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# Novel adenosine-derived inhibitors of *Cryptosporidium parvum* inosine 5'-monophosphate dehydrogenase

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This article is dedicated to the 88th anniversary of the birth of Dr. Kiyoshi Isono.

**Abstract**

**We have found cyclophane-type adenosine derivatives having *p*-quinone amide moieties (1 and 2) as weak inhibitors of *Cryptosporidium parvum* inosine 5'-monophosphate dehydrogenase (CpIMPDH) from the Hokkaido University Chemical Library *via* the luciferase-based high-throughput screening. To obtain more potent inhibitors, we synthesized four new derivatives free from cyclophane rings (3, 4, 5, and 6). The *N*-H derivatives 3 and 5 showed more potent activities (24.4 and 11.1 μM, respectively) in the presence of dithiothreitol (DTT), whereas the *N*-methyl derivative 4 indicated more potent activity (2.1 μM) without DTT. Conformational analysis of compounds 3 and 4 suggested that *N*-H amide 3 binds to IMP binding site in the DTT mediated manner.**

## Introduction

*Cryptosporidium parvum* is a waterborne pathogen, which causes watery or mucoid diarrhea and abdominal pain in many mammal species, including human. The infection is usually self-limiting in immunocompetent patients. Unfortunately, the infections could be severe in immunocompromised patients, such as children, elderly person, or AIDS patients [1–3].

For the prevention of such infections, we have focused on the inhibition of protozoan inosine 5'-monophosphate dehydrogenase (IMPDH) because these microbes rely solely on the IMPDH-mediated guanosine nucleotides biosynthesis. In previous work, we established a luciferase-based high-throughput screening system to identify IMPDH inhibitors from the Pharmakon repositioning library of off-patent US Food and Drug Administration (FAD)-approved compounds (Microsouce Discovery Systems, Inc., CT). The screening of 1,600 compounds resulted in the discovery of three irreversible inhibitors: disulfiram, bronopol, and ebselen. In the efficacy experiment against cryptosporidiosis in severe combined immunodeficiency (DCID) mouse, a decrease in the number of oocyst shed was observed on the oral administration of disulfiram and bronopol [4].

Next, we focused on the chemical library from the Faculty of Pharmacy, Hokkaido University (Hokkaido University Chemical Library). The identified cyclophane-type compounds **1** and **2** as hit-compounds in the first screening, showed relatively weak activities. Therefore, four novel derivatives of **1** and **2** were synthesized and three

compounds **3**, **4**, and **5** showed potent inhibitory activities. In this article, we report synthesis and biological activity of these novel adenosine-derived CpIMPDH inhibitors.

## Results and Discussion

### High–throughput screening of CpIMPDH inhibitors from a chemical library

For the discovery of new protozoan IMPDH inhibitors, a high–throughput screening was performed by using Hokkaido University Chemical Library, which comprises 1,600 compounds derived from Faculty of Pharmacy, Hokkaido University. The screening system providing high accuracy (*Z'*-factor value of 0.7) [4] has been previously established by the authors based on the detection of NADH consumed by luciferase. In addition, the assay was designed so as to sift out $NAD^+$ mimics as hits by adding the excess amount of $NAD^+$ (1.6 mM), because the cofactor ubiquitously functions in diverse biological systems. After screening, the activities of 1,260 were successfully evaluated from 1,600 compounds without mechanical errors, and 44 compounds showed comparable activities to mycophenolic acid, a known IMPDH inhibitor. These hits were next subjected to reproducibility test with triplicating assays. The reproducibility was affirmed only in 19 compounds out of 44. Interestingly, 15 of 19 compounds were adenosine derivatives as typified by cyclophane-type **1** and **2** (Fig. 1), which had been originally developed targeting heat shock protein 90 [5,6]. The ribonucleoside substructures are frequently conserved in IMPDH inhibitors mimicking both IMP and $NAD^+$ [7]. Thus, these compounds could be regarded as the promising lead compounds to develop novel IMPDH inhibitors. On the other hand, the cyclophane skeleton is rarely seen in IMPDH inhibitors, except for naturally occurring halicyclamine A [8–13]. Therefore, we next designed new adenosine derivatives **3**–**6** of which *p*-quinones were liberated from cyclophane and undertook syntheses of these compounds.

Fig. 1

### Synthesis of compounds 3, 4, 5 and 6

Nucleoside unit **12** was first synthesized as shown in Scheme 1. 2',3'-Hydroxy groups and 5'-hydroxy group of adenosine **7** were protected with isopropylidene and TBS groups, respectively to give **9** [14, 15]. After protecting 6-amino group with trityl group, TBS group was effectively deprotected by tetrabutylammonium fluoride (TBAF) to give 5'-alcohol **11**. Dess-Martin oxidation of **11** afforded 5'-aldehyde **12**, which was subjected to Horner-Wadsworth-Emmons (HWE) reaction without further purification.

Phosphonate units, **18** and **21** were synthesized as shown in Scheme 2. Methoxyhydroquinone **13** was protected with MOM group followed by nitration [16] with ammonium nitrate in the presence of trifluoroacetic anhydride to give desired **15** and deprotected **16,** which was converted to **15** by protection with MOM group. Nitro group of **15** was converted to amine to give **17**, which was converted to *N*-H phosphonate **18** by coupling with diethylphosphonoacetic acid in 95% in two steps [17]. To synthesize *N*-methyl phosphonate **21**, compound **15** was converted to trifluoroacetoamide **19** by catalytic hydrogenation followed by protection with trifluoroacetyl group. Methylation, deprotection of trifluoroacetyl group, and acylation with bromoacetyl chloride afforded **20**. Arbuzov reaction of **20** with triethylphosphite gave desired *N*-methyl phosphonate **21** in 84% yield from **15**.

Scheme 1

Scheme 2

Scheme 3

HWE reaction of aldehyde **12** with *N*-H phosphonate **18** gave desired compound **22** under the neutral conditions using zinc triflate, triethylamine, and *N*,*N*,*N'*,*N"*-tetramethylethylenediamine developed by Schauer et al [18]. Similarly, compound **23** was synthesized from **12** and *N*-methyl phosphonate **21**. Compound **22** and **23** were converted to 5'-substituted adenosine **3** and **4**, respectively. Hydrogenation of compound **3** with Pd/C gave saturated amide **5**. Although the same treatment of **4** gave compound **6**, the activity was not tested due to the limited amount (Scheme 3).

