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C6-Substituted Analogues of 8-Azanebularine: Probes of an RNA-Editing Enzyme Active Site

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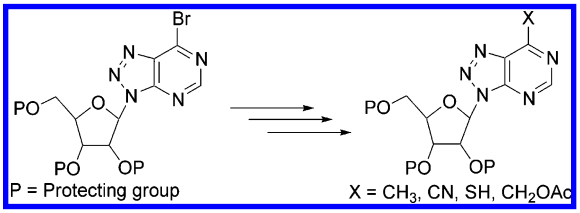
ABSTRACT



We describe the synthesis of derivatives of 8-azanebularine, a known inhibitor of adenosine deaminases including the RNA-editing enzyme ADAR2. 6-Methyl, 6-hydroxymethyl, 6-cyano, and 6-mercapto derivatives were obtained from 6-bromo precursors using different cross-coupling or substitution reactions. The C6-methyl derivative was incorporated into an RNA substrate for ADAR2 via the phosphoramidite. Quantitative gel mobility shift experiments with the resulting RNA indicate that methylation at C6 dramatically reduces the affinity of 8-azanebularine for ADAR2.

Synthetic nucleoside analogues are important tools for the study of biological systems. In particular, they have been used in mechanistic and structural analyses as well as in inhibitors of nucleoside and oligonucleotide modifying enzymes.[1−13] Our laboratory has a continuing interest in the preparation of nucleoside derivatives useful for the study of RNA editing catalyzed by the ADARs (adenosine deaminases that act on RNA).[14−17] ADARs are responsible for adenosine deamination reactions that change codon meaning in mRNAs encoding neurotransmitter receptors, making them important components of the nervous systems of metazoa.[18−24] In a

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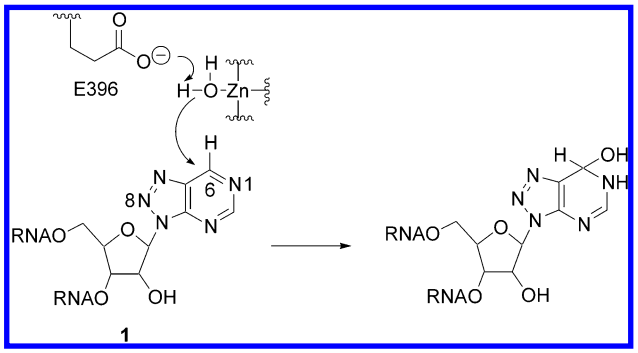
search for an effective mimic of the editing reaction transition state, we previously determined that incorporation of 8-azanebularine (**1**, 8-azaN) into an RNA structure recognized by human ADAR2 results in high-affinity binding (K_D = 2 nM).[15] The propensity of the 8-azapurine ring system to undergo covalent hydration at the C6−N1 double bond, generating a nearly perfect mimic of a proposed high-energy intermediate on the editing reaction pathway, is likely the source of the increased affinity afforded by this compound (Scheme 1).

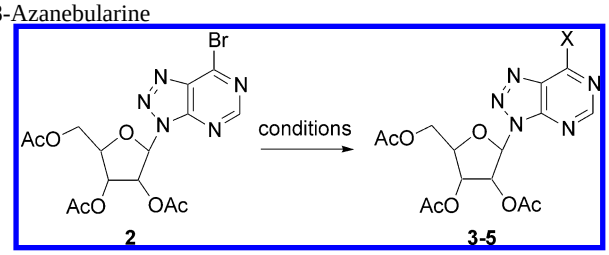
Scheme 1. Hydration of 8-Azanebularine (**1**) Catalyzed by Human ADAR2



In this Letter, we present the synthesis of 8-azaN derivatives with different substituents at the C6 position. We anticipated that structural features added at C6 could affect binding by ADAR2 through new contacts to active site features, such as the catalytic zinc ion.[25]

For the preparation of such compounds, we employed chemistry for purine nucleosides that has been developed and reported by our group and others.[14,15,17,26−28] Halogenated derivatives of purine are versatile reagents for generating analogues.[14,15,17,27,29−31] Thus, we used either acetyl- or *tert*-butyldimethylsilyl-protected 6-bromo-8-azanebularine as an intermediate for the preparation of derivatives via simple substitution or cross-coupling reactions. 2′,3′,5′-Tri-*O*-acetyl-6-bromo-8-azanebularine (**2**) has been reported previously by us.[15] This compound was treated with tetrakis(triphenylphosphine)palladium(0) and tetramethylstannane under conditions similar to those reported for purine nucleosides (Table 1).[28] This protocol afforded 2′,3′,5′-tri-*O*-acetyl-6-methyl-8-azanebularine **3** in good yield after purification. Compound **2** was also subjected to thiourea in ethanol to

