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A Fluorescence-Based Assay for N^5-Carboxyaminoimidazole Ribonucleotide Mutase

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Abstract

The enzyme N^5-carboxylaminoinidazole ribonucleotide (N^5-CAIR) mutase is found in microbial *de novo* purine biosynthesis but is absent in humans making it an attractive antimicrobial target. N^5-CAIR mutase catalyzes the synthesis of carboxyaminoimidazole ribonucleotide (CAIR) from N^5-CAIR which is itself prepared from aminoimidazole ribonucleotide (AIR) by the enzyme N^5-CAIR synthetase. During our research on identifying inhibitors of N^5-CAIR mutase, we developed an innovative, fluorescence-based assay to measure the activity of this enzyme. This assay relies upon our recent serendipitous observation that AIR reversibly reacts with the compound isatin. Reaction of a fluorescently-tagged isatin with AIR resulted in a large increase in fluorescence intensity allowing a measurement of the concentration of AIR in solution. From this observation, we developed a reproducible, non-continuous assay that can replicate the known kinetic parameters of the enzyme and can readily detect a recognized inhibitor of the enzyme. This assay should find utility in screening for inhibitors targeting N^5-CAIR mutase.

Graphical Abstract

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Author Contributions

M.S., C.S., and S.M.F. designed the study. M.S., S.S., and C.S. conducted the study. M.S., C.S., S.S., and S.M.F. analyzed the data. M.S., C.S., S.S., and S.M.F. interpreted the results. M.S., S.S., and S.M.F. wrote the manuscript.

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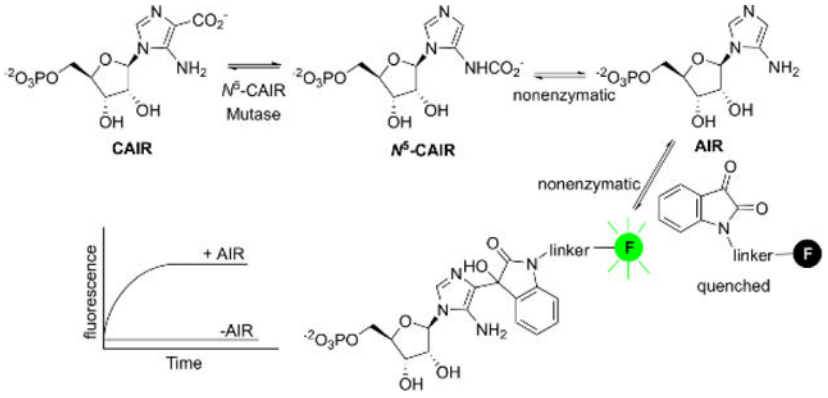

A robust, fluorescence-based assay has been developed that relies on the reaction between the novel istain-fluorecein conjugate and the enzymatically generated compound, AIR. These compounds react non-enzymatically resulting in a highly fluorescent product. This unique reaction provides a new assay for monitoring the activity of the enzyme N^5-CAIR mutase.

Keywords

assay development; enzyme inhibitors; N^5-CAIR mutase; screening assays

Introduction

In 2019, the Centers for Disease Control (CDC) reported that annually in the United States approximately 2.8 million people acquire an antibiotic-resistant infection resulting in 35,000 deaths each year [1]. In the same year, the World Health Organization reported 700,000 deaths worldwide due to antibiotic-resistant infections with a projection of 10 million deaths globally by 2050 [2]. The need for new antibiotics to treat antibiotic-resistant infections is critical, especially for those due to gram-negative bacteria. Unfortunately, there are few new agents in the antibiotic pipeline and pharmaceutical companies have largely abandoned antimicrobial drug discovery [3]. To identify new antimicrobial drugs with activity against resistant infections, there is a crucial need to develop novel antibiotics targeting unique pathways absent in the existing antibiotic armamentarium.

