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NOTE

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Identification of a Naturally Occurring Quinazolin-4(3*H*)-one Firefly Luciferase Inhibitor

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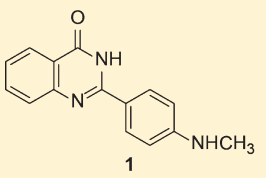
Supporting Information

ABSTRACT: A prefractionated *Streptomyces*-derived extract was initially identified as being active using a luciferase-based AMP-activated protein kinase (AMPK) assay. Bioassay-guided fractionation led to the isolation of the new compound quinazolin-4(3*H*)-one (**1**) as the active component. However, **1** was shown to have potent firefly luciferase inhibitory activity with no effect on AMPK. This is the first report of a natural luciferase inhibitor.



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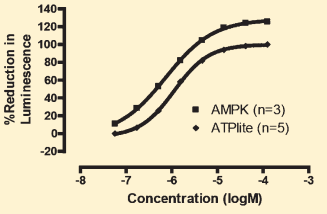

AMP-activated protein kinase (AMPK) is a key enzyme that regulates the energy levels of cells and is considered to be a promising metabolic disease drug target, as well as potential target for the prevention and treatment of cancer.[1−3] Although the type 2 diabetes biguanidine-type drug metformin primarily reduces blood glucose level through hepatic gluconeogenesis, it also has been shown to activate AMPK.[1] Other compounds have been identified that directly or indirectly activate AMPK, but none have advanced to clinical trials.[1,2] We hypothesized that the unique chemical space occupied by many natural products could lead to the identification of a new, druggable AMPK activator.

To probe this target, a luciferase-based high-throughput screen was developed that monitored the utilization of ATP by the AMPK-catalyzed phosphorylation of SAMs (HMRSAMSGLHLVKRR) peptide. The AMPK assay was used to screen a library of 40 296 fractions prepared from 10 074 microbial extracts separated by preparative HPLC into four fractions, PF1 to PF4.[4] A total of 110 active fractions were identified, with many containing AMP, an allosteric activator of AMPK. A PF2 fraction derived from *Streptomyces* sp. A496 was selected for chemical deconvolution. The crude MeOH extract was inactive, which suggested the active compound(s) were present in minor quantities. Bioassay-guided fractionation using the AMPK assay of fraction PF2 led to the isolation of 2-[4′-(methylamino)phenyl]quinazolin-4(3*H*)-one (**1**) (Figure 1) as the active component with an EC_{50} of 0.73 μM (Figure 2).

Compound **1** was obtained as a pale yellow solid, and analysis of its (+)- and (−)-HRESIMS data suggested a molecular formula of $C_{15}H_{13}N_3O$. Analysis of the ^{1}H and COSY NMR data of **1** (Table 1) indicated the presence of three separate spin systems: an *N*-methyl singlet [δ_H 2.85 (3H)], a 1,4-disubstituted aromatic ring [δ_H 7.89 (2H) and 6.70 (2H)], and a 1,2-disubstituted aromatic ring [δ_H 8.17 (1H), 7.43 (1H), 7.77 (1H), and 7.69 (1H)]. The connection of the *N*-methyl to the 1,4-disubstituted aromatic ring to δ_C 154.6 (C-4′) and assignments of C-2 and C-1′ as δ_C 155.1 and 120.6, respectively, were established through HMBC correlations (Table 1). Similarly, HMBC correlations from H-5 and H-8 established the presence of an amide carbonyl at C-4 (δ_C 165.0) and allowed the assignment of C-4a and C-8a as δ_C 121.3 and 150.4, respectively. Joining of these two units to form a quinazolin-4(3*H*)-one skeleton satisfied the proposed molecular formula of $C_{15}H_{13}N_3O$, and the structure of **1** was assigned as 2-[4′-(methylamino)phenyl]quinazolin-4(3*H*)-one (Figure 1). The UV spectrum and NMR data of **1** were in excellent agreement with those previously reported for quinazolin-4(3*H*)-ones.[5−7]