**Evaluation of IMPDH inhibitory activities**

The inhibitory activities of hit compounds **1**, **2**, synthetic compounds **3**, **4** and **5** against recombinant CpIMPDH were evaluated. The apparent $IC_{50}$ values were determined using essentially the same conditions as the luciferase-based high-throughput assay, except that different concentrations were used (Table 1). Interestingly, synthetic compounds **3**–**5** showed remarkable activities ($IC_{50}$: 0.7–1.3 μM) compared with hit compounds (>50 μM) as we expected. Next, we determined $IC_{50}$ values on fluorescence-based *low*-throughput assay (Ex 340 nm/Em 465 nm) to eliminate the possible effect by luciferase in the activity. As shown in Table 2, compounds **3** and **5** showed inferior activities to those in luciferase-based assay ($IC_{50}$: 209.8 and >500.0 μM, respectively), whereas **4** retained same activity. The result would reflect partial inhibitions by **3** and **5** against luciferase activity. Computational conformation analyses predicted that *N*-H amide **3** is in an equilibrium between *trans*-amide (M3-007 in Fig. 2a) and *cis*-amide (M3-078) at the approximate ratio 96:4, while *N*-methyl amide **4** is uniformly present as *cis*-amide in any possible

Table 1

conformer (M4-015, -003, -009, -011 and -071 in Fig. 2b). It can be speculated that *cis* conformation of **4** enables selective inhibition against CpIMPDH.

In the assay of enzymes that require cysteine as a catalytic residue, reducing agents such as dithiothreitol (DTT) or glutathione (GSH) are commonly added to protect the residue from inactivators. Also in IMPDH, the cysteine residue positioned near IMP binding site (*e.g*. Cys331 in human type II IMPDH) is known to play a critical role in the catalysis. Thus, we next evaluated the activities of **3**–**5** in the presence of 1.0 mM DTT. These inhibitors showed the $IC_{50}$ values at 11.1–76.8 μM against CpIMPDH in DTT supplemented conditions, and the values were similar against human type II IMPDH (Table 2). CpIMPDH is known to have a dramatic difference in $NAD^+$ binding site from that of hIMPDH II. Especially the residues related to the binding of adenine ring, such as His253, Phe282, Thr45 and Gln469, are not conserved in CpIMPDH, which discriminates the specificity to $NAD^+$ *versus* nicotinamide hypoxanthine dinucleotide [19]. The similar $IC_{50}$ values between Cp- and hIMPDH II may be due to the less contribution of adenine ring recognition. The addition of reducing agents often attenuates the activity due to the competition or the direct contact with inhibitors [20], and the same phenomena were observed in bronopol, ebselen and disulfiram, previously discovered Cys-targetting inhibitors [4] (Table 2). Among the synthetic compounds, only *N*-methyl derivative **4** behaves according to these precedent examples (76.8 μM with DTT), which in turn suggests that cysteine residue is involved in this inhibition, such as *via* Michael addition to quinone moiety. In contrast, it would be noteworthy that compounds **3** and **5** showed superior activities in the DTT-supplemented conditions. This pronounced difference would stem from the conformational preference of each compound. The “stretched” conformation in compounds **3** and **5** conjures a binding model seen in C2-mycophenolic adenine dinucleotide (C2-MAD), a ligand mimicking $NAD^+$ [21]. Given that **3** and **5** bind in the same manner as C2-MAD, the improved activity may be explained by crosslinking *via* DTT between Cys and *p*-quinone moieties, because they are to be positioned away by 5–10 Å. Meanwhile, these compounds can possibly adopt a different binding manner from C2-MAD considering the screening system set to eliminate $NAD^+$ mimics and the above-discussed similarity in $IC_{50}$ values with hIMPDH. Obviously, this is true also for compound **4**. Although further investigations, such as X-ray crystallography or detailed docking simulation are required, each *N*-H and *N*-methyl amide was proved to have clearly different mode of binding against CpIMPDH. This variance may provide the

Table 2

Fig. 2

optional application of these compounds, such as combined or selective use depending on different physiological conditions.

## Conclusion

By the use of high-throughput screening system, cyclophane-type adenosine derivatives furnishing *p*-quinone moieties (**1** and **2**) were discovered as weak inhibitors of CpIMPDH from the Hokkaido University Chemical Library. To ameliorate the activities, we designed and synthesized four new derivatives of which *p*-quinones were liberated from cyclophane (**3**, **4**, **5** and **6**). All tested compounds (**3**, **4** and **5**) showed more potent activities at the absence of dithiothreitol (DTT) than the hit compounds (**1** and **2**) in luciferase-based high-throughput assay. Interestingly, the supplement of DTT in fluorescence-based *low*- throughput assay differentiated the activity of *N*-methyl amide **4** from those of *N*-H amides **3** and **5**, which would stem from the conformational difference between *cis*- and *trans*-amides. These insights raised a significance of impact by reducing agents in IMPDH assay and will be helpful for the development of novel IMPDH inhibitors.

## Experimental procedures

### Chemistry

Organic solvents were purified by normal methods. Unless otherwise stated, all commercially available chemicals of the highest purity were used without further purification. Thin layer and column chromatography were performed with Merck Silica Gel 60 (0.063–0.020 mm) and Merck Silica Gel 60 (0.040–0.063 mm), respectively. All NMR spectra were measured with JNM-ECA-500, JNM-AL-400 or JNM-ECX-400P (JEOL, Tokyo, Japan). Chemical shifts are defined using tetramethylsilane as the internal standard, except for the measurement with DMSO-$d_6$ in which solvent peaks (2.49 ppm for $^{1}$H and 39.7 ppm for $^{13}$C) were used. Coupling constants (*J*) are given in Hz. Mass spectra were acquired with ESI techniques using JMS-HX 110, JMS-700TZ or JMS-T100LP (JEOL, Tokyo, Japan).

**2',3'-*O*-Isoprppylideneadenosine** (**8**)

Adenosine (20 g, 75 mmol) was dissolved in 800 mL of acetone–DMF (3:1, v/v) and to the mixture was added 14 g of TsOH monohydrate (75 mmol). After 4 hours stirring, 19 mL of 2,2-dimethoxypropane (150 mmol) was added and the reaction was allowed to stir for another 6 hours. The mixture was partially evaporated and partitioned between EtOAc (200 mL) and sat. $Na_2CO_3$ aq.. The water layer was washed with $CHCl_3$ and $CH_2Cl_2$. Combined organic layer was then washed with brine (100 mL) and dried over anhydrous $Na_2SO_4$. After the removal of solvent *in vacuo*, 2',3'-*O*-isoprppylideneadenosine (**8**) was recovered by trituration with EtOAc–hexane as colorless crystals (8.0 g, 35%). $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 8.32 (1H, s, H, H-2), 8.14 (1H, s, H-8), 7.34 (2H, s, H-6), 6.10 (1H, d, *J* = 2.8 Hz, H-1'), 5.33 (1H, dd, *J* = 6.4, 3.2 Hz, H-2'), 4.95 (1H, dd, *J* = 6.2, 2.5 Hz, H-3'), 4.20 (1H, m, H-4'), 3.52 (2H, m, H-5'), 1.53 (3H, s, Me), 1.31 (3H, s, Me).