One underexplored pathway in antibiotic drug discovery is the *de novo* purine biosynthesis pathway. Previous research has shown that the *de novo* pathway is required for dividing cells and multiple lines of evidence have shown that inhibition of the pathway halts bacterial growth [4–5]. The *de novo* purine biosynthesis pathway is responsible for the synthesis of new purines by building the purine heterocycle onto the starting sugar, phosphoribosyl pyrophosphate (PRPP) [6]. Importantly, research conducted in the 1990s revealed that the pathway is different between microbes and humans. Humans require 10 enzymes to accomplish the synthesis of inosine monophosphate (IMP) from PRPP, whereas bacteria, yeast and fungi utilize 11 [6]. The divergence in the pathways is centered on the conversion of the intermediate 5-aminoimidazole ribonucleotide (AIR) to 4-carboxy-5-aminoimidazole ribonucleotide (CAIR) (Figure 1). In higher eukaryotes, AIR is directly carboxylated by the enzyme AIR carboxylase to give CAIR. However, in microbes, two enzymes are needed

to synthesize CAIR from AIR. The first enzyme, N^5-CAIR synthetase utilizes ATP and bicarbonate to carboxylate N^5 of AIR to generate the unstable carbamate, N^5-CAIR [7]. The second enzyme, N^5-CAIR mutase moves the CO_2 group from the N^5 to C^4 position to give CAIR. Genetic studies have validated that deletion of either enzyme results in organisms that are incapable of replicating in environments devoid of exogenous purines, including in animal models of disease [5]. The divergence in the pathway between humans and microbes provides a biochemical rationale for targeting these two enzymes for antibacterial and antifungal drug design.

Our laboratory has a longstanding interest on these enzymes[8–11]. Recently, we have focused on N^5-CAIR mutase as a potential target for antimicrobial drug development since numerous genetic studies have supported the critical role of this enzyme in bacterial cell viability. One of the challenges with drug discovery efforts focused on N^5-CAIR mutase are the limited assays available to monitor the enzyme activity (Table S1). The most common activity assay is the CAIR decarboxylation assay which monitors the conversion of CAIR into N^5-CAIR (and/or AIR) at 260 nm [7, 11–16]. While this assay is highly beneficial for kinetic analysis of the enzyme, the assay can be problematic in inhibition studies since many compounds absorb strongly at 260 nm. Other assays for N^5-CAIR mutase rely on the use of coupling enzymes [14, 16] and a recent metal-stopping assay measures AIR produced from CAIR using the Bratton-Marshal assay [17]. The assay requires heating samples with concentrated sulfuric acid, which presents safety and throughput challenges. A new assay which measures N^5-CAIR mutase activity at a higher wavelength or with a different spectroscopic method would be highly beneficial. In this paper, we will outline a new fluorescence-based assay that relies upon the recently discovered reaction between isatin and AIR to monitor enzymatic activity [8]. We will discuss the assay, its ability to accurately determine the kinetic parameters of the enzyme, as well as validation of the assay using the known inhibitor NAIR.

Results

Design of Assay.

Previously, three enzymatic assays have been developed and optimized for the assessment of N^5-CAIR mutase activity (Table S1). The most common is the CAIR decarboxylase assay in which CAIR is converted into N^5-CAIR (or AIR [a]) by the enzyme [7, 14–16, 18]. The conversion is monitored at 260 nm. We have utilized this assay for our previous inhibition studies; however, substantial problems with high absorbance from the library members were seen [11]. Another assay is a coupled assay in which N^5-CAIR mutase activity is measured based upon the conversion of AIR to SAICAR [14, 16]. This assay uses N^5-CAIR synthetase, SAICAR synthetase, pyruvate kinase, and lactate dehydrogenase and it measures ATP utilization by NADH utilization at 330 nm. The assay involves multiple reagents and enzymes, and requires extensive deconvolution to determine inhibition of N^5-CAIR

[a] N^5-CAIR is chemically unstable and will non-enzymatically decarboxylate to AIR. The rate of conversion of N^5-CAIR to AIR is dependent on pH, temperature, and the amount of dissolved CO_2. The half-life ranges from seconds to minutes depending on conditions. Because of this chemistry, N^5-CAIR mutase will convert CAIR to N^5-CAIR, which then generates AIR. AIR carboxylase will convert CAIR into AIR directly.

mutase versus the other enzymes used in the assay. Finally, a metal-stopping assay has been developed which relies upon the addition of zinc to halt the conversion of CAIR to AIR. The resulting AIR generated from the decarboxylation reaction was detected using a colorimetric Bratton-Marshall assay [14] [19]. While this method has been utilized for over 50 years, it requires treatment with concentrated sulfuric acid and heating to 80°C, both of which are not ideal for screening campaigns [19].

