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## Carbocyclic Pyrimidine Nucleosides as Inhibitors of S-Adenosylhomocysteine Hydrolase

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### Abstract

The design, synthesis and unexpected inhibitory activity against S-adenosylhomocysteine hydrolase (SAHase) for a series of truncated carbocyclic pyrimidine nucleoside analogues is presented. Of the four nucleosides obtained, **10** was found to be active with a  $K_i$  value of 5.0  $\mu\text{M}$  against SAHase.

### Introduction

The significant inhibitory activity exhibited by the carbocyclic nucleosides against S-adenosylhomocysteine hydrolase (SAHase) has, for the most part, been confined to adenosine analogues (Figure 1).<sup>1–3</sup> One notable exception<sup>4</sup> was the unexpected inhibition exhibited by a split guanosine nucleoside “fleximer”, however to date, few non-adenosine analogues have been reported. Inhibition of SAHase and several other enzymes critical for viral replication have been suggested as strategic targets for the inhibition of the orthopox viruses.<sup>5</sup> Given the lack of effective chemotherapeutics against the poxviruses and their potential for use in bioweapons, the search for new and potent inhibitors has increased in intensity.

It is well known that inhibition of SAHase also leads to inhibition of S-adenosyl-L-methionine (SAM) mediated biomethylations.<sup>1</sup> As a result, methylation reactions involved in viral replication become attractive targets for inhibition.<sup>6</sup> Two of the first naturally occurring carbocyclic nucleosides to exhibit activity were aristeromycin (Ari, **1**) and neplanocin A (NpcA, **3**).<sup>7–9</sup> Unfortunately their close resemblance to adenosine rendered them susceptible to recognition and phosphorylation by cellular kinases, resulting in significant toxicity.<sup>10</sup> In response to this, strategic removal of the 4'-hydroxymethyl group of Ari and NpcA to afford the 4',5'-saturated (**2**) and 4',5'-unsaturated (**4**) derivatives proved to be successful, as both were potent inhibitors of SAHase.<sup>11,12</sup> Interestingly, neither served as a substrate for adenosine kinase or adenosine deaminase, thus did not exhibit the toxicity associated with Ari and NpcA.<sup>11,12</sup>

Related to this, a report<sup>5</sup> of potent activity by two carbocyclic pyrimidine nucleosides (Figure 2) against cytidine triphosphate (CTP) synthetase, in addition to numerous substituted pyrimidines against thymidylate synthase, prompted us to consider the design and synthesis of additional carbocyclic pyrimidine analogues (Figure 3), incorporating the truncated scaffold. In that regard, the first of the pyrimidine targets is described.

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## Chemistry

Traditional Mitsunobu coupling was envisioned as a logical route to realize the targets. The cyclopentenyl alcohol precursor precursor<sup>13,14</sup> was obtained in good yield, and, as shown in Scheme 1, coupled to N<sup>3</sup>-Bzuracil. The benzoyl protecting group was removed from the coupled product by stirring with a 1% NaOH solution in MeOH to afford analogue **13** in 45% yield for two steps. This precursor is extremely important to this synthesis because from it three different routes can be employed. The first included deprotection of analogue **13** with a mixture of TFA and H<sub>2</sub>O to result in the first final target **9**. Secondly, in order to get the next immediate target, analogue **13** was first reacted with TIPBSCl then followed with mild ammonolysis conditions to give analogue **14** which was then deprotected in a similar fashion as with analogue **9** to give final target **10**.

In order to synthesize the final two targets in this series, it was necessary to reduce the double bond of the cyclopentyl ring system and as a result analogue **13** was reduced using catalytic hydrogenation to give saturated cyclopentyl analogue **15** (Scheme 2). The isopropylidene group of analogue **15** was removed to give final target **11**. The oxo group of analogue **15** was also converted to amino **16**, which was then deprotected to give final target **12**.