**5'-*O*-*tert*-Butyldimethylsilyl-2',3'-*O*-isopropylideneadenosine** (**9**)

Imidazole (5.4 g, 79 mmol) and **7** were dissolved in 50 mL of DMF and *tert*-butyldimethylchlorosilane (6.0 g, 40 mmol) was added to the solution at 0 ºC under argon atmosphere. The reaction was quenched with MeOH in 1 hour and was partitioned between EtOAc (300 mL) and sat. $Na_2CO_3$ aq. (250 mL). The organic layer was washed with water (150 mL x 3) and brine (100 mL), dried over anhydrous $Na_2SO_4$

and evaporated *in vacuo*. The product **9** was recovered as colorless crystals by filtration, and also from the filtrate after evaporation and trituration with hexane (14 g, quant.). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.39 (lH, s, H-2), 8.05 (1H, s, H-8), 6.17 (1H, d, *J* = 2.8 Hz, H-1'), 5.48 (2H, br s, H-6), 5.27 (lH, dd, *J* = 6.4, 2.3 Hz, H-2'), 4.94 (1H, dd, *J* = 6.4, 2.3 Hz, H-3'), 4.43 (1H, dd, *J* = 6.9, 4.1 Hz, H-4'), 3.88 (1H, dd, *J* = 11.4, 4.1 Hz, H-5'a), 3.76 (1H, dd, *J* = 11.4, 4.1 Hz, H-5'b), l.64 (3H, s, Me), 1.41 (3H, s, Me), 0.84 (9H, s, *t*-Bu), 0.02 and 0.01 (6H, s, Me).

**5'-*O*-*tert*-Butyldimethylsilyl-2',3'-*O*-isopropylide-6-*N*-triphenylmethyladenosine** (**10**)

To a solution of **9** (10 g, 24 mmol) and triethylamine (7.9 mL, 57mmol) in 100 mL of $CH_2Cl_2$, 7.8 g of trityl chloride (28 mmol) was added at 0 ºC. The reaction was allowed to stir at room temperature then 50 ºC under argon atmosphere. After complication of the reaction, sat. $Na_2CO_3$ aq. was added and resulting white precipitate was filtered off. The filtrate was evaporated and dissolved in 200 mL of EtOAc. The organic layer was washed with 200 mL of water, 0.1 M HCl aq., sat. $Na_2CO_3$ aq. and brine. The EtOAc layer was dried over anhydrous $Na_2SO_4$ and evaporated *in vacuo*. The crude product was purified with silica gel column chromatography eluting with EtOAc:hexane = 1:10–3:1 then MeOH:$CHCl_3$ = 1:4 to give 11 g of **10** (71%) and 2.8 g of starting material **9** (20%). $^1$H NMR (400 MHz, $CDCl_3$) δ 8.06 (1H, s, H-2), 7.97 (1H, s, H-8), 7.30 (15H, m, Tr), 6.92 (1H, s, H-6), 6.12 (1H, d, *J* = 2.7 Hz, H-1'), 5.28 (1H, dd, *J* = 6.2, 2.7 Hz, H-2'), 4.93 (1H, dd, *J* = 6.4, 4.1 Hz, H-4'), 3.84 (1H, dd, *J* = 11.5, 4.1 Hz, H-5'a), 3.75 (1H, dd, *J* = 11.5, 4.1 Hz, H-5'b), 1.61 (3H, s, Me), 1.38 (3H, s, Me), 0.83 (9H, s, *t*-Bu), 0.00 and –0.02(6H, s, Me).

**2',3'-*O*-Isopropylidene-6-*N*-triphenylmethyladenosine** (**11**)

To a solution of **10** in THF (150 mL) was added 17 mL of tetra-*n*-butylammonium bromide 1 M solution at 0 ºC under argon atmosphere. After 40 minutes, the mixture was evaporated and directly subjected to a silica gel column chromatography (EtOAc:hexane = 1:1–5:1) to isolate **11** as an yellow foam (9.1 g, quant.). $^1$H NMR (400 MHz, $CDCl_3$) δ 7.98 (1H, s, H-2), 7.79 (1H, s, H-8), 7.30 (15H, m, Tr), 7.05 (1H, s, H-6), 5.83 (1H, d, *J* = 5.0 Hz, H-1'), 5.17 (1H, t, *J* = 5.7 Hz, H-2'), 5.08 (1H, d, *J* = 6.0 Hz, H-3'), 4.52 (1H, s, H-4'), 3.93 (1H, dt, *J* = 12.8, 1.8 Hz, H-5'a), 3.75 (1H, dt, *J* = 12.8, 1.8 Hz, H-5'b),

1.64 (3H, s, Me) , 1.36 (3H, s, Me).

**(*E*)-*N*-(3-Methyl-1,4-bismethoxymethylphenyl)-3-{(3a*R*,4*R*,6*R*,6a*R*)-6-(6-triphenylmethylamino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl}prop-2-enamide** (**22**)

Alcohol **4** (550 mg, 1.0 mmol) was dissolved in $CH_2Cl_2$ (10 mL) and excess amount (>4.0 eq.) of Dess–Martin periodinane was added at 0 ºC under argon atmosphere. After complication of the reaction, sat. $Na_2S_2O_3$ aq. and sat. $Na_2CO_3$ aq. were added. To the mixture was added 20 mL of water and extracted with EtOAc (30 mL). The organic layer was washed with sat. $Na_2CO_3$ aq. (20 mL) and brine (20 mL), dried over anhydrous $Na_2SO_4$ and evaporated *in vacuo.* Resulting crude product (330 mg) was used in next step without further purification.

The aldehyde **18**, $Zn(OTf)_2$ (440 mg, 1.2 mmol), triethylamine (560 μL, 4.0 mmol) and TMEDA (180 μL, 1.2 mmol) was added to 3.0 mL of THF. A solution of **11** in THF (12 mL) was added to the mixture, which was allowed to stir for 15 under argon atmosphere. The mixture was evaporated and partitioned between EtOAc (40 mL) and water (40 mL). The organic layer was washed with 20 mL of water, 0.1 M HCl aq., sat. $Na_2CO_3$ aq. and brine. The EtOAc layer was dried over anhydrous $Na_2SO_4$ and evaporated *in vacuo.* Subsequent silica gel column chromatography eluting with EtOAc:hexane = 2:3–2:1 gave 14 as brown foam (420 mg, 67%). $^1$H NMR (400 MHz, $CDCl_3$) δ 8.05 (1H, s, purinyl H-2), 7.85 (1H, s, purinyl H-8), 7.58 (1H, s, phenyl), 7.30 (15H, m, Tr), 6.99 (1H, dd, *J* = 15.6, 5.0 Hz, olefinic H-3), 6.95 (1H, s, purinyl H-6), 6.77 (1H, s, phenyl), 6.13 (1H, dd, *J* = 15.6, 1.4 Hz, olefinic H-2), 6.10 (1H, d, *J* = 6.1 Hz, H-6), 5.49 (1H, dd, *J* = 6.1, 2.5 Hz, H-6a), 5.18 (2H, s, $OC\underline{H}_2OCH_3$), 5.05 (3H, m, H-3a and $OC\underline{H}_2OCH_3$), 4.83 (1H, t, *J* = 3.4 Hz, H-4), 3.83 (3H, s, OMe), 3.53 (3H, s, $OCH_2OC\underline{H}_3$), 3.41 (3H, s, $OCH_2OC\underline{H}_3$), 1.63 (3H, s, Me) , 1.38 (3H, s, Me); LR-ESI-MS *m*/*z* = 814.33 $[M]^+$.