There is a clear need for a new enzymatic assay that can detect inhibitors of N^5-CAIR mutase. Ideally, we would like to have an assay that readily monitors enzymatic activity at a wavelength distant from the absorption of most organic compounds. Recently, we found that the compound isatin rapidly and non-enzymatically reacts with AIR and hypothesized that this reaction may allow for the determination of the concentration of AIR in solution (Figure 2A) [8]. Previous work indicated a UV change in isatin upon reaction with AIR, but the extinction coefficient was too low to be useful [8]. As part of our studies on the reactivity of isatin, we prepared an isatin analog in which the fluorescent dye, fluorescein, was added to the nitrogen of isatin (isatin-fluorescein, I-F, compound **4**, scheme 1). We investigated the reaction between I-F and AIR to see whether a change in fluorescence occurred upon reaction (Figure 2B).

Incubation of I-F with excess AIR resulted in a large increase in fluorescence intensity (Ex: 485 nm; Em: 528 nm) (Figure 3). This change was not observed when fluorescein was incubated with AIR (data not shown). The maximum change in fluorescence took approximately 15 minutes followed by a slow decrease in signal over many hours. We speculate that the decrease in fluorescence is due to precipitation of the AIR-isatin-fluorescein complex. Previous work on the reaction of AIR with isatin indicated that AIR attacked the C3 position of isatin (Figure 2A). To determine whether the change in fluorescence was due to reaction of AIR with isatin at the same location, we protected the C3 position as the cyclic ketal. Incubation of AIR with the protected I-F (**6**) resulted in no change in fluorescence compared to the isatin analog alone (Figure 3). This indicates that reaction at C3 is needed for the fluorescence change. In relation to this, it is interesting to note that the overall fluorescence of the protected I-F (**6**) is much larger than that of the I-F or the reacted AIR-I-F complex. This suggests that the conversion from sp^2 to sp^3 at C^3 triggers the increase of fluorescence of I-F.

Concentration of AIR versus Fluorescence Intensity.

A key question is whether the signal intensity of the assay was proportional to the concentration of AIR. To determine this, varying concentrations of AIR (8–80 μM) were incubated with 20 μM I-F and the change in fluorescence was monitored over 120 minutes. As shown in Figure 4, varying the concentration of AIR resulted in proportional changes in the maximal fluorescence intensity. At lower concentrations of AIR, it took approximately 60 minutes to reach equilibrium.

A plot of the concentration of AIR versus the fluorescence intensity at 60 minutes gave a linear line with a R^2 value of 0.99 indicating that the fluorescence intensity is proportional to the concentration of AIR (Figure S1).

Enzymatically Generated AIR and its Reactions with I-F.

The previous experiments utilized chemically synthesized AIR. For this reaction to be useful in the determination of enzyme activity, a large fluorescence change must be observed for AIR generated from CAIR by the enzyme. As mentioned earlier, N^5-CAIR mutase generates N^5-CAIR from CAIR and not AIR. However, N^5-CAIR is chemically unstable and will non-enzymatically and rapidly decarboxylate to generate AIR [7]. Another challenge in a discontinuous enzymatic assay is the requirement for a method to stop the enzymatic reaction at a specified time. Other researchers have utilized acid or base to halt the enzymatic reaction [17]; however, initial studies revealed that the presence of either reagent altered the chemistry between AIR and isatin and thus changed the fluorescence intensity. Fortunately, a 2014 paper from the Kappock Lab[17] discovered that zinc inhibits N^5-CAIR mutase by chelating to CAIR preventing it from interacting with the enzyme.