The four targets were then screened for their ability to inhibit SAHase, DNA methyltransferase (MTase), and CTP synthetase. No activity was seen for any of the analogues against DNA methyltransferase, or CTP synthetase (data not shown). For SAHase, in the first experiment the concentration of inhibitor was varied while the concentration of substrate (SAH) was held constant at 10  $\mu$ M. Conversion of SAH to adenosine and then to inosine was monitored at 265 nm (Figure 4). In the second experiment the concentration of inhibitor was held constant at 45  $\mu$ M while the concentration of substrate was varied. Initial rates in the presence and absence of inhibitors were measured (Figure 5). There was no change in absorbance at 265 nm in the absence of SAH. These experimental data best fit a competitive inhibition mechanism. The  $K_i$  value for compound **10** was found to be  $5.0 \pm 0.9 \mu$ M against SAHase in the hydrolysis direction, based on a  $K_m$  value of 7.9  $\mu$ M for the SAH substrate.<sup>15</sup> Ongoing studies suggest that the mechanism may be more complicated than a simple competitive inhibition mechanism, so that the “true” inhibitory form probably binds to the enzyme tighter than SAH. Detailed mechanistic studies will be reported in the future.

The four compounds were also screened for their ability to inhibit S-adenosylhomocysteine (SAH) nucleosidase (EC 3.2.2.9). SAH nucleosidase irreversibly cleaves SAH and 5'-methylthioadenosine (MTA) to form adenine and the analogous thioribose. This enzyme plays an important role in microbial pathways such as quorum sensing, biological methylation, polyamine synthesis, and methionine recycling. Inhibition of SAH nucleosidase has been shown to lead to a buildup of SAH and MTA, which are feedback inhibitors to various enzymes. This enzyme, encoded by the *pfs* gene, is presented in about half of all bacterial species, including *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, *Salmonella typhimurium*, *Haemophilus influenzae*, *Vibrio cholerae*, and *Bacillus anthracis*; but is not found in mammals.<sup>16,17</sup> Knockout of the *pfs* gene has been found to be lethal or marked impaired in cell growth. All together, SAH nucleosidase represents an attractive target for antimicrobial drug therapy. For SAH nucleosidase inhibition, concentration of inhibitor was varied while the concentration of substrate (SAH) was held constant at 10.8  $\mu$ M. Consumption of substrate was monitored at 265 nm. Compound **10** inhibited about 50% of the activity at approximately a 13-fold excess (data not shown). Given the limited structural similarity between the SAH substrate and compound **10**, the finding was unexpected. Thus in spite of its moderate inhibitory activity, compound **10** may serve as a lead compound for different approach to inhibit this nucleosidase.

## EXPERIMENTAL

### General

Melting points are uncorrected.  $^1\text{H}$  and  $^{13}\text{C}$  spectra were operated at 300 and 75 MHz, respectively, all referenced to internal tetramethylsilane (TMS) at 0.0 ppm. The spin multiplicities are indicated by the symbols s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and b (broad). Reactions were monitored by thin-layer chromatography (TLC) using 0.25 mm Whatman Diamond silica gel 60-F<sub>254</sub> precoated plates. Column chromatography was performed on Whatman silica, 200–400 mesh, 60 Å and elution with the indicated solvent system. Yields refer to chromatographically and spectroscopically ( $^1\text{H}$  and  $^{13}\text{C}$  NMR) homogeneous materials.