**(*E*)-*N*-methyl-*N*-(3-Methyl-1,4-bismethoxymethylphenyl)-3-{(3a*R*,4*R*,6*R*,6a*R*)-6-(6-triphenylmethylamino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl}prop-2-enamide** (**23**)

Following the same procedure as synthesis of **22**, *N*-methyl product **23** was prepared (1.7 g, 57%) from the reaction between crude **12** and 1.6 g of **21** (3.6 mmol).

$^1$H NMR (400 MHz, $CDCl_3$, mixture of rotamers) δ 8.05 (1H, s, purinyl H-2), 8.03 (1H, s, purinyl H-2), 7.73 (1H, s, purinyl H-8), 7.71 (1H, s, purinyl H-8), 7.30 (30H, m, Tr), 6.90 (6H, m, purinyl H-6, olefinic H-3 and phenyl), 6.79 (1H, s, phenyl), 6.78 (1H, s, phenyl), 6.02 (4H, m, H-6 and olefinic H-2), 5.33 (1H, dd, *J* = 6.4, 2.8 Hz, H-6a), 5.26 (1H, dd, *J* = 6.4, 2.8 Hz, H-6a), 5.12 (8H, m, $OC\underline{H}_2OCH_3$), 4.88 (2H, m, H-3a), 4.59 (2H, m, H-4), 3.87 (6H, s, OMe), 3.49 (3H, s, $OCH_2OC\underline{H}_3$), 3.46 (3H, s, $OCH_2OC\underline{H}_3$), 3.39 (3H, s, $OCH_2OC\underline{H}_3$), 3.35 (3H, s, $OCH_2OC\underline{H}_3$), 3.22 (3H, s, NMe), 3.21 (3H, s, NMe), 1.56 (3H, s, Me), 1.55 (3H, s, Me), 1.32 (6H, s, Me); ESI-MS *m*/*z* = 828 .35 $[M]^+$.

**(*E*)-*N*-(4-Methoxy-*p*-benzoquinonyl)-3-{(3a*R*,4*R*,6*R*,6a*R*)-6-(6-triphenylmethylamino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl}prop-2-enamide** (**3**)

A precursor **22** was dissolved in 80% trifluoroacetic acid aqueous solution. After stirring for 25 minutes, dilution with EtOH and evaporation were repeated several times. The concentrate was dissolved in 15 mL of MeOH and Pd/C (Pd 10%) (31 mg) was added to the solution. The suspension was allowed to stir at $O_2$ atmosphere. After 25 minutes, Pd/C was removed by celite filtration and solvent was removed *in vacuo*. Subsequent recrystallization from MeOH gave 82 mg of **3** as yellow crystals (73%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.49 (1H, s, purinyl H-2), 8.26 (1H, s, purinyl H-8), 7.80 (2H, br s, purinyl H-6), 7.42 (1H, s, quinonyl), 7.01 (1H, dd, *J* = 16.0, 5.5 Hz, olefinic H-3), 6.82 (1H, dd, *J* = 16.0, 1.5 Hz, olefinic H-2), 6.15 (1H, s, quinonyl), 5.99 (1H, d, *J* = 5.0 Hz, H-6), 4.62 (1H, t, *J* = 5.0 Hz, H-6a), 4.53 (1H, t, *J* = 5.5 Hz, H-4), 4.22 (1H, t, *J* = 5.0 Hz, H-3a), 3.79 (3H, s, OMe); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 183.3, 182.8, 165.7, 159.9, 158.9, 153.5, 149.4, 144.1, 142.0, 140.7, 124.8, 119.6, 113.0, 105.8, 88.6 , 83.5, 74.2, 73 .7, 57.3; ESI-MS *m*/*z* = 442.12 $[M]^+$.

**(*E*)-*N*-(4-Methoxy-*p*-benzoquinonyl)-3-{(3a*R*,4*R*,6*R*,6a*R*)-6-(6-triphenylmethylamino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl}propanamide** (**5**)

Amide **3** (15 mg, 27 μmol) was dissolved in 6.0 mL of MeOH and to the solution was added 10 mg of Pd/C (Pd 10%). The reaction was allowed to stir under $H_2$ atmosphere for 8 hours followed by $O_2$ atmosphere for another 70 minutes. Pd/C was removed by celite filtration and solvent was evaporated *in vacuo.* Recrystallization with the mixed

solvent of MeOH, EtOAc and hexane gave propanamide **5** (8.1 mg, 54%) as yellow crystals.

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.45 (1H, d, $J$ = 3.6 Hz, purinyl H-2), 8.24 (1H, d, $J$ = 3.2 Hz, purinyl H-8), 7.86 (2H, br s, purinyl H-6), 7.24 (1H, s, quinonyl), 6.09 (1H, s, quinonyl), 5.85 (1H, d, $J$ = 5.6 Hz, H-6), 4.63 (1H, t, $J$ = 5.0 Hz, H-6a), 4.05 (1H, t, $J$ = 5.0 Hz, H-3a), 3.87 (1H, m, H-4), 3.79 (3H, s, OMe), 2.63 (2H, t, $J$ = 7.5 Hz, olefinic H-2), 1.93 (2H, m, olefinic H-3); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 182.7, 182.2, 173.8, 159.2, 156.1, 152.7, 149.4, 139.8, 139.8, 119.2, 111.6, 105.1, 87.4, 83.0, 73 .1, 73.0, 56.6, 32.9, 28.4, 22.1; ESI-MS $m/z$ = 467.13 $[M+Na]^+$.

**(*E*)-*N*-Methyl-*N*-(4-methoxy-*p*-benzoquinonyl)-3-{(3a*R*,4*R*,6*R*,6a*R*)-6-(6-triphenylmethylamino-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl}prop-2-enamide** (**4**)

Following the same procedure as synthesis of **3**, *N*-methyl product **4** was prepared in 77% yield. Recrystallization was performed with the combination of MeOH and EtOAc. $^1$H NMR (500 MHz, DMSO-$d_6$) δ 8.35 (1H, s, purinyl H-2), 8.20 (1H, s, purinyl H-8), 7.75 (2H, br s, purinyl H-6), 6.83 (1H, dd, $J$ = 14.9, 6.3 Hz, olefinic H-3), 6.78 (1H, s, quinonyl), 6.16 (1H, dd, $J$ = 14.9, 1.5 Hz, olefinic H-2), 6.12 (1H, s, quinonyl), 5.92 (1H, d, $J$ = 5.2 Hz, H-6), 4.64 (1H, t, $J$ = 5.2 Hz, H-6a), 4.46 (1H, t, $J$ = 6.3 Hz, H-4), 4.15 (1H, t, $J$ = 5.2 Hz, H-3a), 3.77 (3H, s, OMe), 3.12 (3H, s, NMe); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 182.9, 181.5, 165.3, 159.1, 153.9, 150.0, 149.0, 147.2, 141.6, 140.9, 127.1, 123.5 ,119.1, 106.7 , 87.8, 83.0, 73.7, 72.8, 56.8, 36.4; ESI-MS $m/z$ = 479.13 $[M+Na]^+$.