To measure the production of AIR from CAIR, a time-dependent experiment was conducted using 100 μM CAIR and 89 ng of enzyme in Tris, pH 8 buffer. At various time points, 50 μL of the reaction mixture was removed, diluted with buffer and the reaction was stopped by the addition of 150 μM $ZnCl_2$. After stopping the enzymatic reaction, the AIR produced was allowed to react with 10 μM I-F and the fluorescence was monitored over one hour using excitation/emission wavelengths of 485 nm/528 nm. Figure 5 shows a single enzymatic reaction at varying time points.

Fluorescence values at 60 minutes for each stoppage time were plotted to examine the linearity of the enzymatically produced AIR (Figure S2). As seen in Figure S2, the production of AIR from the enzymatic decarboxylation of CAIR is linear.

Dependence of I-F on N^5-CAIR Mutase Concentration

Since the conversion of CAIR to AIR by the enzyme was linear, we next sought to determine if the conversion was also proportional to the concentration of the enzyme. Assays were conducted in which the amount of N^5-CAIR mutase was varied from 0 to 102 ng while the concentration of CAIR was held fixed at 62 μM. After 5 minutes, the enzymatic reaction was stopped by the addition of 300 μM $ZnCl_2$. To measure the amount of AIR produced, 20 μM I-F was added and fluorescence was monitored over 60 minutes. As shown in Figure 6, increasing the concentration of enzyme resulted in an increase in the conversion of CAIR to AIR.

Determining Z-factor.

To measure assay robustness, the Z’ was calculated. To do this, we conducted a screen using eight non-inhibited replicates of the enzymatic assay and eight 300 μM zinc-inhibited replicates. The enzymatic reaction was initiated upon the addition of enzyme to each well. The reaction was stopped at 10 minutes by the addition of $ZnCl_2$, I-F was added and the reaction with AIR was followed over 60 minutes (Figure 7). The mean and standard deviations of the fluorescence maxima at 60 minutes were determined and the calculated Z’ is 0.79 which is indicative of a robust assay [20].

NAIR as Control.

Though a simulated inhibition study was done using zinc to show dose dependent inhibition, another control was to test a known inhibitor of N^5-CAIR mutase to further verify the effectiveness of the assay. NAIR is a potent inhibitor of AIR carboxylase with a Ki of 0.34 nM, but is also a micromolar inhibitor of N^5-CAIR mutase [9]. As shown in Figure 8, NAIR results in a dose-dependent decrease in fluorescence intensity validating the ability of the assay to detect enzyme inhibition.

V_{max} and K_m Studies.

With a reproducible and functional assay, we next sought to determine whether the assay could reproduce the V_{max} and K_m values of the enzyme as determined by the standard UV-based CAIR decarboxylation assay. One of the challenges with this comparison is the fact that the chemistry occurring in each assay is different. In the UV assay, the change in absorbance is directly related to the conversion of CAIR to N^5-CAIR. The non-enzymatic decarboxylation of N^5-CAIR to AIR is modest in the time frame of the experiment and the low surface area of the 1000 μL cuvette hinders the loss of CO_2 from the reaction. In contrast, the fluorescence assay requires the conversion of N^5-CAIR to AIR before reaction with I-F. The fluorescence-based assay is conducted in a much shallower 96-well plate with a final volume of 200 μL. The smaller volume and larger surface area affects the amount of AIR formed during the experiment because it alters the amount of CO_2 in solution [21].

Initial studies revealed substantial differences in the values from both assays; however, limiting the exposure of air in the microplate by sealing the plate yielded V_{max} and K_m values that are comparable between both assays. The V_{max} and K_m for the UV assay are 58 ± 0.5 μmoles/min•mg and 8.3 ± 3 μM (Figure S9), respectively. For the fluorescence assay, the V_{max} and K_m values are 14 ± 1.3 μmoles/min•mg and 27.8 ± 14 μM (Figure S10).

Discussion

Although a significant amount of research has been conducted on the discovery of inhibitors of N^5-CAIR mutase, no selective, potent drug-like molecule against the enzyme is known. We have speculated that one reason for this is the lack of a suitable activity-based high-throughput assay. Existing activity assays rely upon changes in absorption at 260 nm or the use of multiple coupling enzymes. Most small organic compounds absorb at 260 nm and coupling assays require extensive deconvolution. Binding assays such as thermal shift and surface plasmon resonance have been used previously, yet do not report on activity and could identify binding agents that target pockets distal from the active site [13, 15].