Quinazolinones have been isolated from various plants and microorganisms and are often the central core of complex natural products.[8] Closely related, naturally occurring compounds (Figure 1) include the plant-derived quinazolin-4(3*H*)-one (**2**),[9] the fungal-derived chrysogine (**3**)[10] and 2-acetyl-quinazolin-4(3*H*)-one (**4**),[7,11,12] and the bacterial-derived 2-methylquinazolin-4(3*H*)-one (**5**).[6] These simple quinazolin-4(3*H*)-ones are thought to be produced through cyclization of amide β-ketoamides such as 2-pyruvoylaminobenzamide (**6**),[13] which often co-occurs with chrysogine (**3**) and 2-acetylquinazolin-4(3*H*)-one **4**,[7,10−12] and 2-acetylaminobenzamide (**7**), which was isolated from *Streptomyces aurantiogriseus*.[14]

With this potential lead in hand, we began efforts to further validate the quinazolin-4(3*H*)-one motif as an AMPK activator. Further biological testing of **1** was carried out using the ATPlite luciferase test kit, to determine its effect on luciferase, independent of AMPK. Unfortunately, this testing revealed that **1** displayed a comparable activity against the luciferase reporter itself and, as a consequence, was identified as a luciferase inhibitor with no effect on the AMPK enzyme. Quninazolinone **1** displayed a reduction in luminescence (EC_{50} 1.1 μM) in the ATPlite assay that was comparable to that obtained in the AMPK assay (Figure 2). Thus the observed activity of **1** in the AMPK assay was due to the inhibition of

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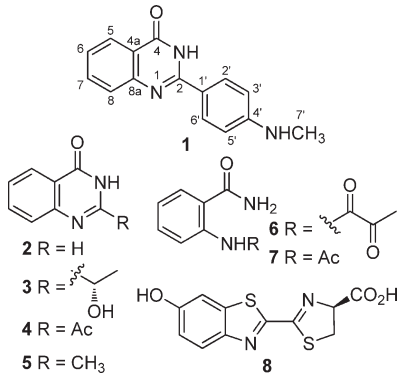

Figure 1. Structures of the new quinazolin-4(3*H*)-one **1**, related natural products **2**−**7**, and luciferin (**8**).

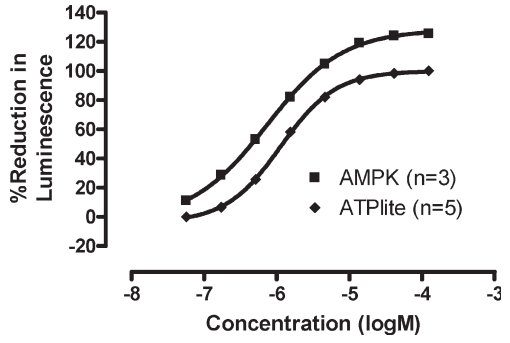

Figure 2. Activity titration of **1** in the AMPK assay and ATPlite system.

Table 1. NMR Data for 1 (CD_3OD, 500 MHz)

position	δ_C [a]	δ_H, mult.(*J* in Hz)	COSY	gHMBC [b]
2	155.1			
4	165.0			
4a	121.3			
5	127.1	8.17, d (8.2)	6, 7	4, 7, 8a
6	126.9	7.43, ddd (8.2, 8.2, 1.6)	5, 7	4,[c] 7, 8
7	135.9	7.77, ddd (8.2, 8.2, 1.6)	5, 6, 8	5, 8a
8	127.2	7.69, d (8.2)	7	4,[c] 6, 4a
8a	150.4			
1′	120.6			
2′, 6′	130.3	7.89, d (8.8)	3′, 5′	2, 2′, 4′, 6′
3′, 5′	112.6	6.70, d (8.8)	2′,6′	2,[c] 1′, 3′, 5′
4′	154.6			
7′	30.0	2.85, s		4′

[a] Obtained at 125 MHz. [b] H to C, J_{CH} = 7.6 Hz. [c] Weak correlation.

luciferase; however, the nature of the inhibitory activity of **1** (competitive, noncompetitive) on the luciferase or another component(s) in the luciferase/luciferin system has not been determined.