**Conformational conformation analysis**

All calculations were performed using the Spartan'16 (Wavefunction Inc., Irvine, CA, USA) in gas conditions. The structures of compound **3** and **4** were built and the conformer distributions were first analyzed by MMFF. For the given conformers of **3** and **4**, IDs were assigned as "M3-NNN" or "M4-NNN", respectively, where the numbers after prefixes are the order of relative energies after molecular mechanics calculation. The conformers within relative energies of 40 kJ/mol were geometrically optimized by Hartree–Fock/3-21G, and energies of resulting conformers within 40 kJ/mol were evaluated using density functional (DF)/ωB97 X-D/6-31G* calculation.

The conformers with >15 kJ/mol relative energies were discarded and geometry optimization was again performed using DF/ωB97 X-D/6-31G*. This series of DF calculations returned M3-007 and M4-015 as the lowest-energy conformers together with the conformers within 10 kJ/mol relative energies (one conformer for M3-007 and four conformers for M4-015; see Fig. 2). Finally, energies and Boltzmann distribution at 297 K of these conformers were calculated using DF/ωB97 X-V/6-311+G (2df, 2p).

**Biology**

**Expression and purification of recombinant CpIMPDH and human IMPDH II**

The PCR product coding CpIMPDH amplified with the primer sets 5'-TTTTGGATCCTCAAACATGGGTACA-3' and 5'-TTTTGAATTCCTATTTACT-ATAATT-3' was cloned into pCR2.1-TOPO vector (Invitrogen Japan KK, Tokyo, Japan). The target gene was digested by BamHI and EcoRI, and inserted into a pGEX-6P-2 plasmid (GE Healthcare Bio-Science Corp., UK). The plasmid was then transformed to ECOS *E. coli* BL21 (DE3) (Wako Pure Chemical Ind., Ltd., Japan). The cells were grown overnight at 30 °C in 50 mL 2 x YT broth containing 100 μg/mL ampicillin. Afterwards, the broth was subcultured to 700 ml of medium containing a final concentration of 1.0 mM isopropyl-1-thio-β-galactopyranoside (Sigma-Aldrich Japan, Tokyo, Japan) and 100 μg/mL ampicillin. After 5 h incubation at 25 °C, cells were harvested by centrifugation, washed with PBS solution, and stored frozen at –80 °C until usage.

hIMPDH II plasmid was a generous gift from Prof. Lizbeth Hedstrom, Brandeis University, USA. The plasmid was transformed in the same manner as CpIMPDH.

**High-Throughput Screening of IMPDH inhibitors**

The HTS assay was established using NAD(P)H-Glo Assay Kit (Promega, Madison, WI, USA). Briefly, 10μL of substrate solution containing 50 mM Tris-HCl pH 8.0, 200 mM KCl, 0.1 mg/mL BSA, 1.6 mM β-$NAD^+$ (Oriental Yeast Co., Tokyo, Japan), 100 μM IMP (Sigma-Aldrich Japan, Tokyo, Japan), and assay kit was added to white 384-well plates. Mycophenolic acid (APAC Pharmaceutical LLC, Hangzhou, China), a known IMPDH inhibitor, or the chemical library from the Faculty of Pharmacy, Hokkaido University were also added to the wells in concentration of 10 μM. Reaction was started by addition of 10 μL enzyme solution containing 50 mM Tris-HCl pH 8.0, 0.1 mg/mL BSA, and CpIMPDH. Reaction was carried out at 30 °C for 30 minutes in dark. Solution handlings

was carried out using Mosquito LCP (TTP LabTech Ltd., Melbourn, UK) and Multidrop Combi (Thermo Scientific, Waltham, MA, USA). The production of NADH was monitored by the measurement of luminescence (RLU) using either VERITAS Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA) or EnSpire Multimode Reader (Perkin-Elmer, Waltham, MA, USA). The experiments were performed without repetition in 1st screening, and in triplicate in other assays.

**Determination of $IC_{50}$ values of hit and synthetic compounds.**

Standard IMPDH assay solution containing 50 mM Tris-HCl pH 8.0, 100 mM KCl, 3 mM EDTA, 0.1 mg/mL BSA, 1.0 mM dithiothreitol (DTT) and appropriate amounts of inhibitors with a total volume of 270 μL. Substrate concentrations in the solution were 500 μM $NAD^+$ and 250 μM IMP for CpIMPDH. Reaction was started by the addition of 30 μL enzyme solution (CpIMPDH: 0.15 μg/mL; hIMPDH: 0.75 μg/mL). The activity of the enzyme was measured by monitoring NADH production in either absorbance at 340 nm or fluorescence emissions at 465 nm. $IC_{50}$ values were calculated by plotting the recorded NADH yield against log of compound concentration in GraFit ver. 7 (Erithacus Software, UK). All screening assays in this study was conducted with 0.5% DMSO as vehicle and library compound concentration of 10 μM unless stated otherwise.

**Conflict of interest**

The authors declare no conflict of interest.

**Acknowledgement**

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**Legend to Figures**

**Figure 1** Structures of hit compounds in high-throughput screening of CpIMPDH and designed derivatives.

**Scheme 1** Synthesis of adenosine unit **12**.

**Scheme 2** Synthesis of phosphonate units **18** and **21**.

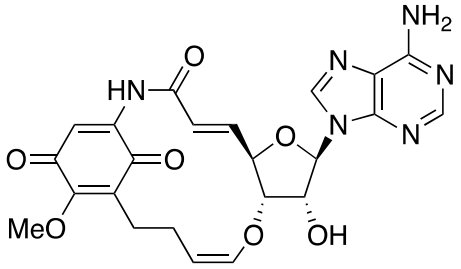
**Scheme 3** Synthesis of adenosine derivatives **3**, **4, 5** and **6**.

**Table 1.** Apparent $IC_{50}$ values of hit compounds **1**, **2**, synthetic compounds **3**, **4** and **5** measured by luciferase-based assay.

