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A Potent and Selective AMPK Activator That Inhibits de Novo Lipogenesis

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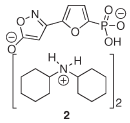
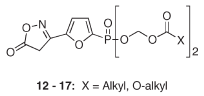
ABSTRACT AMP-activated protein kinase (AMPK) is a heterotrimeric kinase that regulates cellular energy metabolism by affecting energy-consuming pathways such as de novo lipid biosynthesis and glucose production as well as energy-producing pathways such as lipid oxidation and glucose uptake. Accordingly, compounds that activate AMPK represent potential drug candidates for the treatment of hyperlipidemia and type 2 diabetes. Screening of a proprietary library of AMP mimetics identified the phosphonic acid **2** that bears little structural resemblance to AMP but is capable of activating AMPK with high potency (EC_{50} = 6 nM vs AMP EC_{50} = 6 μM) and specificity. Phosphonate prodrugs of **2** inhibited de novo lipogenesis in cellular and animal models of hyperlipidemia.

KEYWORDS 5-(5-Hydroxy-isoxazol-3-yl)-furan-2-phosphonic acid, AMPK activator, lipogenesis, hepatocytes



2

Potent AMPK activator



12 - 17: X = Alkyl, O-alkyl

Esterase-sensitive prodrugs of **2** Inhibit *de novo* lipogenesis in cellular and animal models

Diseases associated with obesity, such as type 2 diabetes, hypertension, and dyslipidemia, are growing worldwide health concerns. These conditions are frequently associated with impaired regulation of glucose and lipid metabolism, leading to increased cardiovascular risks. Current therapies for the treatment of dyslipidemia and type 2 diabetes are only moderately effective, and even in combination, many patients still do not meet the recommended guidelines.[1] Therefore, new pharmacological approaches that can address one or multiple components related to these diseases are under investigation. One approach that has recently elicited considerable interest is the activation of 5′-AMP-activated protein kinase (AMPK).[2−5]

AMPK is a heterotrimeric kinase expressed in a variety of tissues, particularly the liver, brain, and skeletal muscle. It has been dubbed a "metabolic master switch"[6] that putatively regulates fatty acid synthesis, sterol synthesis, and glucose production.[7] AMPK acts as an energy sensor that is activated by AMP under conditions where the intracellular ratio of AMP to ATP is increased, such as hypoxia, exercise, or glucose deprivation. As a result of this activation, processes where ATP is consumed (e.g., fatty acid synthesis, gluconeogenesis, and cholesterol synthesis) are inhibited through targets downstream of AMPK, while pathways where ATP is generated (e.g, fatty acid oxidation, ketogenesis, and glycolysis) are stimulated, resulting in restoration of energy homeostasis. Because of its important role in the regulation of lipid and carbohydrate metabolism, activators of AMPK could potentially provide a multifaceted approach to the treatment of metabolic diseases.

5-Aminoimidazole-4-carboxamide riboside (AICAR) is a nucleoside that undergoes intracellular phosphorylation to the corresponding 5′-mono-, di-, and triphosphate (Chart 1). AICAR-monophosphate (ZMP) is an AMP mimetic that binds to AMP binding sites on various intracellular proteins[8,9] and induces the associated functional response such as inhibition of fructose-1,6-bisphosphatase and the activation of glycogen phosphorylase (GPPase).[10] While ZMP is a modestly potent and poorly selective activator of AMPK, it has been used in hundreds of studies attempting to elucidate the role of AMPK in energy homeostasis.[11−16] In addition, it has been suggested that AMPK activation may contribute to the glucose-lowering activity of marketed diabetes drugs metformin[17] and the thiazolidinediones.[18,19] These findings as well as the report of a specific AMPK activator[20,21] (A-769662, **1**) suggest that a small molecule activator of AMPK may be a viable approach to treat diseases associated with dysregulation of energy homeostasis.

Herein, we disclose the discovery of compound **2**, a potent and selective activator of human AMPK. We also report the synthesis of esterase-sensitive phosphonate prodrugs of **2** and demonstrate the ability of these compounds to inhibit de novo lipogenesis in rat hepatocytes and in rodents.

Compound **2** was identified as a potent activator of AMPK after screening a proprietary library containing ca. 1200 AMP mimetics. Its synthesis is depicted in Scheme 1.

