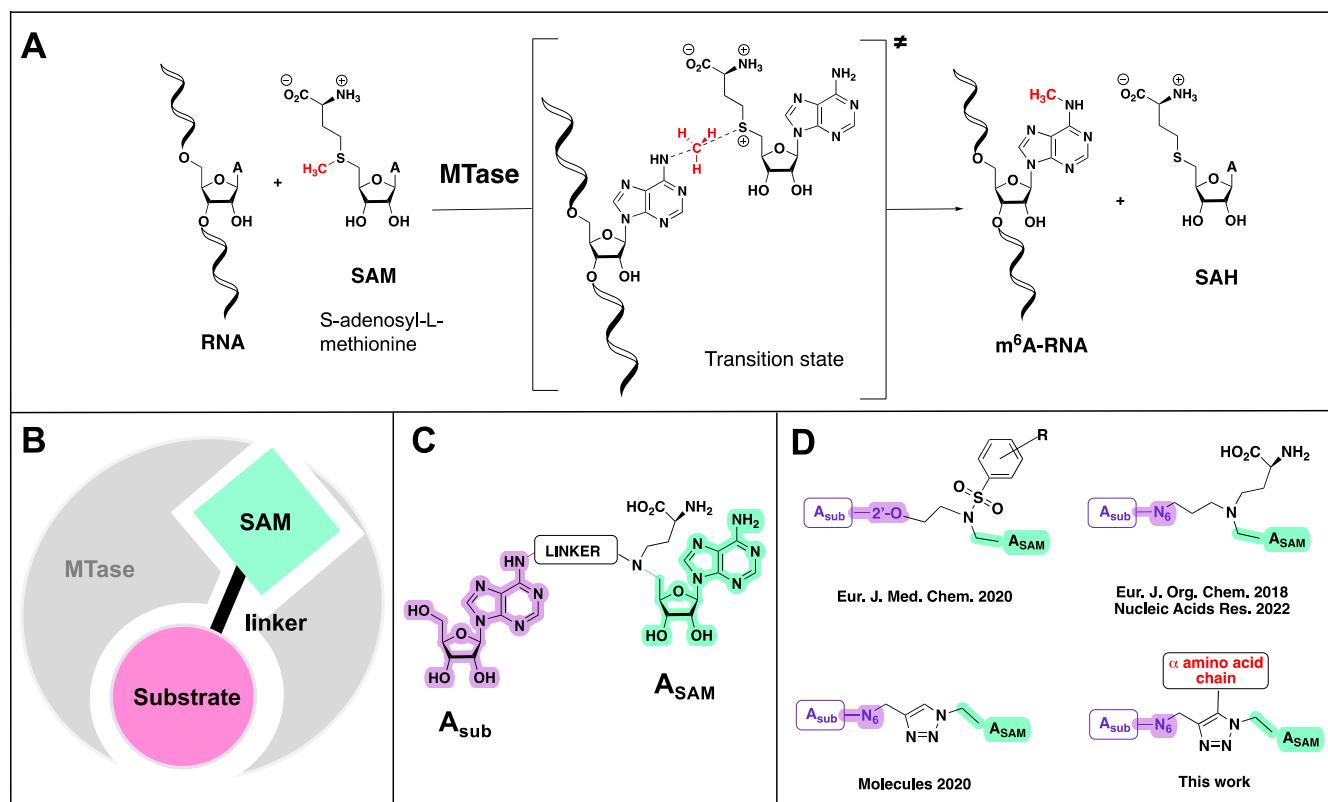


Figure 1. A) Mechanism of methyl transfer catalyzed by m^6A RNA MTases. B) Bisubstrate binding mode. C) General structure of bisubstrate analogues. D) Linkers used for RNA MTases bisubstrate analogues.