#### (1'S, 2'R, 3'S)-1-[(2',3'-O-isopropylidene)-4'-cyclopenten-1'-yl]uracil (**13**)

To cyclopenten-ol (6.50 g, 41.67 mmol), *N*<sup>3</sup>-Bzuracil (18.02 g, 83.33 mmol) and PPh<sub>3</sub> (21.88 g, 83.33 mmol) in anhydrous CH<sub>3</sub>CN (500 mL) at 0 °C was added dropwise DIAD (16.85 g, 83.33 mmol, 16.52 mL). After stirring at rt for 15 h the mixture was concentrated and purified by column chromatography (EtOAc:hexanes, 3:1) to afford 9.74 g of 3-benzoyl-1'-(2',2'-dimethyl-4',6'-dihydro-3'H-cyclopenta[1',3']dioxol-4'-yl)-1H-pyrimidine-2,4-dione as a white solid.  $^1\text{H}$  NMR (DMSO-*d*<sub>6</sub>): δ 1.23 (3H, s), 1.3 (3H, s), 4.71 (1H, d), 5.27 (1H, s), 5.29 (1H, d), 5.81 (1H, d), 5.84 (1H, dd), 6.23 (1H, dt), 4.71 (1H, d), 5.29 (2H, d), 5.84 (1H, d), 5.85 (1H, d), 6.22 (1H, m), 7.51 (1H, d), 7.57 (2H, t), 7.78 (1H, t), 7.97 (2H, m).  $^{13}\text{C}$  NMR (DMSO-*d*<sub>6</sub>): δ 24.4, 26.2, 69.1, 83.2, 84.6, 101.1, 111.8, 128.9, 129.0, 130.1, 131.6, 135.0, 139.0, 143.0, 149.9, 163.0, 168.8.

NaOH (10 mL, 1% in MeOH) was added to the coupled product and the mixture was allowed to stir at rt for 12 h. After this time the mixture was neutralized with HCl (1N), the solvent removed and the resulting residue was dissolved in EtOAc (50 mL), washed with H<sub>2</sub>O (25 mL), dried (MgSO<sub>4</sub>), filtered, and concentrated to give a solid. This solid was purified by column chromatography (EtOAc:hexanes, 4:1) to afford **13** (7.50 g, 71%) as a white solid.  $^1\text{H}$  NMR (DMSO-*d*<sub>6</sub>): δ 1.22 (3H, s), 1.31, (3H, s), 4.53 (1H, d), 5.27 (2H, m), 5.52 (1H, dd), 5.77 (1H, dd), 6.19 (1H, d), 7.23 (1H, d), 11.31 (1H, s).  $^{13}\text{C}$  NMR (CD<sub>3</sub>OD): δ 24.4, 26.2, 68.4, 83.4, 84.6, 101.2, 111.7, 129.3, 138.5, 142.5, 151.3, 165.0. Anal. Calcd. For C<sub>12</sub>H<sub>14</sub>N<sub>2</sub>O<sub>4</sub>: C, 57.59; H, 5.64; N, 11.19. Found: C, 57.92; H, 5.75; N, 11.18.

#### (1'S, 2'R, 3'S)-1-[(2',3'-Dihydroxy)-4'-cyclopenten-1'-yl]uracil (**9**)

A mixture of **13** (0.15 g, 0.60 mmol) in TFA/H<sub>2</sub>O (2:1, 4 mL) was allowed to stir for 12 h at rt upon which time the TFA/H<sub>2</sub>O was removed by evaporation. The resulting residue was coevaporated with MeOH (3 × 10 mL) to remove trace amounts of TFA. The resulting residue was then purified using C-18 HPLC eluting 90:10, H<sub>2</sub>O:MeOH → 50:50, H<sub>2</sub>O:MeOH → 10:90, H<sub>2</sub>O:MeOH afford **9** (0.106 g, 83 %) as a white solid.  $^1\text{H}$  NMR (CD<sub>3</sub>OD): δ 4.10 (1H, t), 4.55–4.60 (1H, dd), 5.45–5.50 (1H, dd), 5.70 (1H, d), 5.90 (1H, d), 6.20–6.23 (1H, dd), 7.40 (1H, dd).  $^{13}\text{C}$  NMR (CD<sub>3</sub>OD): δ 66.6, 73.0, 76.5, 101.6, 132.4, 136.7, 142.3, 151.8, 165.0. HRMS: calcd for C<sub>9</sub>H<sub>10</sub>N<sub>2</sub>O<sub>4</sub> (M + H)<sup>+</sup>, 211.19; found, 211.07.