Luciferase has been used in many high-throughput screening assays as an activity reporter due to its availability and strong light emission. However, in the past few years, there has been a growing list of luciferase inhibitors identified from synthetic compound libraries, which have been classified as false positives, as they are not active against the desired biological target.[15−18] Most of the inhibitors identified to date are nitrogen or nitrogen- and sulfur-containing heterocycles related to luciferin (**8**).[15−18] The identification of the naturally occurring quinazolin-4(3*H*)-one alkaloid **1** as a relatively potent luciferase inhibitor (EC_{50} 0.73 μM) showed that luciferase inhibitors are also produced by bacteria. It is important to eliminate these false positive assay hits as soon as possible in the screening cascade, which can be achieved by repeating the primary assay without the enzyme, as was undertaken in this study. Finally, the identification of a submicromolar active compound from a fractionated inactive extract demonstrates the usefulness of this strategy for identifying active compounds present in minor amounts.[4]

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were scanned on a Pharmacia Biotech Ultrospec 2000. NMR spectra were collected on a Bruker Avance DRX-500 NMR spectrometer, using 5 mm BBI (^{1}H, G-COSY, multiplicity-edited G-HSQC, and G-HMBC) or BBO (^{13}C spectra) probeheads equipped with z-gradients. Spectra were calibrated to residual protonated solvent signals of CD_3OD (δ_H 3.30 and δ_C 49.0). HRESIMS values were collected on a Bruker micrOTOF mass spectrometer, using sodium trifluoroacetate as an internal standard for both positive- and negative- ionization modes. Preparative HPLC was performed on a Gilson HPLC with a Gilson 322 pump, Gilson UV/vis-156, with a Gilson 215 liquid handler using UNIPOINT software.

Bacterial Strain Isolation and Initial Characterization. The producing strain was isolated from terrestrial soil on the edge of a pond in Stoke Poges, England, and has been deposited in the MerLion Pharmaceuticals culture collection as A496. The strain was grown on Bennett's agar and sporulated within 10−15 days of inoculation. Samples were mounted in lactoglycerol or water and observed using an Olympus BX50 microscope. A496 was initially identified as a member of the actinomycete genus *Streptomyces* based on the morphology of the aerial mycelium and spore chains. This was confirmed by 16S rDNA analysis using MicroSeq 500 16S rDNA sequencing kit (Applied Biosystems) using standard manufacturer's protocol and sequenced by a third party (1st Base, Singapore) using an ABI Prism Big Dye Terminator Cycle sequencing kit (Perkin-Elmer). Sequences generated from A496 were used to perform a BLAST search to determine if possible taxonomic matches were present in the GenBank database. While the search did not provide an unambiguous match, it gave a high degree of sequence similarity to the genus *Streptomyces* with a number of possible matches at the species level (including *S. griseus* and related species). Further molecular work would need to be undertaken to confirm species level identification, including comparison with appropriate type strains, which was beyond the scope of the current research.

Fermentation. A496 was subcultured on Bennett's agar (composed of 1% glucose, 0.2% casitone, 0.1% yeast extract, 0.1% Lab Lemco, 5% glycerol, and 1.5% agar) for 7−10 days at 28 °C. The actively growing subculture was used to inoculate 250 mL Erlenmeyer flasks each containing 50 mL of seed medium composed of 1.5% glucose, 1.5% glycerol, 1.5% peptone (soy neutralized), and 0.1% $CaCO_3$. The pH of the medium was adjusted to 7 prior to sterilization and autoclaved at 121 °C for 30 min. The seed flasks were incubated for 3 days at 28 °C on a rotary shaker with a 50 mm orbit, at 200 rpm. A volume of 2.5 mL of seed culture was used to inoculate approximately 50 mL of solid medium in 250 mL flasks. The solid medium was composed of brown rice (16.7 g/flask), cornmeal (4.8 g/flask), and oat bran (2.4 g/flask), which was moistened with 26 mL of reverse osmosis water. The pH was unadjusted prior to sterilization and autoclaved at 121 °C for 30 min. Fermentation was undertaken for 11 days at 28 °C.