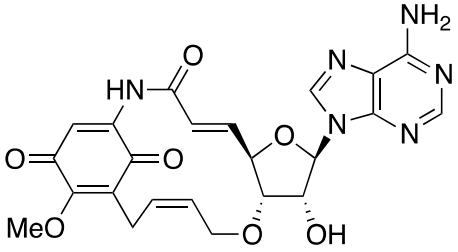
**Table 2.** $IC_{50}$ values of previously discovered Cys-targetting compounds and synthetic compounds **3**–**5** measured by luciferase-free low-throughput assay.

**Figure 2** Predicted stable conformers of a) compound **3** and b) compound **4**. The numbers in parentheses are relative energies to the most stable conformers M3-007 and M4-015.

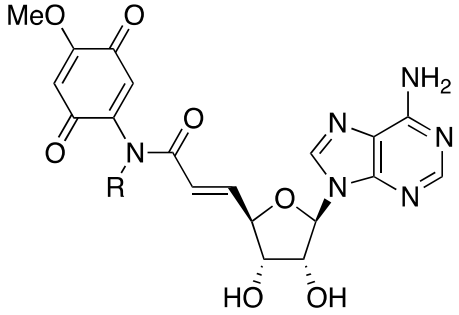
Fig 1



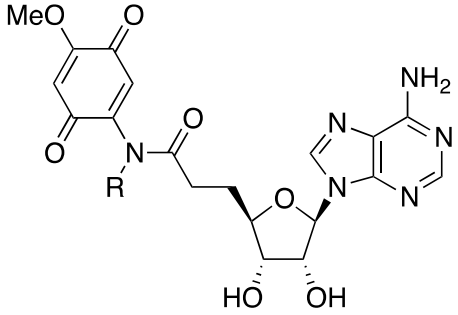

Hit compound **1**




Hit compound **2**

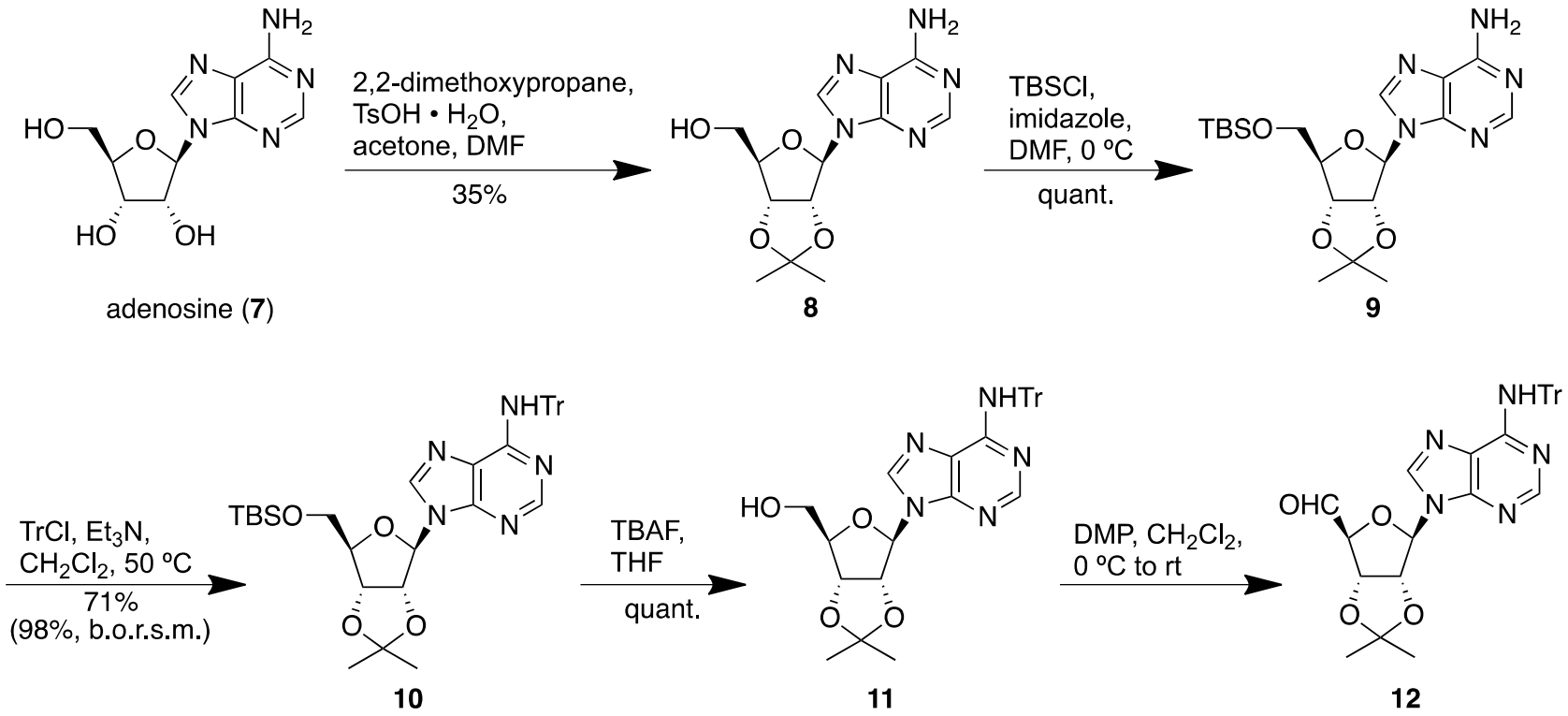



Derivatives **3** (R = H) and **4** (R = Me)