#### (1'S, 2'R, 3'S)-1-[(2',3'-O-isopropylidene)-4'-cyclopenten-1'-yl]cytosine (**14**)

To a solution of **13** (0.75 g, 3.00 mmol), DMAP (1.47 g, 12.00 mmol) and Et<sub>3</sub>N (20 mL) in anhydrous CH<sub>3</sub>CN (100 mL) at 0 °C was added portionwise TIPBSCI (3.63 g, 12.00 mmol). After stirring at rt for 3 h NH<sub>4</sub>OH (50 mL) was added and the mixture continued to stir for 12 h at rt. The solvent was then removed and the resulting residue was purified via column chromatography (EtOAc:acetone:EtOH:H<sub>2</sub>O, 6:1:1:0.5) to afford **14** (0.58 g, 78%) as a white solid.  $^1\text{H}$  NMR (CD<sub>3</sub>OD): δ 1.27 (3H, s) 1.38 (3H, s), 4.53–4.54 (1H, d), 5.32–5.34 (1H, m),

5.43 (1H, m), 5.76–5.78 (1H, m), 5.81–5.83 (1H, d), 6.19–6.21 (1H, m), 7.30–7.32 (1H, m). <sup>13</sup>C NMR (CD<sub>3</sub>OD): δ 24.6, 26.3, 69.3, 83.8, 84.7, 94.8, 111.54, 130, 138.1, 142.8, 157.4, 166.3. Anal. Calcd. For C<sub>12</sub>H<sub>15</sub>N<sub>3</sub>O<sub>3</sub> (0.75 H<sub>2</sub>O): C, 54.88; H, 6.33; N, 16.00. Found: C, 54.64; H, 5.83; N, 15.73.

**(1'S, 2'R, 3'S)-1-[(2',3'-Dihydroxy)-4'-cyclopenten-1'-yl]cytosine, 3'-(10)**

See procedure **9** for other reaction details. Compound **14** (0.15 g, 0.60 mmol) and TFA (4 mL) afforded **10** (0.11 g, 87%) as a white solid. <sup>1</sup>H NMR (CD<sub>3</sub>OD): δ 4.03–4.05 (1H, t), 4.58–4.59 (1H, m), 5.47–5.50 (1H, m), 5.90–5.98 (2H, dd), 6.19–6.21 (1H, m), 7.55–7.56 (1H, d). <sup>13</sup>C NMR (CD<sub>3</sub>OD): δ 67.8, 73.7, 76.9, 95.1, 132.6, 136.4, 142.6, 157.9, 166.0. Anal. Calcd. For C<sub>9</sub>H<sub>11</sub>N<sub>3</sub>O<sub>3</sub>: C, 51.67, H, 5.30, N, 20.09. Found: C, 51.78, H, 5.39, N, 19.70.

**(1'S, 2'R, 3'S)-1-[(2',3'- O-isopropylidene)-cyclopent-1'-yl]uracil -(15)**

To **13** (0.25 g, 1 mmol) in MeOH (10 mL) was added Pd/C (10 mol %, 0.025 g). This mixture was hydrogenated at a pressure of 25 psi for 20 min. The Pd/C was removed by filtration and the filtrate was concentrated to afford 0.26 g of **15** in quantitative yield as a white solid. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 1.20 (3H, s), 1.35 (3H, s), 1.65–1.82 (2H, m), 1.95–2.15 (2H, m), 4.63 (1H, m), 4.64 (1H, m), 4.71 (1H, dd), 5.52 (1H, d), 7.58 (1H, d) and 11.26 (1H, s). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) δ 24.4, 27.3, 29.1, 31.2, 63.9, 80.3, 84.4, 101.8, 110.0, 143.9, 152.6, 163.8. Anal. Calcd. For C<sub>12</sub>H<sub>16</sub>N<sub>2</sub>O<sub>4</sub> (0.25 H<sub>2</sub>O): C, 56.30; H, 6.50; N, 10.95. Found: C, 56.42; H, 6.44; N, 10.88.