Extraction and Isolation. Freeze-dried mycelia derived from a 2 L fermentation were shaken overnight with MeOH (4 L), followed by MeOH−water (1:1) (4 L) and water (2 L). An aliquot of each extract

was fractionated[4] and tested together with extracts. Activity was found only in the fractions derived from the MeOH and MeOH−H_2O extracts. The MeOH extract (23 g) was separated using a C_{18} vacuum liquid chromatograph (VLC) (column 15 × 7 cm) with a 20% stepwise elution of MeOH in H_2O from 100% H_2O to 100% MeOH (1 L each). Activity was identified in the 60% MeOH (240 mg) and 80% MeOH fractions (200 mg), which were combined and separated by preparative HPLC (Waters Prep LC 25 mm module fitted sequentially with a guard column and a 25 × 100 mm Prep Nova-Pak C18 6 μm 60 Å cartridge, solvent A (0.1% HCO_2H in H_2O): solvent B (0.1% HCO_2H in CH_3CN) isocratic 90:10 for 2 , then linear gradient to 75:25 over 68 min, flow rate 12 mL/min) with activity detected in fractions with retention times around 35 min. The active fractions were combined (45 mg) and separated repeatedly using Sephadex LH20 (eluent MeOH, 45 × 4.5 cm) to obtain a small amount of the quinazolin-4(3*H*)-one alkaloid **1** (0.7 mg). The MeOH−H_2O (1:1) extract (23 g) was separated using a C_{18} VLC column in a similar manner to the 60% and 80% MeOH fractions, which were further separated using a Sephadex column (eluent MeOH, 45 × 4.5 cm). The active fractions were combined and separated using C_{18} preparative HPLC (YMC-Pack-ODS-Aqueous column, 5 μm, 150 × 20 mm, solvent A:solvent B 95:5 → 90:10 for 2 min, then linear gradient to 80:10 over 60 min, flow rate 12 mL/min) to give **1** (1.0 mg).

2-[4′-(Methylamino)phenyl]quinazolin-4(3*H*)-one 1: pale yellow solid; UV (MeOH λ_{max} (log ε) 205 (4.22), 225 (4.13), 340 (4.24) nm; NMR data, see Table 1; (+)-HRESIMS *m*/*z* 252.1129 (calcd for $C_{15}H_{14}N_3O$, 252.1136); (−)-HRESIMS *m*/*z* 250.0985 (calcd for $C_{15}H_{12}N_3O$, 250.0980).

AMPK Assay. A luciferase-based assay was performed using 0.2 mg/mL AMPK (Abbott Laboratories), 20 μM SAMs (HMRSAMS-GLHLVKRR) peptide (Abbott Laboratories), and 2 μM ATP (Roche, 10519979001) in a 384-well microtiter plate (Griener, 781095) to identify potential activators of AMPK. The buffer system contained 40 mM HEPES pH 7.0 (Sigma, H4034), 1 mM DTT (Boehringer Mannheim, 708992), 80 mM NaCl (BDH, 10241), 5 mM $MgCl_2$ (Sigma, M-2670), 0.8 mM EDTA (Sigma, ED2SS), 8% glycerol (Sigma, G-5516), and 0.18% Triton X-100 (Fluka, 93443). The reaction was allowed to incubate at room temperature for 120 min before the addition of luciferase/luciferin solution (Easylite-Kinase, Perkin-Elmer) to determine the amount of ATP left in the reaction mixture. AMPK activity will result in low luminescence, as the ATP will be used up by the kinase reaction, while the complete inhibition of AMPK activity will result in high luminescence. The percent reduction in luminescence was calculated as [(sample luminescence − high-luminescence control)/(low-luminescence control − high-luminescence control)] × 100. The assay had a Z' value of 0.60 ± 0.05, and the standard activator AMP had an EC_{50} of 0.7 ± 0.5 μM over the period of primary screening.

ATPlite Assay. To analyze whether **1** was an inhibitor against the luciferase/luciferin system, it was tested in a simple luminescence ATP detection system (ATPlite 1 Step, Perkin-Elmer), which uses the same firefly luciferase but without any components from the AMPK assay. The assay was performed according to the manufacturer's protocol with the same volume and concentration of ATP; **1** was added in serial dilution as in the AMPK assay for equivalent comparison.

■ ASSOCIATED CONTENT

Supporting Information. (+)- and (−)-HRESIMS and ^1H, ^{13}C, COSY, HSQC, and HMBC NMR spectra for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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