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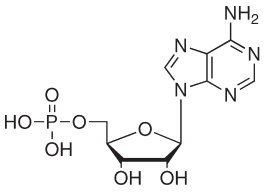
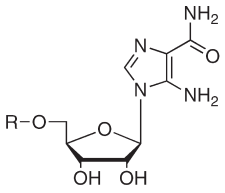
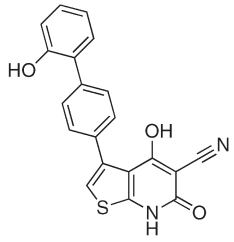
Chart 1



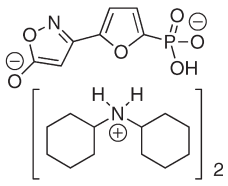
AMP



R = H: AICAR
R = PO_3H_2: ZMP

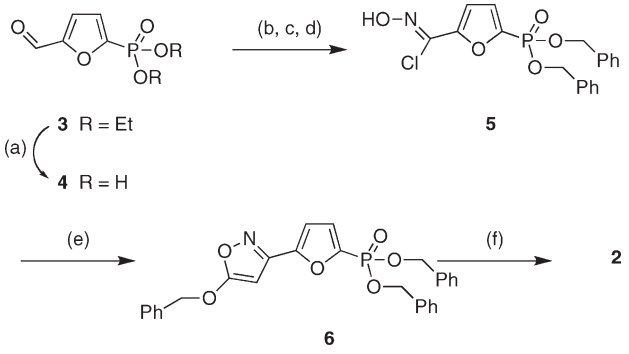


A-769662 (**1**)



2

Scheme 1[a]

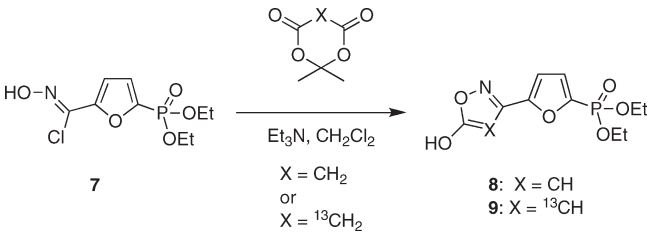


[a] Reagents and conditions: (a) TMSBr, CH_3CN, room temperature. (b) $PhCH_2OC(=N(cyclohexyl))NH(cyclohexyl)$, DMF, toluene, 100 °C. (c) $H_2NOH \cdot HCl$, NaOAc, THF, room temperature. (d) NCS, DMF, room temperature. (e) $PhCH_2OCCH$, Et_3N, CH_2Cl_2, room temperature. (f) H_2, $Pd(OH)_2$ on C, *N*,*N*-dicyclohexylamine, EtOH.

The diethyl aldehyde **3**[8] was deprotected using bromotrimethylsilane in acetonitrile at room temperature. The resulting phosphonic acid **4** was reprotected as the dibenzyl phosphonate using the DCC adduct of benzyl alcohol in DMF/toluene.[22] The crude aldehyde was converted to a mixture of isomeric oximes (cis/trans, 34% yield over two steps) by treatment with hydroxylamine hydrochloride. Chlorination of the intermediate oximes with *N*-chlorosuccinimide (NCS) in DMF yielded chlorooxime **5** (88%). [3 + 2] Cycloaddition of **5** with benzyloxyacetylene[23] regioselectively produced isoxazole **6**. Hydrogenation of **6** using palladium hydroxyde on carbon in ethanol in the presence of *N*,*N*-dicyclohexylamine gave compound **2**.

Alternatively, chlorooxime **7** (prepared from **3** using the methods described above) was used to generate the diethylphosphonate analogue **8** by reaction with Meldrum's acid in dichloromethane (Scheme 2). However, attempts to remove the ethyl groups in phosphonate **8** under a variety of reaction conditions failed to yield phosphonic acid **2**, generating instead a complex mixture of products.