Table 1. Synthesis of Acetyl-Protected C6 Derivatives of 8-Azanebularine

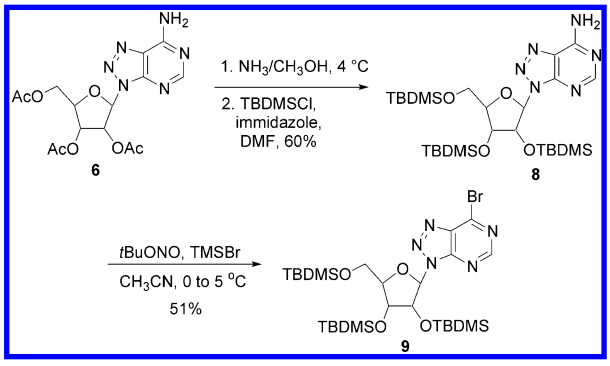


product	X	reagent(s)	solvent	temp (°C)	yield (%)
3	−CH_3	$Pd(Ph_3P)_4$, $Sn(CH_3)_4$	NMP	85	57
4	−SH	NH_2CSNH_2	EtOH	reflux	78
5	−CN	Et_4NCN, DABCO	CH_3CN	0 to rt	61

obtain the 2′,3′,5′-tri-*O*-acetyl-6-mercapto-8-azanebularine derivative (**4**) in 78% yield (Table 1). In addition, we synthesized acetyl-protected 6-cyano-8-azanebularine nucleoside **5** by displacement of the bromide with tetraethylammonium cyanide utilizing chemistry previously reported for the purine analogue (Table 1).[26] This compound should prove useful for the generation of additional C6 analogues such as the carboxylate, methylamine, or carboxamide derivatives.

Introduction of a hydroxymethyl substituent would also allow access to a variety of analogues with carbon substitution at C6 of the 8-azapurine ring. However, procedures described in the literature toward the synthesis of 6-hydroxymethylpurine suffer from numerous disadvantages including difficult purification and low yields.[4,13,32] Recently, an efficient synthesis of 6-hydroxymethylpurines was reported by Hocek and co-workers utilizing Negishi cross-coupling reaction conditions.[27] Because we desired an analogue with the C6 hydroxymethyl group differentiated from hydroxyls of the ribose, we generated a silyl-protected substrate for the cross-coupling reaction. 2′,3′,5′-Tri-O-acetyl-8-azaadenosine was deacylated in a solution of ammonia in methanol followed by silylation to give 2′,3′,5′-tris-*O*-(*tert*-butyldimethylsilyl)-8-azaadenosine **8**. 2′,3′,5′-Tris-*O*-(*tert*-butyldimethylsilyl)-6-bromo-8-azanebularine **9** was synthesized from **8** employing diazotiozation/bromination conditions (Scheme 2).[29]

Scheme 2. Synthesis of 2′,3′,5′-Tris-*O*-(*tert*-butyldimethylsilyl)-6-bromo-8-azanebularine



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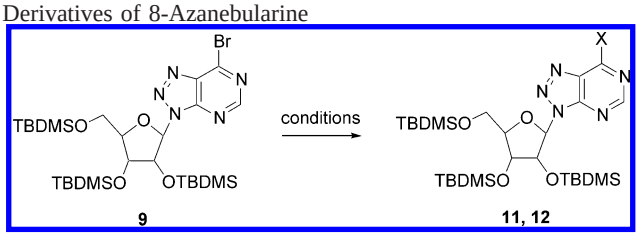
2′,3′,5′-Tris-*O*-(*tert*-butyldimethylsilyl)-6-acyloxymethyl-8-azanebularine **11** was then prepared using a Negishi-type coupling with acetyl-protected organozinc bromide **10** and silyl-protected 6-bromo-8-azanebularine derivative **9** as the electrophile. The required organozinc reagent **10** for this coupling was prepared in situ by introduction of zinc into commercially available bromomethyl acetate at 15 °C and subsequent addition to the reaction mixture of tetrakis-(triphenylphosphine)palladium(0) and **9** in THF.[27] The coupling reaction went smoothly to completion at room temperature in 9 h. Compound **11** was isolated in 77% yield after purification (Table 2). Interestingly, the reaction worked

Table 2. Synthesis of *tert*-Butyldimethylsilyl-Protected C6 Derivatives of 8-Azanebularine

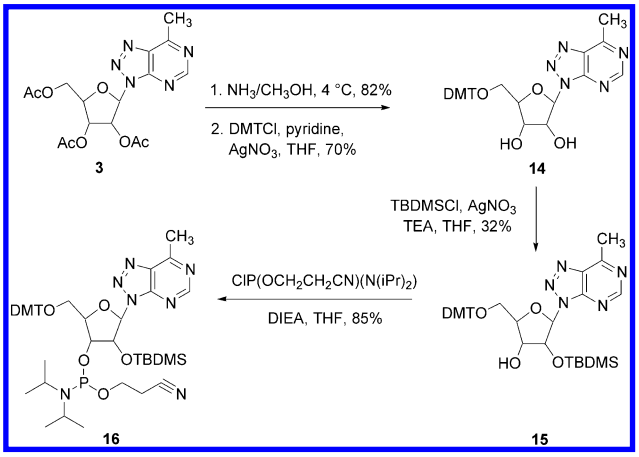


product	X	reagent(s)	solvent	temp (°C)	yield (%)
11	$-CH_2OCOCH_3$	$Pd(Ph_3P)_4$, $BrZnCH_2OCOCH_3$(10)	THF	rt	77
12	−SH	NH_2CSNH_2	EtOH	reflux	51