**(1'S, 2'R, 3'S)-1-[(2',3'-Dihydroxy)-cyclopent-1'-yl]uracil (11)**

See procedure **9** for other reaction details. Compound **15** (0.25 g, 0.99 mmol) and TFA (15 mL) afforded **11** (0.19 g, 91%) as a white solid. <sup>1</sup>H NMR (CD<sub>3</sub>OD): δ 1.69–1.79 (2H, m), 2.06–2.21 (2H, m), 4.06 (1H, m), 4.22–4.25 (1H, dd), 4.61–4.67 (1H, q), 4.86 (1H, s), 5.65–5.67 (1H, d), 7.62–7.64 (1H, d). <sup>13</sup>C NMR (CD<sub>3</sub>OD): δ 19.9, 26.2, 59.5, 77.2, 85.9, 102.4, 141.3, 150.9, 163.6. Anal. Calcd. For C<sub>9</sub>H<sub>12</sub>N<sub>2</sub>O<sub>4</sub> (0.6 mol H<sub>2</sub>O): O: C, 48.49, H, 5.96, N, 12.57. Found: C, 48.79, H, 5.51, N, 12.20.

**(1'S, 2'R, 3'S)-1-[(2',3'-O-isopropylidene)-cyclopent-1'-yl]cytosine -(16)**

See procedure **14** for reaction details. Compound **15** (0.42 g, 1.67 mmol), DMAP (0.81 g, 6.66 mmol), Et<sub>3</sub>N (12 mL) and CH<sub>3</sub>CN (75 mL) afforded **16** (0.34 g, 81%) as a white solid. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 1.18 (s, 3H), 1.36 (s, 3H), 1.64–1.80 (m, 2H), 1.94–2.15 (m, 2H), 4.40–4.50 (m, 1H), 4.60–4.68 (dd, 1H), 4.69 (m, 1H), 5.65 (d, 1H), 7.10 (br s, 1H), 7.30 (br s, 1H), 7.50 (d, 1H). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 22.3, 23.8, 29.5, 30.5, 64.9, 80.6, 84.7, 110.7, 144.9, 163.0.

**(1'S, 2'R, 3'S)-1-[(2',3'-Dihydroxy)-cyclopent-1'-yl]cytosine (12)**

See procedure for **9** for other reaction details. Compound **16** (0.10 g, 0.40 mmol) and TFA (5 mL) afforded **12** (0.07 g, 85%) as a white solid. <sup>1</sup>H NMR (DMSO-*H* *d*<sub>6</sub>): δ 1.4–1.6 (m, 2H), 1.8–2.1 (m, 2H), 3.85 (s, 1H), 4.05 (s, 1H), 4.3–4.6 (m, 2H), 4.61–4.8 (m, 1H), 5.65 (s, 1H), 6.95 (s, 2H), 7.55 (d, 1H). <sup>13</sup>C NMR (DMSO-*C* *d*<sub>6</sub>): δ 39.4, 39.6, 63.1, 71.1, 75.5, 93.8, 144.7, 156.6, 165.8. Anal. Calcd. For C<sub>9</sub>H<sub>13</sub>N<sub>3</sub>O<sub>3</sub> (0.5 mol H<sub>2</sub>O): C, 49.08, H, 6.41, N, 19.08. O): Found: C, 49.21, H, 6.03, N, 18.73.