Scheme 2



While the diethyl phosphonate **8** was not an AMPK activator, it was a useful tool compound that allowed us to evaluate some of the properties of the 5-hydroxyisoxazole portion of the molecule. The hydroxyisoxazole ring of **8** exists in the keto form in $CDCl_3$, as determined by proton NMR, which shows a 2H signal at 3.81 ppm corresponding to the methylene group (C-4) of the isoxazolone ring. However, in DMSO-d_6, also **8** exists as the enol form, as indicated by the presence of two 1H signals at 5.60 and 4.20 ppm. Compound **9**, the ^{13}C-labeled version of **8**, was similarly generated by using 5-^{13}C-Meldrum's acid. Consistent with the results observed for compound **8**, the ^{1}H NMR for **9** in $CDCl_3$ showed a 2H signal (d, J = 138 Hz) at 3.84 ppm. The ^{13}CNMR spectrum of **9** in the same solvent showed a signal at 33.85 ppm. When **9** was dissolved in DMSO-d_6, it showed two signals at 77.27 and 34.25 ppm, indicating the presence of both the enol and the keto form, respectively, in this solvent. The addition of triethylamine to this solution caused the complete disappearance of these two signals and the appearance of a single peak at 67.11 ppm, suggesting that conversion to the triethylammonium enolate of the hydroxyisoxazole ring in DMSO-d_6 had occurred. In an aqueous solution of sodium hydroxide (pH > 10), the ^{13}C NMR of **9** showed a single peak at 71.37 ppm, again indicating the presence of the enolate form of **9** in this highly basic medium. The ^{13}C NMR spectrum of **9** in pH 7.4 phosphate buffer also shows a single peak at 71.37 ppm, indicating that it exists almost exclusively as the enolate under these conditions. This keto−enol tautomerization is evidence of the relatively high acidity of the 5-hydroxyisoxazole group. Indeed, potentiometric titration of **8** in mixtures of methanol/water provided pK_a values of 3.23. Similarly, potentiometric titration of **2** in the same solvent revealed pK_a values of 3.32, 3.93, and 6.94. Consistent with these findings, the log $D_{7.4}$ for **2** was found to be −4.3.

The highly anionic nature of **2** was an impediment for its transport across cell membranes and likely was responsible for its lack of effect in cell-based and in vivo models; thus, prodrug strategies were explored[27] to improve cell penetration for compound **2**.

For the synthesis of esterase-sensitive prodrugs of **2**, the formal [3 + 2] cycloaddition reaction using Meldrum's acid was utilized. Scheme 3 illustrates the application of this method to the synthesis of compound **12**, a bis-pivaloyloxymethyl (bis-POM) prodrug of **2**. Alkylation of **4** with the

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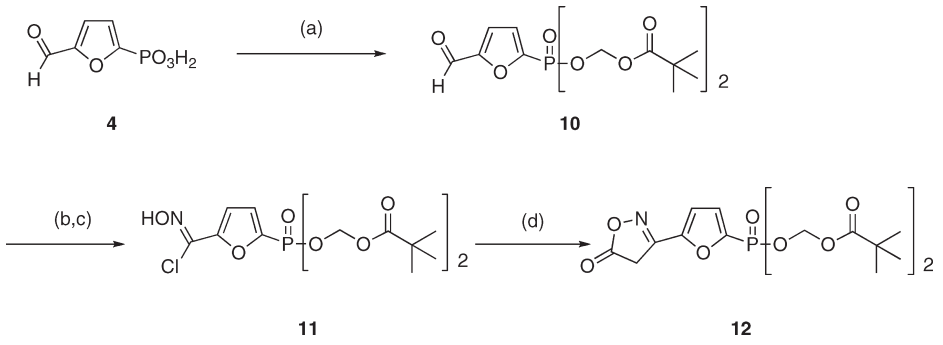
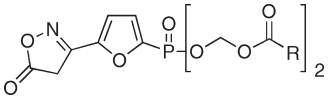
Scheme 3[a]



[a] Reagents and conditions: (a) Iodomethyl pivalate, *N*,*N*-diisopropyl ethylamine, acetonitrile, 23 °C. (b) Hydroxylamine hydrochloride, THF, sodium acetate, 23 °C. (c) NCS, DMF, 23 °C. (d) Meldrum's acid, Et_3N, CH_2Cl_2, 23 °C.

Table 1. In Vitro and in Vivo Inhibition of de Novo Lipogenesis (DNL) by Phosphonate Prodrugs of **1**



compound no.	R	EC_{50} (nM)[a]	in vivo DNL inhibition[b] (%)
2	N/A	> 10000	ND[c]
12	*t*-butyl	100 (r) 13 (m)	65
13	iso-propyl	20 (r)	78
14	cyclopentyl	30 (r)	ND
15	*O*-ethyl	42 (r) 17 (m)	73
16	*O*-benzyl	609 (r)	34
17	*O*-iso-propyl	27 (r) 8 (m)	73
18	$O-C(CH_3)_2\ CH_2CH_3$	390 (r)	ND