Given these challenges, we sought to develop a new assay that measured activity yet was compatible with the absorption of organic compounds found in commercial libraries. Our fluorescence assay takes advantage of our recent observation that AIR reacts with isatin [8]. Reaction of I-F with chemically synthesized AIR resulted in an increase in fluorescence which plateaued at 60 minutes. This reaction was considerably slower than reaction with isatin alone which completely reacted with AIR in only a few minutes [8]. We speculate that the presence of the large fluorescein molecule sterically hinders the reaction at C^3 of

isatin. We have been unable to determine the exact reason for the fluorescence increase upon the reaction. UV and fluorescence measurements of isatin, fluorescein and AIR did not indicate obvious spectral overlap. Fluorescein has a strong pH dependence with an increase in fluorescence as the pH increases [22]. However, all reactions take place in millimolar buffer which should negate a pH effect on fluorescence. The fact that the signal increases upon reaction indicates that the fluorescein is quenched in I-F. Previous studies have noted that fluorescein binding to proteins results in quenching due to interactions with aromatic amino acids [23]. We hypothesize that an intramolecular interaction between isatin and fluorescein results in quenching of the dye. Reaction of AIR with isatin disrupts this interaction leading to an increase in fluorescence. This hypothesis is supported by the larger fluorescent signal of the C^3 ketal protected I-F (compound **6**). In this molecule, the sp^3-hybridized C^3 contains the ketal group which is orthogonal to the plane of the ring which could disrupt the interaction between isatin and fluorescein. The interaction between these two groups could also explain the reduced reactivity of I-F with AIR.

Once we verified that a reaction could be detected, various dose dependence studies were done involving different concentrations of chemically synthesized AIR. All of these showed a linear dependence between the fluorescence signal and AIR concentration. AIR generated via an enzymatic reaction also gave a signal change. The enzymatic reaction required a means to stop the conversion so that the amount of AIR in solution can be accurately measured at a fixed timepoint. Different methods of stopping enzymatic reactions include altering the pH, heating, or freezing; however, these methods either were not practical or resulted in changes in fluorescence independent of reaction with AIR. Fortunately, a 2014 paper from the Kappock Laboratory [17] found that zinc can be used in the application of discontinuous assays involving CAIR as the substrate. Zinc was found to chelate CAIR preventing either binding to the enzyme or decarboxylation [17]. After verifying this stopping method, zinc was chosen to halt the N^5-CAIR mutase mediated reaction.

Since there are more components to the N^5-CAIR mutase mediated reaction, each individual component was tested with I-F to make sure any unwanted reactions were not observed. When CAIR and I-F are incubated in buffer, it was observed that there was also a large increase in fluorescence over time. Fortunately, the addition of zinc needed to halt the enzymatic reaction also prevented CAIR from reacting with I-F [17]. No other components reacted with I-F. Finally, we demonstrated that the assay was reproducible and robust with a Z’ of 0.79.

Our next goal is to conduct a pilot screen using this assay. We anticipate that we will have to deal with quenching of the signal by organic compounds and/or the reaction of library compounds with isatin. Unfortunately, uncatalyzed reactions in water of various functional groups with isatin have had limited study [24–26]. The few papers that do discuss these reactions typically involve elevated temperatures along with thiols and hydrazines [24, 27–29]. Undoubtedly, we will discover other reactions during screening, and we will report the results of these future studies in due course.

Experimental Section

General Comments.