**SAHase Inhibition Assay**

A reported enzyme-coupled continuous assay in the hydrolysis direction was employed. In this assay, SAH is hydrolyzed to homocysteine and adenosine, which is subsequently converted by adenosine deaminase into ammonia and inosine, a process associated with an absorbance

decrease around 265 nm.<sup>15</sup> All assays were performed in thermostatted 1 cm quartz cuvettes at 37 °C maintained by a Peltier unit on a Cary 100 ultraviolet-visible photospectrometer. In a total volume of 928  $\mu$ L, a typical enzyme assay solution contained 50 mM potassium phosphate at pH 7.4, 0.39 units of adenosine deaminase (Worthington Biochemical, catalog number LS009043), 132 nM of SAHase (final concentration; provided by Lynne Howell), 10  $\mu$ M of SAH (final concentration) and various concentrations of inhibitors. The reactions were initiated by the addition of SAH. Under these conditions, SAH hydrolysis catalyzed SAHase was rate limiting (data not shown). The kinetic data were analyzed using KaleidaGraph 4.0 (Synergy). Based on a competitive inhibition mechanism, the  $K_i$  value was determined using the equation,  $v = k_{cat} \times [S] \times [E] / \{K_m \times (1 + [I]/K_i) + [S]\}$ ; and  $v$ ,  $k_{cat}$ ,  $[S]$ ,  $[E]$ ,  $K_m$ ,  $[I]$  and  $K_i$  stand for the initial reaction rates, rate constant, substrate concentration, enzyme concentration, Michaelis-Menten constant, inhibitor concentration and dissociation constant of the enzyme-inhibitor complex, respectively. The  $K_m$  value for SAH in the hydrolysis direction was assumed to be 7.9  $\mu$ M as previously reported.<sup>15</sup>

### SAH Nucleosidase Assay

In this assay<sup>18</sup>, SAH is hydrolyzed into S-ribosylhomocysteine ribosylhomocysteine and adenine, which is subsequently converted by adenine deaminase into ammonia and hypoxanthine, a process associated with a decrease in absorbance around 265 nm. Assays were performed in thermostatted 1-cm quartz cuvettes at 37 °C maintained by a Peltier unit on a Cary 100 ultraviolet-visible photospectrometer. In a total volume of 920  $\mu$ L, a typical enzyme assay solution contained 50 mM KPi (pH 7.4), 100  $\mu$ M MnSO<sub>4</sub>, 30.0 nM adenine deaminase, 4.2 nM SAH nucleosidase, 10.8  $\mu$ M SAH, and various concentrations of inhibitors. The reaction was initiated by the addition of SAH. Under the assay conditions, SAH hydrolysis catalyzed by the nucleosidase was rate limiting (data not shown). Manganese or other divalent ions (e.g., zinc) are required for adenine deaminase activity.<sup>19,20</sup>