[a] EC_{50} for the inhibition of de novo lipogenesis in rat (r) or mouse (m) hepatocytes. [b] After intraperitoneal administration of the compound at a 30 mg/kg dose to C57Bl/6 mice. [c] ND, not determined.

iodomethyl ester of pivalic acid in the presence of Hunig's base generated **10**. Chlorooxime **11** was generated from **10** in two steps: oxime formation by reaction with 1.05 equiv of hydroxylamine hydrochloride and sodium acetate, followed by conversion to **11** by reaction with NCS in DMF. Cycloaddition with Meldrum's acid gave the desired prodrug **12**. This method was used for the synthesis of additional prodrugs (compounds **13**−**18**, Table 1) where the phosphonic acid was functionalized with other esterase sensitive groups.

Compound **2** was identified by screening a library of proprietary AMP mimetics[8] against both human and rat AMPK by monitoring the phosphorylation of the SAMS peptide, an extensively utilized substrate for AMPK.[24] Compound **2** was found to be a full activator of human AMPK, with an EC_{50} of 6.3 nM (Figure 1), which is > 900-fold more potent than the endogenous activator AMP (EC_{50} = 5.9 μM). The potency of **2** is similar against rat AMPK (EC_{50} = 21 nM), but interestingly, it was a partial activator

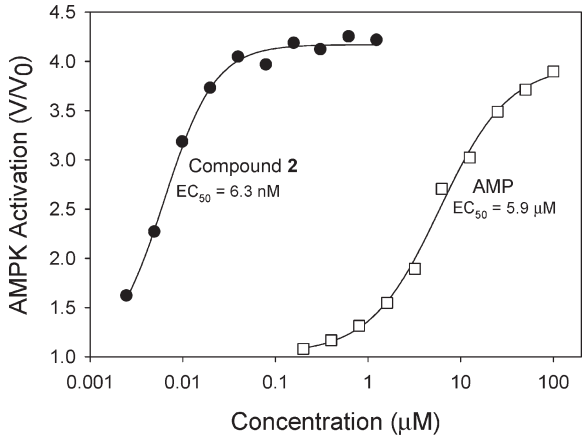


Figure 1. Activation of human AMPK by AMP and **2**. Activation was determined by monitoring the phosphorylation of the SAMS peptide by AMPK.

of rat AMPK, reaching a maximum of 51 % of the activation observed with AMP. In selectivity assessments, screening of **2** against a panel of 64 targets (MDS Pharma Services) indicated no significant interactions with these targets at a test concentration of 10 μM.

AMP allosterically modulates other key enzymes: It activates GPPase (EC_{50} = 1.4 μM), while it inhibits FBPase (IC_{50} = 1.0 μM). ZMP is an AMP mimic that activates rat liver AMPK with an EC_{50} = 84 μM. However, it is not specific toward AMPK. For example, it activates GPPase with an EC_{50} = 115 μM and inhibits FBPase with an IC_{50} = 9.4 μM. In contrast, **2** had no activity against GPPase and FBPase at concentrations of 100 μM, about 1000-fold its EC_{50} for activation of AMPK. These results suggest that, unlike ZMP, **2** is a selective AMPK activator.

Compounds **8** and **12** were not activators of the isolated enzyme, suggesting that the phosphonic acid group was essential for the in vitro activation of AMPK by **2**. Acetyl-CoA carboxylase (ACC) catalyzes the rate-limiting step in free fatty acid biosynthesis. Phosphorylation of ACC by AMPK decreases its activity, resulting in a reduction in the ACC product malonyl-CoA and, correspondingly, in the inhibition of de novo lipogenesis. Because malonyl-CoA is a potent inhibitor of carnitine

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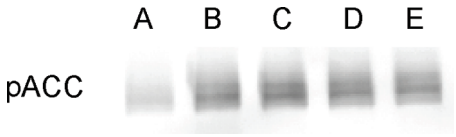

Figure 2. Western blot analysis of the phosphorylation of ACC by **13** or AICAR. Western blot analysis of phosphorylated ACC in cell lysates from primary rat hepatocytes treated for 4 h with 1% DMSO (line A), 1000 μM AICAR (line B), or compound **13** at 10, 3, and 1 μM (lines C, D, and E, respectively).

palmitoyltransferase-1 (CPT-1), activation of AMPK also results in inactivation of CPT-1 and in an increase in free fatty acid oxidation. In addition to its effects on fatty acid production and metabolism, AMPK activation also results in the phosphorylation of HMGCoA reductase and, consequently, in the inhibition of cholesterol biosynthesis.[25,26]