efficiently with organozinc bromide whereas the more reactive iodide was required for the reaction with the purine system previously reported by Hocek.[27] Apparently, the increased electrophilicity at C6 of 8-azapurine compared to purine allows for Negishi coupling to proceed with the less-reactive organozinc reagent. Indeed, we have observed that simple substitution reactions with 6-bromo-8-azanebularine proceed under conditions where little product can be observed for the 6-bromonebularine derivative. For instance, we previously reported that protection of 6-bromonebularine with *tert*-butyldimethylsilyl groups on ribose had an inhibitory effect on substitution reactions whereas efficient substitution is observed with silyl-protected 6-bromo-8-azanebularine.[31] This was demonstrated by the generation of a 6-mercapto compound (**12**) from reaction of compound **9** with thiourea in ethanol in 51% (Table 2).

We chose to evaluate the 6-methyl derivative as an initial test of the effect C6 substitution has on ADAR binding. For this purpose, analogue **3** was elaborated to the 5′-DMT, 2′-TBDMS, and *β*-cyanoethyl phosphoramidite. Protecting acetyl groups were removed from **3** by ammonia in methanol to produce 6-methyl-8-azanebularine ribonucleoside **13**. Compound **13** was transformed to the 5′-*O*-(4,4′-dimethoxytrityl)-6-methyl-8-azanebularine **14** followed by silylation in the presence of *tert*-butyldimethylsilyl chloride and silver nitrate in THF to generate compound **15**. Compound **15** was allowed to react with $ClP(OCH_2CH_2CN)(N(^iPr)_2)$ to produce 6-methyl-8-azanebularine phosphoramidite **16** as shown in Scheme 3.

Scheme 3. Synthesis of 6-Methyl-8-azanebularine Phosphoramidite



Standard automated RNA synthesis and deprotection conditions were used to generate a modified RNA oligonucleotide using the new phosphoramidite unit **16** (Figure 1). The RNA used in these experiments is a model substrate

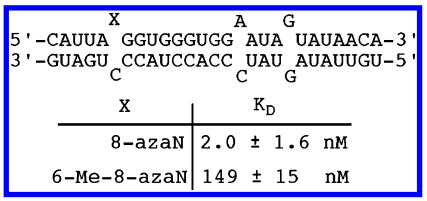


Figure 1. Sequence of duplex RNA ligands for ADAR2 based on the human GluR-B R/G editing site and dissociation constants (K_D) measured by gel mobility shift experiments with an active deletion mutant of ADAR2.[15]

based on the duplex secondary structure found in the glutamate receptor B subunit pre-mRNA containing the R/G editing site.[33] MALDI mass spectrometric analysis of the modified oligonucleotide after deprotection and purification confirmed the successful incorporation of 6-methyl-8-azanebularine (6-Me-8-azaN). RNA binding affinity was then measured using gel mobility shift experiments as previously described.[15] Introduction of a methyl group at the 6 position of 8-azaN results in a large decrease in binding affinity with an increase in the dissociation constant of nearly 2 orders of magnitude (8-azaN, K_D = 2 nM; 6-Me-8-azaN, K_D = 149 nM) (Figure 1). The affinity measured for the 6-Me-8-azaN-containing duplex is near that observed for nonspecific binding by this enzyme to duplex RNA and suggests that

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the 6-methyl group has prevented productive interactions with the deaminase active site.[15] This may be due to a steric effect, but the similarity in size of the C6 amino group of the substrate and a methyl group argues against this. In addition, the ADAR2 active site structure does not suggest obvious steric clashes with a purine C6 methyl group.[25] However, methylation at the site of hydration is known to inhibit covalent hydration of a number of nitrogen-containing heterocycles.[34] Indeed, methylation at C6 of 8-azapurine reduces covalent hydration at that site.[34] Furthermore, 6-methylnebularine is a weaker inhibitor than nebularine of the nucleoside-modifying enzyme adenosine deaminase (ADA).[13,35] Nebularine has been shown by X-ray crystallography to be hydrated in the active site of ADA.[12] Thus, we believe the large inhibitory effect on ADAR2 binding observed from C6 methylation of 8-azaN most likely arises from its effect on the formation of the covalent hydrate in the ADAR2 active site. This effect must be taken into account in the design of any new ADAR inhibitors based on 8-azaN.

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In conclusion, we have shown that the 6-bromo derivative of 8-azanebularine is a key intermediate that allows access to a variety of C6 analogues often under conditions where the nebularine derivative is not formed. In particular, 6-methyl-8-azanebularine and 6-acyloxymethyl-8-azanebularine have been successfully synthesized using palladium-catalyzed cross-coupling methodology. We described the synthesis of a 6-methyl-8-azanebularine phosphoramidite and evaluated the effect of this modification on RNA binding by ADAR2 using a gel mobility shift assay. Methylation at C6 of 8-azanebularine has a large inhibitory effect on editing enzyme binding and must be considered in the design of new ADAR inhibitors based on this ring system.

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Supporting Information Available: The experimental procedures and spectral data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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