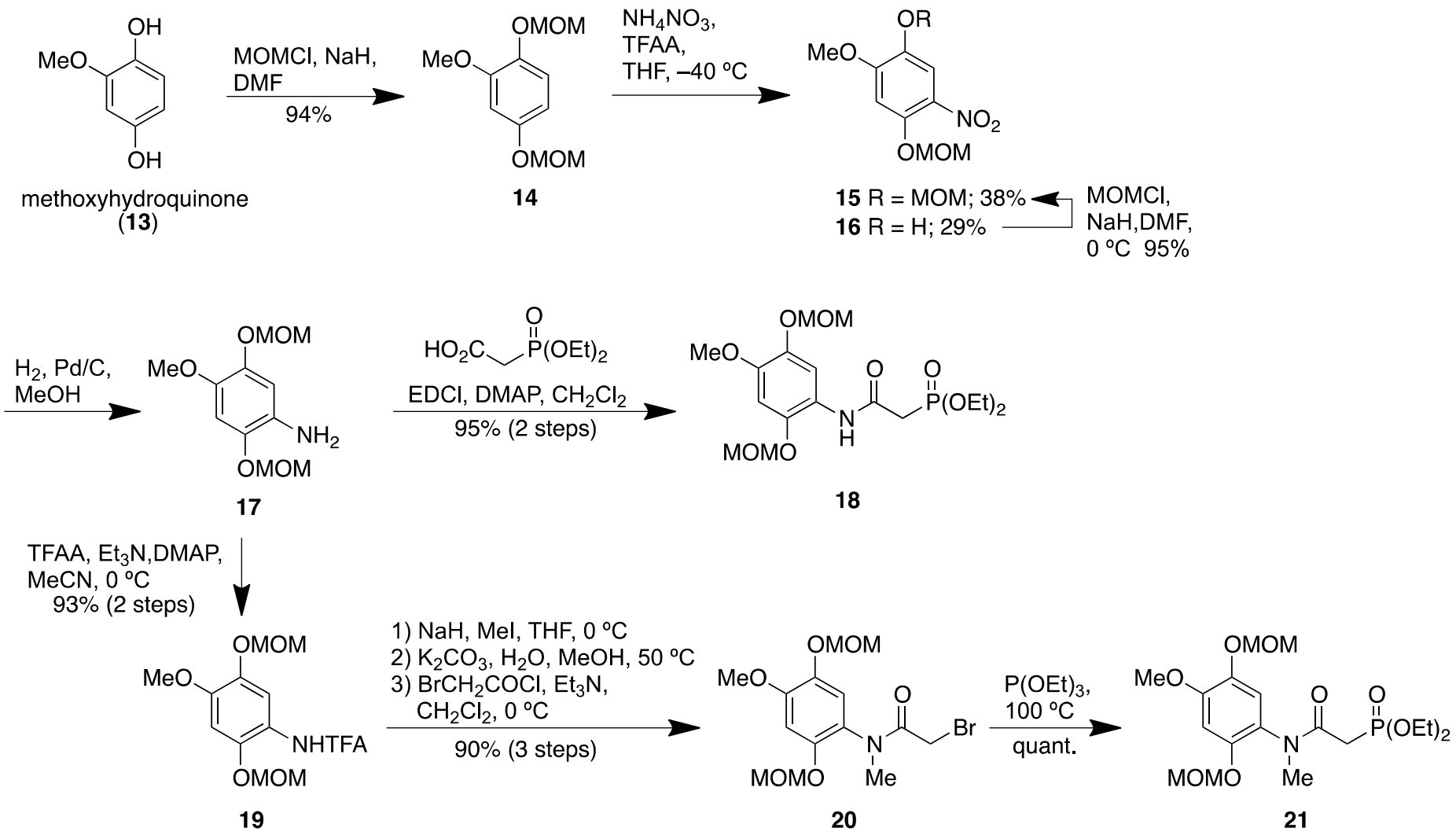



Derivatives **5** (R = H) and **6** (R = Me)

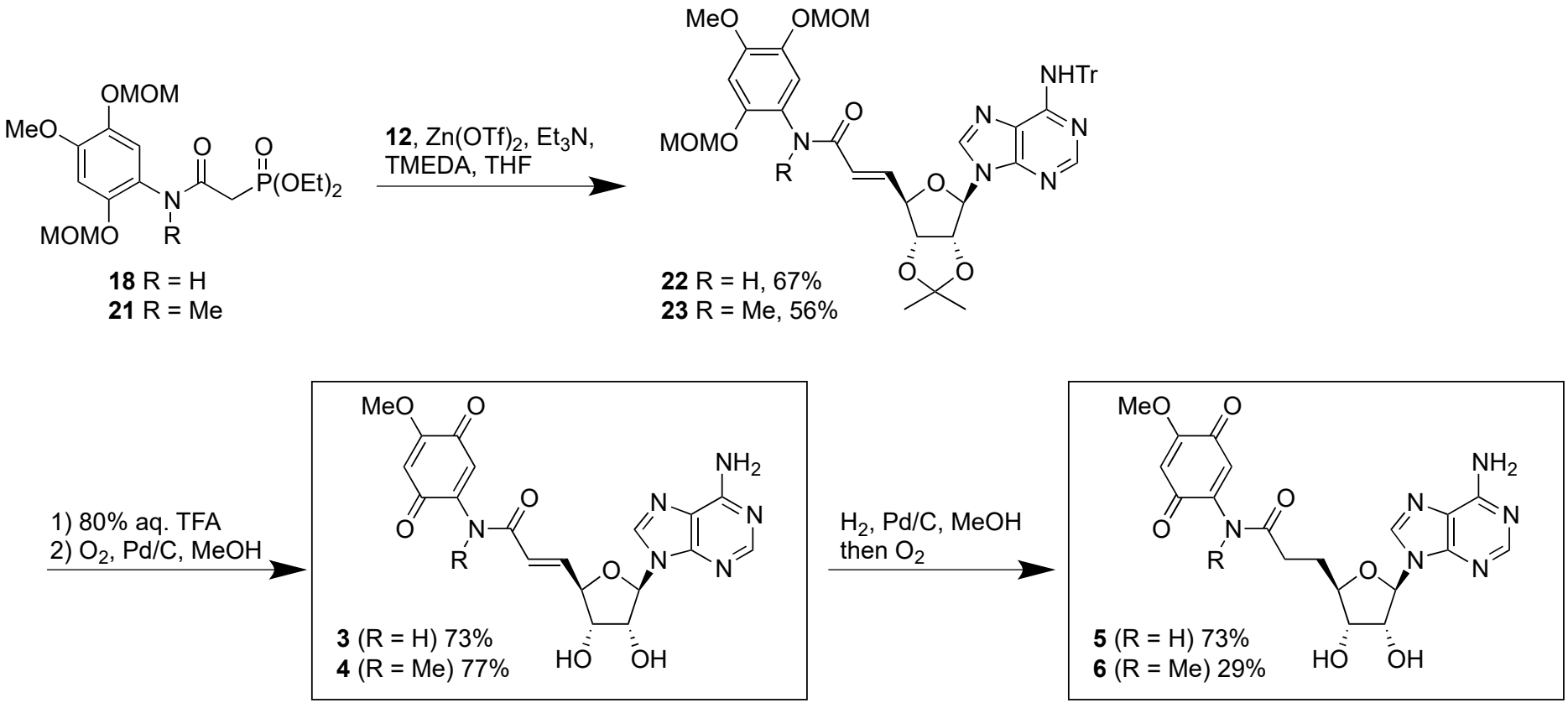
Scheme 1


NH2
HO
HO
OH
adenosine (7)
2,2-dimethoxypropane,
TsOH • H2O,
acetone, DMF
35%
8
TBSCl,
imidazole,
DMF, 0 °C
quant.
TBSO
9
TrCl, Et3N,
CH2Cl2, 50 °C
71%
(98%, b.o.r.s.m.)
NHTr
10
TBAF,
THF
quant.
11
DMP, CH2Cl2,
0 °C to rt
OHC
12