All reagents and solvents were purchased from either Sigma-Aldrich, Acros Organics, Alfa Aesar or TCI Chemicals and were used without further purification. Fluorescein 5-isothiocyanate (5-FITC 'Isomer I') was purchased from Thermo Fisher Scientific, Inc (Invitogen). Microwave vials were obtained from Biotage. Microwave reactions were conducted using a Biotage Initiator with settings dependent on the solvent or as listed. Silica gel for column chromatography was purchased from Sorbent Technologies, Inc. Thin layer chromatography (TLC) were performed on pre-coated (0.25 mm) silica gel plate (Sorbtech, 60 F-254), and visualization was done either by UV (254 nm), or iodine staining. Column chromatographic purifications of compounds were performed on silica gel (Sorbtech, 60–230 mesh, 0.063–0.20mm). ^{1}H and ^{13}C NMR spectra were recorded on a Varian 600 MHz or Bruker 400 MHz spectrometer by dissolving the compounds in deuterated solvents as chloroform-*d* ($CDCl_3$), methanol-d_4 (CD_3OD) or dimethyl sulfoxide-d_6 (DMSO-d_6) and all peaks were referenced with TMS as an internal standard or to the residual solvent peak. Chemical shifts are expressed in ppm (δ) whereas coupling constants (*J*) are listed in hertz (Hz) and the multiplicities are recorded by the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), p (quintet/pentet), m (multiplet), and bs (broad signal).

Protein Expression and Purification.

E. coli protein expression and purification was done according to previously described methods [8].

Synthesis of CAIR.

CAIR was synthesized according to previously published methods [16, 30]. Solid CAIR was dissolved in 100 mM Tris pH 8 buffer and the concentration of CAIR was determined by absorbance at 260 nm using the extinction coefficient ε_{260}=10,500 $M^{-1}cm^{-1}$. Stocks were stored at −80°C and were thawed on ice before use. CAIR solutions were stored on ice during the day and the remaining stock was discarded at the completion of all assays.

Synthesis of AIR.

AIR was prepared by published methods. Solid AIR was dissolved in water and the concentration of AIR was determined by absorbance at 260 nm using the extinction coefficient ε_{260}=1570 $M^{-1}cm^{-1}$. AIR stocks were stored at −80°C and were thawed on ice before use. AIR stocks were stored on ice and discarded at the completion of all assays.

Synthesis of Isatin-Fluorescein Analogs (4,6)

Compound 2.: In a microwave vial, isatin (**1**, 295 mg, 2.0 mmol) and K_2CO_3 (830 mg, 6.0 mmol) were added along with anhydrous DMF (10 mL). The mixture was stirred for 5 minutes at room temperature. A solution of 3-(*tert*-butyloxycarbonyl)aminopropylbromide[31] (478 mg, 2.0 mmol) in 2 mL of DMF was added and the vial was placed into the microwave reactor and heated to 50°C using the "very high absorbance" setting for 1 minute. The reaction vessel was cooled to room temperature and

the pressure from the vessel was released via a needle inserted into the tube's rubber septa. The above process was repeated an additional four times with varying lengths of time (3, 15, and 60 minutes respectively) while maintaining a temperature of 50°C. The progress of the reaction was monitored by TLC (n-hexane-ethyl acetate (2:1 and 1:1)). After completion of the reaction, DMF was decanted from the tube and the residue was washed with 15 mL of ethyl acetate. The DMF and ethyl acetate soluble materials were combined, and the solvent was removed using a rotary evaporator to produce a viscous brown-red material. The product was purified by silica column chromatography using a gradient of n-hexane-ethyl acetate (2:1, 1:1, 1:2 and 0:1) to afford 492 mg (81%) of an orange-red solid. R*f*. 0.43 (n-hexane-ethyl acetate, 1:1); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.58–7.56 (m, 2H), 7.11 (t, 1H, *J* = 7.8 Hz), 6.90 (d, 1H, *J* = 7.8 Hz), 5.05 (bs, 1H), 3.78 (t, 2H, *J* = 6.6 Hz), 3.15 (m, 2H), 1.86 (m, 2H), 1.41 (s, 9H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 183.22, 158.56, 156.01, 150.50, 138.45, 125.57, 123.93, 117.64, 110.11, 79.41, 37.50, 28.36, 27.21.