To evaluate the effect of **2** on de novo lipogenesis, plated rat hepatocytes were incubated with the test compound and then coincubated with ^{14}C-sodium acetate as a lipogenic substrate. De novo lipogenesis was evaluated by measuring the radiolabeled counts coming from ^{14}C-acetate incorporated into organic soluble lipids. Compound **2** did not affect de novo lipogenesis in primary rat hepatocytes (data not shown). The highly anionic nature of **2** suggested that its lack of activity may have been due to poor cellular permeability. Indeed, upon incubation of plated rat hepatocytes with **2**, at a concentration of 100 μM for up to 6 h, the intracellular levels of **2** were below the limits of detection (55 nmol/g). To improve cell permeability, the phosphonic acid group was masked with esterase-sensitive prodrugs.[27] Incubation of plated rat hepatocytes with a 10 or 100 μM solution of **12** led to 254 ± 104 and 2151 ± 712 nmol/g intracellular concentrations of **2**, respectively, after a 2 h incubation period. The levels of **2** were maintained at >180 nmol/g for up to 8 h and detected as early as 15 min (see the Supporting Information).

Shown in Table 1 are ester and carbonate prodrugs of **2** that inhibited de novo lipogenesis in plated rat hepatocytes with EC_{50} values below 1 μM. Given that the prodrug **12** is not an activator of isolated AMPK (data not shown), these results are consistent with conversion to **2** by intracellular esterases. It is clear from the data that relatively unhindered esters and carbonates had very similar potencies in this assay, reflecting similar rates of intracellular generation of **2** and subsequent AMPK activation in plated rat hepatocytes.

As evidence pointing to the activation of AMPK as the source of the inhibition of the de novo lipogenesis, we evaluated the ability of compound **13** to induce phosphorylation of ACC. Primary plated rat hepatocytes were incubated for a 4 h period with **13** (1, 3, and 10 μM) or AICAR (1000 μM) as the positive control. Immunoblot analysis of the lysate using a specific antibody that recognizes ACC phosphorylated at serine-79 shows increased phosphorylation of ACC (Figure 2). There was no change in total ACC protein levels following treatment with either of the agents used, reprobing the blot with a total ACC antibody, or in total protein loaded by staining the blot with Ponceau S (data not shown). These results are consistent with the activation of AMPK as the source for the inhibition of the de novo lipogenesis.

To determine whether the cellular effects of compounds **12**–**18** would translate to the in vivo setting, de novo lipogenesis was measured in mice following treatment with potential AMPK activators. Because preliminary results showed that oral dosing of prodrugs **12**–**18** resulted in low plasma levels of **2** as well as the corresponding prodrugs (data not shown), the compounds were administered by intraperitoneal injection. Compounds were dosed (generally 30–100 mg/kg) 1 h prior to intraperitoneal administration of ^{14}C-sodium acetate in saline. One hour later, animals were anesthetized, and plasma and liver were harvested. Newly synthesized lipids and sterols were extracted using a modified Folch method[28] and quantified relative to those in vehicle-treated mice. At a dose of 30 mg/kg, compounds **12**–**17** achieved plasma concentrations of 200–300 μM and inhibited de novo lipogenesis by more than 30% (Table 1).

In conclusion, we have described the synthesis and evaluation of **2**, a furan phosphonic acid derivative containing an acidic 5-hydroxyisoxazole group. Compound **2** activates AMPK with an impressive $EC_{50} < 10$ nM and is the most potent direct AMPK activator reported to date.[29−31] Esterase-sensitive prodrugs of **2** enhanced the intracellular delivery of this highly anionic compound into hepatocytes. These tool compounds inhibited de novo lipogenesis both in plated rat hepatocytes and in mice. It should be noted that while **2** is only a partial activator of rat liver AMPK, it was able to fully inhibit de novo lipogenesis in rat hepatocytes. Given the high specificity of compound **2** relative to ZMP, prodrugs of **2**, for example, **12**–**18**, may be useful tools to elucidate the role that AMPK activation plays in whole body energy homeostasis. Additional studies are ongoing that utilize these and related compounds in disease-relevant models to understand the therapeutic potential of AMPK activators.

SUPPORTING INFORMATION AVAILABLE

Synthesis and characterization of new compounds reported and description of biological protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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