The usefulness of triazole derivatives in chemical biology and medicinal chemistry has led to the development of synthetic methodologies to access 1,4,5-trisubstituted-1,2,3-triazole analogues. A first strategy lies on condensation reactions between 1) azides and activated methylene compounds¹¹ or 2) diazo derivatives and amines.¹² The functionalization of disubstituted triazoles has also been reported in the last decade by development of *N*-alkylation reactions¹³ or C-H activation approaches using palladium¹⁴ or copper catalysts.¹⁵ The most common strategy remains however the functionalization of 1,2,3-triazoles obtained by copper catalyzed cycloaddition between azide and alkyne. Reported to promote the cycloaddition of internal alkyne, ruthenium¹⁶ and nickel¹⁷ catalysts have also been used to directly access trisubstituted derivatives. In particular, the reactivity of iodo-alkynes has been studied in Cu(I)-catalyzed cycloaddition reaction to furnish iodo-triazoles (iCuAAC).^{19,20} The cycloaddition of metallo-alkyne in the presence of azide allows the formation of 5-metallotriazoles and gives access to 1,4,5-trisubstituted-1,2,3-triazoles by reaction with electrophiles²¹⁻²³ or after an additional metallo-catalyzed cross-coupling step.²⁴ Halo-triazole derivatives represent a key intermediate regarding the synthesis of 1,4,5-trisubstituted-1,2,3-triazoles. In 2005, Wu and co-workers depicted the use of a stoichiometric amount of copper(I) in the presence of I_2 or ICl as electrophilic trapping reagent.²⁵ Addition of oxidant such as NXS ²⁶ or selectfluor²⁷ to the system could be consid-

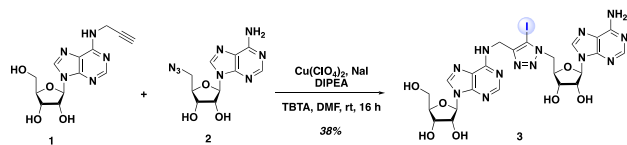
ered for the preparation of 5-halo-1,4-disubstituted-1,2,3-triazoles. In 2012, the group of Zhu developed a Cu(I)-mediated cycloaddition approach in which copper(II) salt is reduced by NaI to generate *in situ* the copper(I) species and I_3^- as the iodinating source.²⁸ The synthetic utility of halo-triazoles has been highlighted by a number of examples describing their functionalization by palladium-catalyzed cross-coupling reactions.^{29, 12} More recently, the development of multicomponent (and multicatalyst) reactions for the construction of fully substituted 1,4,5-trisubstituted-1,2,3-triazoles has been reported.³⁰⁻³⁴ These strategies have been mainly applied to standard organic building blocks and to the best of our knowledge, no example depicts an application to nucleic acids modifications.

We propose here to develop a double metal-catalyzed procedure to access to bisubstrate analogues of RNA MTases. We first examined the reactivity of adenosine derivatives to access to SAM-adenosine conjugates and then optimized the synthetic route to apply the methodology to short oligonucleotides (from 2 to 5 nt).