1-(3-Aminopropyl)-isatin hydrochloride (3).: Compound **2** (1.75g, 5.75 mmol) was dissolved into 40 mL of anhydrous ethyl acetate under argon atmosphere and 5 mL of 1M HCl in ethyl acetate was added dropwise. The reaction was stirred at room temperature for 24h followed by the addition of 5 mL of 1M HCl in ether. Over approximately 1 hr, an orange-red solid separated to the bottom of the flask and the solvent was decanted leaving the orange-red solid. The solid was washed with 5 mL of ether (3X) and dried under high vacuum (~0.01 Torr) to give 1.32 g (95% yield) of compound **3**. ^{1}H NMR (DMSO-d_6, 600 MHz): δ 8.14 (bs, 3H), 7.64 (dt, 1H, *J* = 7.8 and 1.2 Hz), 7.52 (dd, 1H, *J* = 7.2 and 0.6 Hz), 7.27 (d, 1H, *J* = 7.8 Hz), 7.10 (t, 1H, *J* = 7.2 Hz), 3.74 (t, 2H, *J* = 7.2 Hz), 2.86 (q, 2H, *J* = 6.0 Hz), 1.89 (p, 2H, *J* = 7.2 Hz); ^{13}C NMR (DMSO-d_6, 150 MHz): δ 183.29, 158.40, 150.41, 137.99, 124.44, 123.18, 117.73, 110.69, 37.06, 36.58, 25.15.

Compound 4 (I-F).: Compound **3** (121 mg, 0.50 mmol) and 5-FITC (194 mg, 0.50 mmol) were placed in a 100 mL round bottom flask followed by the addition of 10 mL of anhydrous DMF. The reaction was placed under argon atmosphere. The solution was stirred vigorously and a solution of DIPEA (258 mg, 2.0 mmol) in DMF (2 mL) was added dropwise over 30 min. The reaction continued to stir at room temperature for 48h and the progress of the reaction was monitored by silica TLC using CH_2Cl_2-MeOH (9:1). After completion, DMF was removed by high vacuum rotary evaporation (~5 Torr) to give a viscous oily product. The product was purified by silica column chromatography using a gradient of CH_2Cl_2-MeOH (90:10, 85:15, 80:20, 70:30, and 50:50). The orange-red solid obtained after column chromatography was dried under high vacuum (~0.01 Torr) while heating to 50 °C for 12 hrs to give 102 mg (34% yield) of the desired product. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.10 (s, 2H), 9.97 (bs, 1H), 8.20 (s, 1H), 8.06 (bs, 1H), 7.73 (d, 1H, *J* = 8.0 Hz), 7.69–7.65 (m, 1H), 7.56 (d, 1H, *J* = 7.6 Hz), 7.23–7.12 (m, 3H), 6.68–6.56 (m, 6H), 3.75 (t, 2H, *J* = 6.8 Hz), 3.60 (b, 2H), 1.96 (p, 2H, *J* = 6.8 Hz); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 183.39, 180.60, 168.48, 159.46, 158.30, 151.88, 150.54, 147.39, 141.18, 138.10, 129.82, 129.03, 126.57, 124.45, 124.09, 123.16, 117.62, 116.85, 112.58, 110.63, 109.71, 102.24, 83.02, 41.36, 37.38, 26.24. HRMS (ESI, m/z): calculated for $C_{32}H_{24}N_3SO_7$ (M+H) ± : 594.1329; found, 594.1326

C3-protected isatin (5).: In a round bottom flask equipped with a stir bar, isatin **1** (2.97 g, 20.18 mmol) was added to 70 mL of toluene. The resulting suspension was stirred while 4.0 mL of ethylene glycol was added followed by a catalytic amount of *p*-toluenesulfonic acid, PTSA (35 mg). The reaction was stirred at room temperature for 2 hr. A Dean-Stark apparatus was added to the reaction and the reaction was refluxed for 4.5 hrs to remove water from the reaction. After TLC analysis indicated the absence of starting material, toluene was removed by rotary evaporation leaving a viscous material. The product was dissolved in CH_2Cl_2 (100 mL) and stirred with 10% $NaHCO_3$ solution (50 mL). The organic layer was separated, washed with water and the organic layer was concentrated by rotary evaporation to give 3.43 g of solid spiro[indoline-3,2'-[1,3]dioxolan]-2-one (**5**, 89% yield). R*f*. 0.48 (n-hexane-ethyl acetate, 1:1); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.64 (bs, 1H), 7.35 (d, 1H, *J* = 7.8 Hz), 7.31–7.29 (m, 1H), 7.07–7.05 (m, 1H), 6.81 (d, 1H, *J* = 8.4 Hz), 4.57–4.55 (m, 2H), 4.33–4.31 (m, 2H).