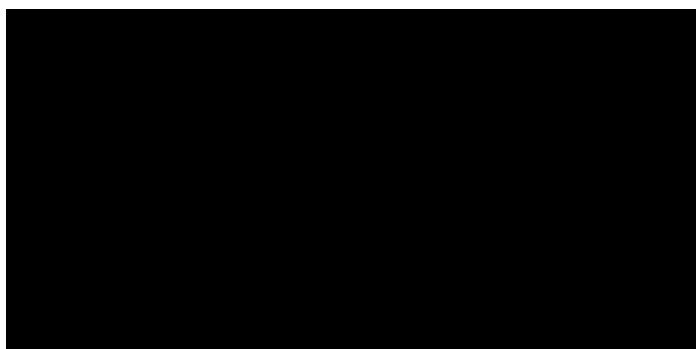
### Acknowledgements

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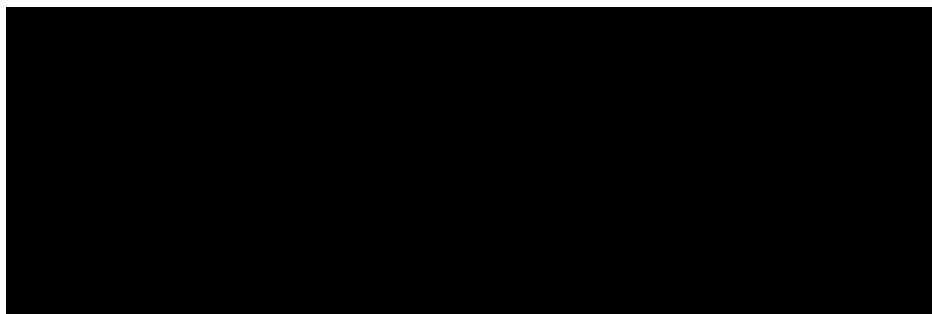
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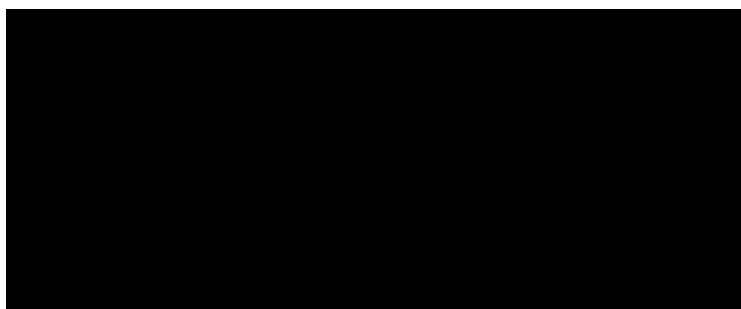