RESULTS AND DISCUSSION

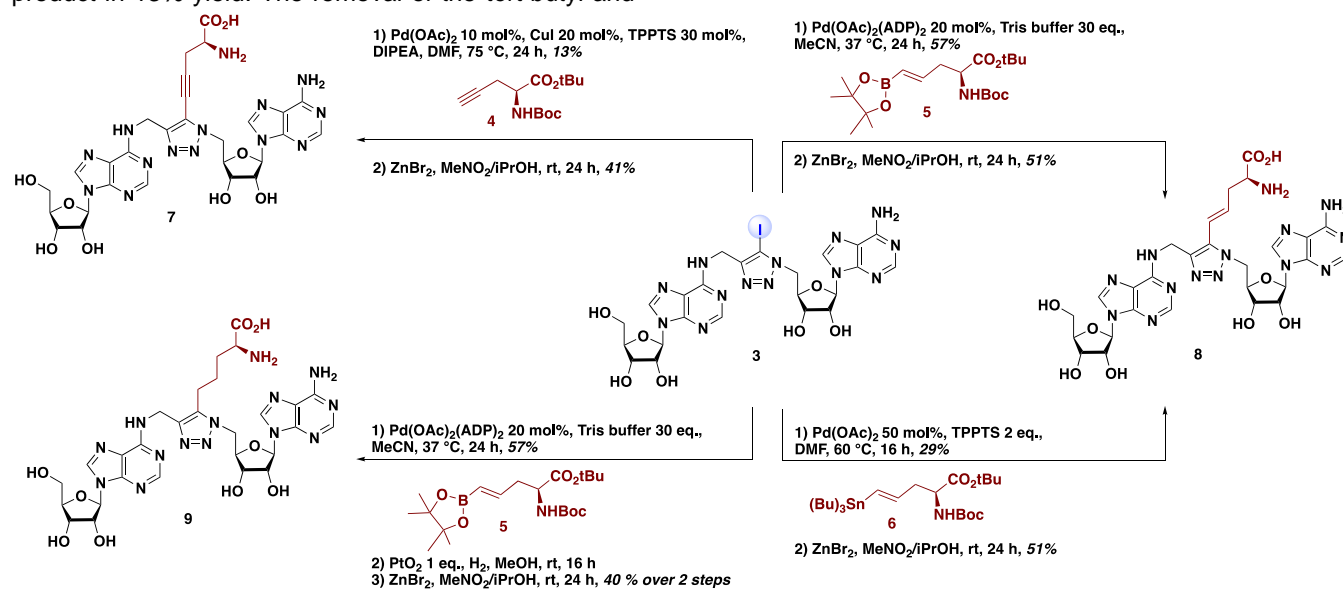
We first started to explore the reactivity of adenosine derivatives in iCuAAC reactions. Adenosine **1**¹⁰ substituted at the N6-position with an alkyne function was treated with a substoichiometric amount of $Cu(ClO_4)_2$, an excess of NaI as iodinating source and N,N-

diisopropylethylamine in DMF in the presence of 5'-azido-adenosine **2**. The addition of TBTA ligand in the reaction medium was mandatory to access to the 5-iodotriazole **3** (Scheme 1).



Scheme 1. Synthesis of 5-iodo-triazole **3**.

A second step of metal-catalyzed promoted reaction was then developed in which we took advantage of the reactivity of the 5-iodo-triazole derivative **3** to introduce the α -amino acid motif mimicking the methionine side chain of the SAM cofactor. A two-step procedure was assessed using palladium-catalyzed cross-coupling reaction followed by the removal of the amino acid protecting groups (Scheme 2). Compound **3** was subjected to Sonogashira cross-coupling reaction conditions using Pd(OAc)₂, CuI, TPPTS ligand and DIPEA in DMF in the presence of alkyne **4** (See supporting information S3) to afford the corresponding coupling product in 13% yield. The removal of the *tert*-butyl and



Scheme 2. Post-functionalization of 5-iodo-triazole **3**.

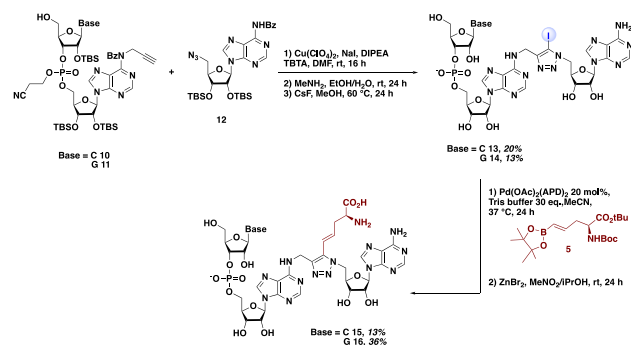
We have recently demonstrated the effectiveness of bisubstrate analogues containing a di or a tri-nucleotide to mimic the RNA part to probe MTases active site.⁹ To synthesize SAM-RNA conjugates, we investigated the opportunity to prepare a 5-iodo-triazole compound from a dinucleotide as alkyne-substrate. The previously described methodology was optimized to apply the iCuAAC reaction conditions to the dinucleotides **10** and **11** with 5'-azido-adenosine **12**. The use of copper(II) salt to generate *in situ* the copper(I) species and I₃⁻ in the presence of NaI led to the formation of the expected 5-iodo-1,4-disubstituted-1,2,3-triazoles. This step was then directly followed by the deprotection of the phosphotriester function and the nucleic bases. The