Scheme 2


OH
MeO
OH
methoxyhydroquinone
(13)
MOMCl, NaH,
DMF
94%
OMOM
MeO
OMOM
14
NH4NO3,
TFAA,
THF, –40 °C
OR
MeO
NO2
OMOM
15 R = MOM; 38%
16 R = H; 29%
MOMCl,
NaH,DMF,
0 °C 95%
H2, Pd/C,
MeOH
OMOM
MeO
NH2
OMOM
17
HO2C
O
P(OEt)2
EDCI, DMAP, CH2Cl2
95% (2 steps)
OMOM
MeO
O
O
N
H
P(OEt)2
MOMO
18
TFAA, Et3N,DMAP,
MeCN, 0 °C
93% (2 steps)
OMOM
MeO
NHTFA
OMOM
19
1) NaH, MeI, THF, 0 °C
2) K2CO3, H2O, MeOH, 50 °C
3) BrCH2COCl, Et3N,
CH2Cl2, 0 °C
90% (3 steps)
OMOM
MeO
O
N
Br
MOMO
Me
20
P(OEt)3,
100 °C
quant.
OMOM
MeO
O
O
N
P(OEt)2
MOMO
Me
21

Scheme 3


OMOM
MeO
O
O
P(OEt)2
N
MOMO
R
18 R = H
21 R = Me
12, Zn(OTf)2, Et3N,
TMEDA, THF
MeO
OMOM
MOMO
NHTr
22 R = H, 67%
23 R = Me, 56%
1) 80% aq. TFA
2) O2, Pd/C, MeOH
NH2
3 (R = H) 73%
4 (R = Me) 77%
HO
OH
H2, Pd/C, MeOH
then O2
5 (R = H) 73%
6 (R = Me) 29%

| Compounds | $IC_{50}$ (μM) against CpIMPDH |
|---|---|
| | without DTT |
| **1** | >50 |
| **2** | >50 |
| **3** | 1.1 |
| **4** | 0.7 |
| **5** | 1.3 |

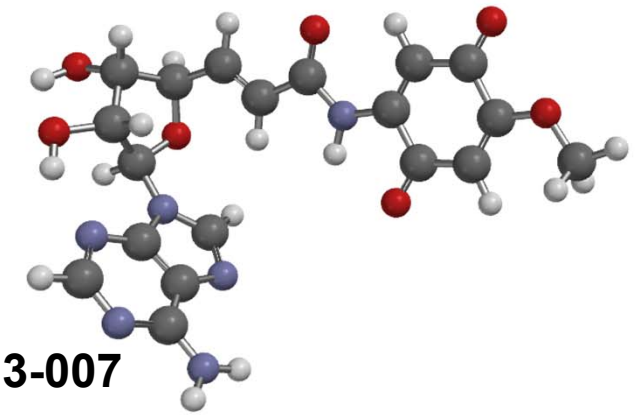
Table1

| Compounds | $IC_{50}$ (μM) against CpIMPDH (0.15 μg/mL) | | $IC_{50}$ (μM) against hIMPDH (0.75 μg/mL) |
|---|---|---|---|
| | with DTT | without DTT | with DTT |
| Disulfiram[4] | >500.0 | 1.4 | >500.0 |
| Ebselen[4] | 148.7 | 1.1 | >500.0 |
| Bronopol[4] | 291.0 | 1.3 | >500.0 |
| **3** | 24.4 | 209.8 | 30.3 |
| **4** | 76.8 | 2.1 | 43.0 |
| **5** | 11.1 | >500.0 | 11.3 |

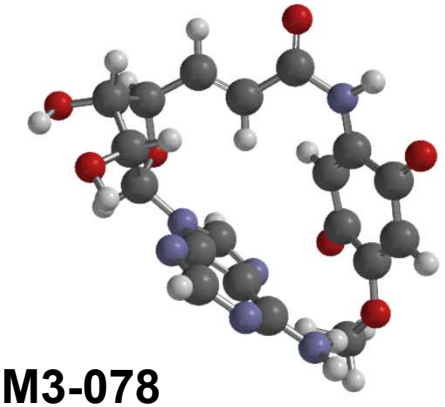
Table2

Fig 2

a)

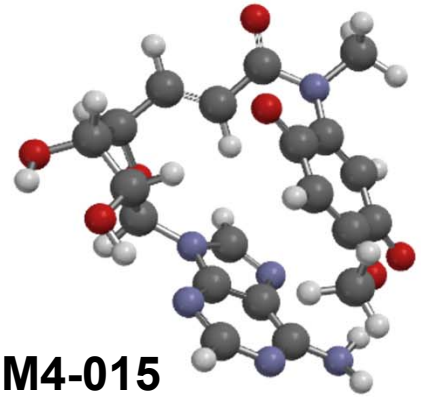


**M3-007**

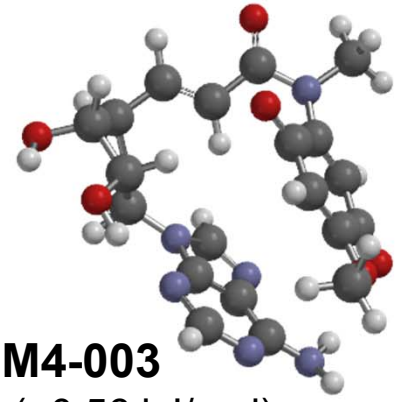


**M3-078**
(+7.99 kJ/mol)

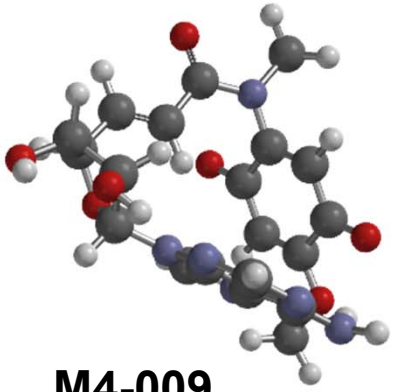
b)



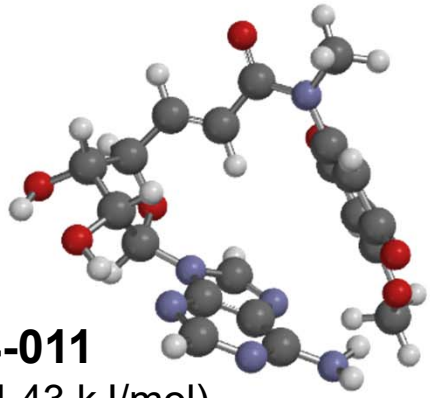
**M4-015**



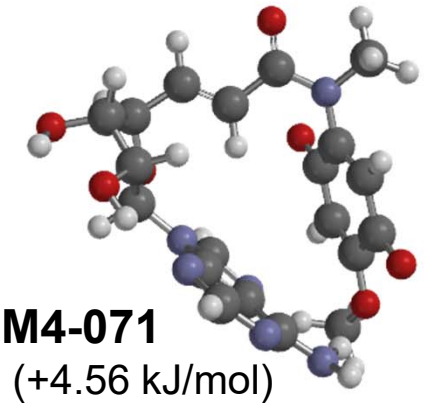
**M4-003**
(+0.56 kJ/mol)



**M4-009**
(+3.74 kJ/mol)



**M4-011**
(+4.43 kJ/mol)



**M4-071**
(+4.56 kJ/mol)