**Figure 1.**  
Potent Carbocyclic SAHase Inhibitors.

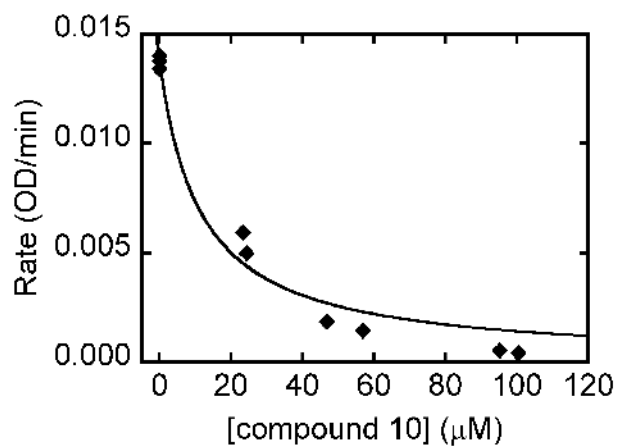


**Figure 2.**  
Carbocyclic derivatives of uridine and cytidine

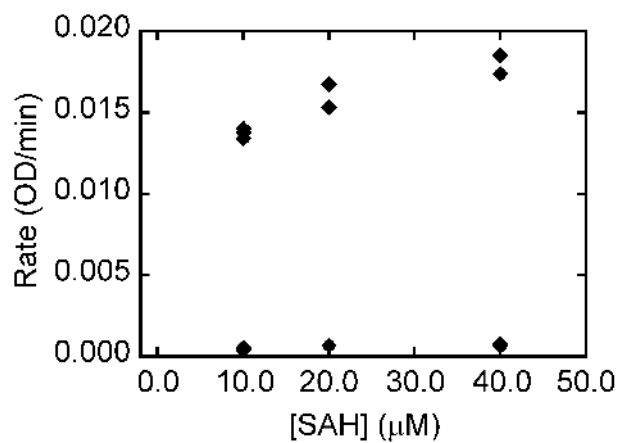




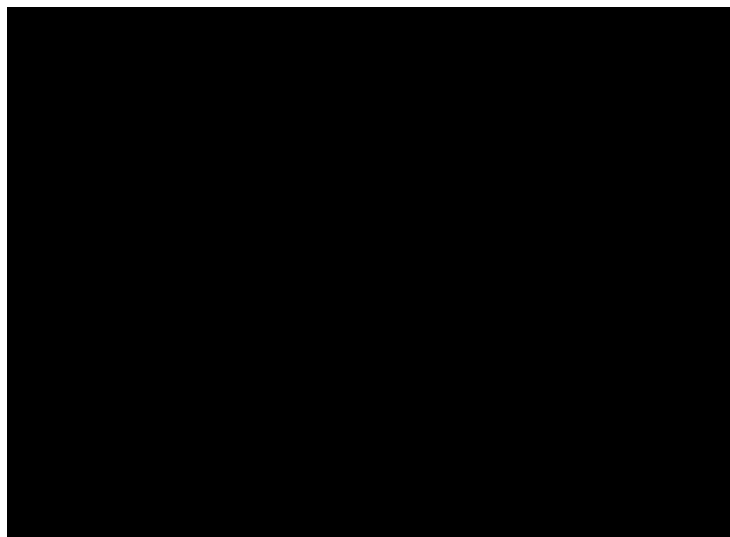
**Figure 3.**  
Carbocyclic Targets.



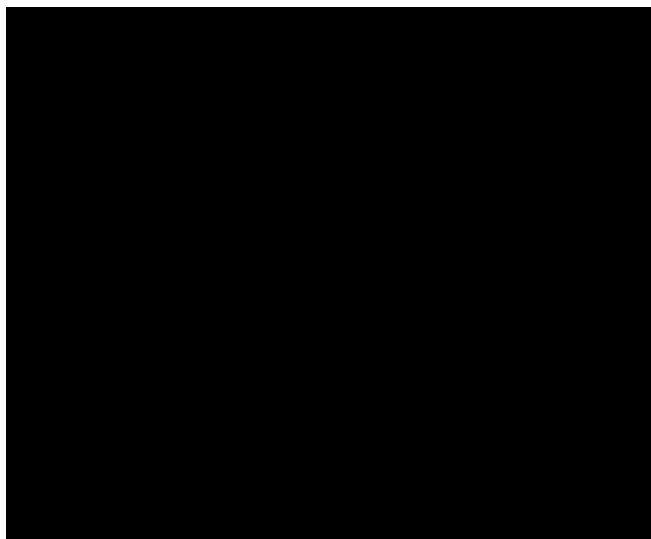
**Figure 4.** Inhibition of SAHase by **10**. Assay solutions contained 50 mM potassium phosphate at pH 7.4, 0.39 units of adenosine deaminase, 132 nM of SAHase, 10 μM of SAH (substrate), and various concentrations of **10**. Consumption of substrate was monitored at 265 nm.



**Figure 5.** Inhibition of SAHase by **10**. In the assay solution, the concentration of **10** was held constant at 45  $\mu\text{M}$  while the concentration of substrate was varied. Rates in the presence (data points at bottom of plot) and absence (data points at top of plot) of inhibitors were measured.

**Scheme 1.**

Reagents and conditions: (a) i. N<sup>3</sup>-Bz-uracil, PPh<sub>3</sub>, DIAD, CH<sub>3</sub>CN, 0 °C to rt, 70%, ii. NaOH in MeOH (1%),rt, 15h, 75%; (b) TFA:H<sub>2</sub>O (2:1), rt, 3h, 95%; (c) i. TIPBSCI, NEt<sub>3</sub>, DMAP, CH<sub>3</sub>CN, rt, 4 h; ii. NH<sub>4</sub>OH, rt, 15 h, 65%.

**Scheme 2.**

Reagents and conditions: (a) H<sub>2</sub> (25 psi), Pd/C, EtOH, rt, 30 min, quant.; (b) TFA/H<sub>2</sub>O (2:1), rt, 3 h, 95%; (c) i. TIPBSCI, NEt<sub>3</sub>, DMAP, CH<sub>3</sub>CN, rt, 4 h; ii. NH<sub>4</sub>OH, rt, 15 h, 65%.