removal of the silyl ether groups was then achieved using ZnBr₂ salts in a mixture of isopropanol and nitromethane.^{35,36} Compound **7** was obtained in 41% yield after HPLC purification. To expand the synthetic potential of 5-iodo-triazole **3**, Suzuki-Miyaura cross-coupling reaction conditions were assessed. In the presence of pinacolboronic ester **5** (See supporting information S4) with a water-soluble palladium species^{37,38} in tris buffer, the corresponding cross-coupling product was obtained in 57% yield. A second step of deprotection afforded the bisubstrate analogue **8** substituted by a vinyl side chain. Following a similar approach, compound **8** was likewise synthesized by a Stille cross-coupling in the presence of vinylstannane **6** in 29% yield (See supporting information S5). In order to access to compound **9** with an alkyl chain, we performed a three-step procedure beginning with a Suzuki-Miyaura cross-coupling reaction in the presence of compound **5**. The alkene was then reduced under hydrogen atmosphere in the presence of PtO₂ and the hydrogenated compound was subjected to deprotection conditions to afford compound **9** in 40% yield over 2 steps.

removal of the silyl ether groups was performed using cesium fluoride to afford the SAM-dinucleotide conjugates **13** and **14** in 20 and 13% yields respectively over three steps (Scheme 3). The moderate yields observed during the iCuAAC could be explained by the formation of the 5-proto-triazole **13'** and **14'** in competition with the expected 5-iodo-triazole derivative (See supporting information S12-14).

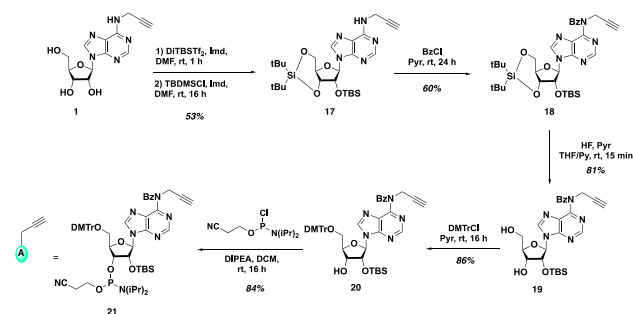
Dinucleotides **13** and **14**, obtained by iCuAAC reaction were then post-modified by a palladium-mediated Suzuki-Miyaura cross-coupling reaction in the presence of pinacolboronic ester **5** in the conditions previously described. The Suzuki-Miyaura cross-coupling was cho-

sen since it gives the best yield in our previous study. The removal of the amino acid protecting groups led to the formation of SAM-dinucleotide conjugates **15** and **16** in 13 and 36% yields respectively (Scheme 3). We then applied the methodology to the modification of 5 nt-RNA to access SAM-RNA conjugate.



Scheme 3. Double metal-catalyzed reaction to access functionalized dinucleotide **15** and **16**.

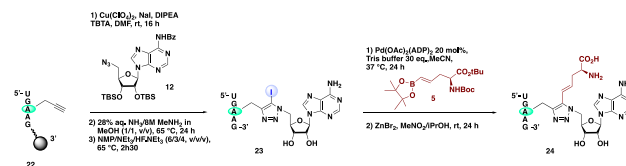
The synthesis of modified RNA sequences with an alkyne at internal position required the preparation of an adenosine phosphoramidite building block substituted at the N6 position with an alkyne function (compound **21**, Scheme 4). First, the modified adenosine **1** was silylated in 2'- 3'- and 5'-positions to afford compound **17** in 53% yield over 2 steps. The protection of the exocyclic amine at the N6-position was then performed with benzoyl chloride and led to the formation of compound **18** in 60% yield. A selective deprotection followed by the tritylation of the alcohol in 5'-position gave compound **20**. A phosphorylation step, in the presence of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, finally produced compound **21** in 84% yield.



Scheme 4. Synthesis of the phosphoramidite compound **21**.

Phosphoramidite **21** was incorporated in a 5-nt RNA sequence using solid-phase synthesis (SPS) (See supporting information S20). The resulting propargyl-N6-A-RNA **22** was modified by iCuAAC in the presence of 5'-azido-adenosine **12** under conditions previously optimized for dinucleotide modification. The cycloaddition reaction was performed on the solid support, followed by cleavage of the RNA from the support and standard deprotection steps of the cyanoethoxy groups, nucleobases, and 2'-hydroxyls to yield the desired 5-iodo-triazole conjugate **23** (Scheme 5). For-

mation of the 5-iodotriazole **23** competed with the formation of a 5-proto-triazole byproduct **23''** (See supporting information S48), which was detected by HPLC and characterized by mass spectrometry. The p6A-RNA **23'** was also detected in the reaction mixture, complete conversion of the starting material being not achieved (See supporting information S48). To further our synthetic efforts, the SAM-RNA conjugate **23** was post-modified by Suzuki-Miyaura cross-coupling with pinacolboronic ester **5**. The desired compound **24** was obtained and isolated after HPLC purification (Scheme 5).



Scheme 5. Double metal-catalyzed functionalization of RNA **22**.

Molecular modeling studies were performed to predict the binding positions of compounds **7**, **8** and **9** in the catalytic site of a m⁶A RNA MTase and to evaluate the contribution of the triazole linker and the α -amino acid side chain in the stabilization of the complexes bisubstrate/MTases (See supporting information S50). Docking of the bisubstrate containing the simple 1,4 triazole as a linker (compound **A-triaz-SAM**)¹⁰ was also performed. The docking was based on the RlmJ complex structure solved in the presence of a bisubstrate analogue containing an alkyl chain as a linker (molecule **GA-3C-SAM**, Figure 2).⁸ RlmJ is used as a model for the structural studies of m⁶A RNA MTases.⁹ Docking was performed with CDOCKER, a CHARMM-based MD docking algorithm using a rigid receptor and a flexible ligand.³⁹ Poses were selected based on the docking score (CDOCKER interaction Energy) and their ability to bind the methionine side chain pocket. Compound **9** adopts a similar binding mode to the one observed for the bisubstrate analogue **GA-3C-SAM** with a docking score of -110.1 kcal/mol (Figure 2A and supporting information S51). The methionine part of compound **9** is buried deep in the active site pocket surrounded by charged residues H19, D40, H42 and D164 (Figure 2B). The two adenosine moieties of **9** established the same H-bond interactions with N12, K18, E118, D143, G144 and hydrophobic contacts with H9, H42, L119, P166, W195 as observed for compound **GA-3C-SAM** (See supporting information S51). In addition, the triazole linker is stabilized through hydrophobic contacts with P166 and electrostatic interactions with D164 (Figure 2C). Although docking suggested that the two adenosine moieties of compound **A-triaz-SAM** may occupy the same regions as observed for compound **9** (See supporting information S51), the lack of the α -amino acid part in this bisubstrate led to a compound with reduced affinity as indicated by the docking score -74.9 kcal/mol. For rigid compounds **7** and **8**, the α -amino acid is bound to the active site of the SAM cofactor but the anchoring of the triazole moiety is modified, and the two adenosines failed to properly

orientate in their respective pockets (docking score of -80.9 kcal/mol and -87.3 kcal/mol, respectively) (See supporting information S52 and S53).

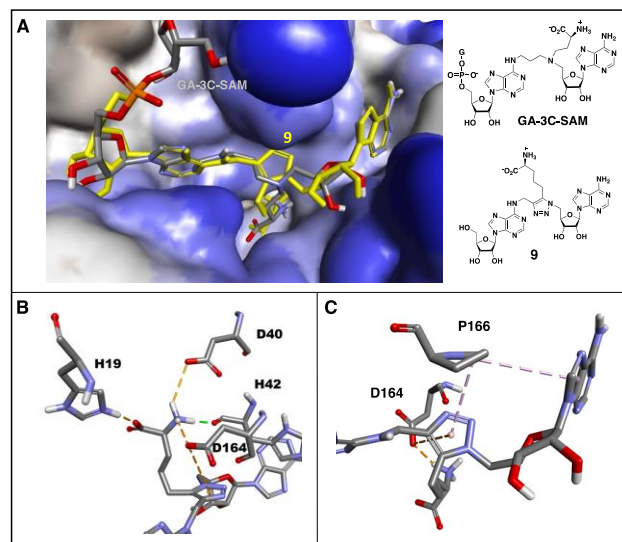


Figure 2. *In silico* modeling of bisubstrates in the SAM binding pocket of RlmJ (PDB: 7P8Q, resolution 2.29 Å). A) Superimposition of the bisubstrate **GA-3C-SAM** and compound **9** in the catalytic site of RlmJ. Compound **9** is represented as yellow sticks, The protein surface is shown: Hydrophobic regions are in grey, hydrophilic regions are in blue. B) α -amino-acid moiety of compound **9** in interaction with H42, D40, D164 and H19 of RlmJ. C) Triazole ring of **9** in interaction with P166 and D164 of RlmJ. Hydrophobic interactions are represented in purple, hydrogen-bonding in green and electrostatic interactions in orange (salt bridge) and brown (π -anion). Laurent, Peut-on mettre les interaction selector en même couleur ?? Je pense qu'il est préférable de préciser dans la catégorie des interactions électrostatiques le type d'interaction précis (π -anion, salt bridge). Non polar hydrogens are omitted for clarity.

CONCLUSION

We have reported in this study a double metal-catalyzed pathway to access bisubstrate molecules to study RNA MTases. In a first step, an 5-iodo-triazole motif was synthesized through a copper(I)-catalyzed alkyne-azide iodo-cycloaddition (iCuAAC). The introduction of the α -amino acid moiety was then promoted by the development of palladium-catalyzed cross-coupling reactions. As a result, three SAM-adenosine conjugates containing the 1,4,5-trisubstituted triazole linkage were obtained through Sonogashira, Suzuki-Miyaura, and Stille coupling. This synthetic pathway was then applied to the modification of two dinucleotides (CA and GA). The extension of our methodology to longer RNA sequence required the synthesis of an adenosine phosphoramidite substituted at the N6 position with an alkyne. Its incorporation in a 5 nt-RNA followed by the double metal-mediated functionalization of the corresponding RNA afforded a SAM-RNA bisubstrate. These results validate the compatibility of

the double post-functionalization strategy based on iCuAAC followed by Suzuki-Miyaura cross-coupling on RNA molecules. More widely, it could be of broad interest for the development of original bioconjugation approach of biomolecule based on the reactivity of 5-iodo-triazole.

The docking of these molecules in the active site of a methyltransferase shows a binding of our bisubstrate to both SAM and RNA binding pockets. The triazole ring is stabilized through hydrophobic contacts and electrostatic interactions and the presence of the α -amino acid is essential for the stabilization of the compounds. The bisubstrate **9**, containing the more flexible carbon chain, possess all the major interactions to be in a suitable orientation in the binding site of RNA methyltransferase.

Structural studies are underway to obtain structures of methyltransferase/bisubstrate complexes with the aim of elucidating the mechanism of RNA recognition by methyltransferases and better understanding the RNA methylation reaction.

Finally, RNA methyltransferases being promising new therapeutic targets, our synthetic strategy allows to expand the possibilities of structural variation of these bisubstrates to obtain more efficient and selective molecules.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

General procedure for the preparation of starting materials as well as the protocol for the iCuAAC reaction and the palladium-mediated cross-coupling (PDF).

NMR spectra (PDF).

HPLC chromatogram for compounds **7**, **8**, **9**, **13'**, **14'**, **15** and **16** (PDF).

LC-HRMS analysis for compounds **23**, **23'**, **23''** and **24** (PDF).

Molecular docking (PDF)

FAIR Data is available as Supporting Information for Publication and includes the primary NMR FID files for compounds **3-11**, **13**, **13'**, **14**, **14'**, **15-21**.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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