Compound 6.: Compound **5** and 3-(*tert*-butyloxy-carbonyl)aminopropylbromide were reacted according to the method described for compound **2** with the following changes; microwave heating to 60°C for 2 min, then 65°C for 45 min, then 65°C for 1h, and finally 75°C for 30 min. Product formation was monitored after each microwave run by TLC (1:1 ethyl acetate:hexane). The product was purified by silica column chromatography using a gradient of n-hexane-ethyl acetate (4:1, 2:1,1:1 and 1:2) to afford a viscous product which contained a small amount (~5%) of unreacted **5**. This mixture was used in the next step without additional purification.

Compound **5** was dissolved in CH_2Cl_2 (85 mL), cooled in an ice-bath and trifluoroacetic acid (4.5 mL) was added dropwise. The reaction was brought to room temperature and stirred until TLC analysis showed complete reaction (approximately 5–6h). After completion, the solvent was removed *in vacuo* to give a viscous TFA salt of the amine. This was used in the next step without purification.

The TFA salt (211 mg, 0.58 mmol) and 5-FITC (196 mg, 0.50 mmol) were dissolved in dry DMF (10 mL) containing DIPEA (0.40 mL). The reaction was stirred at room temperature for 48 hr and treated according to the protocols outlined for compound **4**. The crude product was purified by silica column chromatography using a gradient of CH_2Cl_2-MeOH (97.5:2.5, 95:05, 90:10, 80:20, and 70:30) to afford an orange-red solid. This material was subjected to a second chromatographic purification using a step gradient of ethyl acetate-MeOH (90:10: 80:20 and 70:30). The fractions containing pure product were collected and concentrated to dryness by rotary evaporation. The resulting solid was dried for 12 hr while heating to 50 °C under high vacuum (~0.01 Torr) to give 83 mg (26% yield) of the desired product. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.10 (s, 2H), 9.99 (bs, 1H), 8.19 (s, 1H), 8.10 (bs, 1H), 7.75 (d, 1H, *J* = 7.6 Hz), 7.47–7.39 (m, 2H), 7.19 (d, 1H, *J* = 8.4 Hz), 7.15–7.09 (m, 2H), 6.68 (d, 2H, *J* = 2.0 Hz), 6.63–6.55 (m, 4H), 4.39 (t, 2H, *J* = 6.8 Hz), 4.29 (t, 2H, *J* = 6.4 Hz), 3.69 (t, 2H, *J* = 7.2 Hz), 3.55 (2 peaks, 2H), 1.91 (p, 2H, *J* = 6.8 Hz); ^{1}H NMR (CD_3OD, 400 MHz): δ 8.07 (d, 1H, *J* = 1.6 Hz), 7.72 (dd, 1H, *J* = 8.0 and 1.6 Hz), 7.39 (d, 1H, *J* = 8.0 Hz), 7.34 (d, 1H, *J* = 6.8 Hz), 7.16 (d, 1H, *J* = 8.4 Hz), 7.09 (t, 1H, *J* = 7.6 Hz), 7.02 (d, 1H, *J* = 7.6 Hz), 6.70–6.67 (m, 4H), 6.54 (d, 1H, *J* = 2.4 Hz), 6.52 (d, 1H, *J* = 2.4 Hz), 4.43–4.40 (m, 2H), 4.27–4.24 (m, 2H), 3.70 (t, 2H, *J* = 7.2 Hz), 3.64 (t, 2H, *J* = 6.0 Hz),

1.98 (m, 2H); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 182.82, 175.74, 171.06, 161.49, 154.20, 150.20, 144.62, 141.77, 132.89, 132.49, 130.38, 129.35, 125.97, 124.61, 120.85, 113.68, 11.46, 110.47, 103.58, 103.51, 67.06, 42.39, 37.92, 27.44. HRMS (ESI, m/z): calculated for $C_{34}H_{28}N_3SO_8$ (M+H) ± : 638.1592; found, 638.1590.

Fluorescence Assay for N^5-CAIR Mutase.: The assay was conducted using non-treated Nunc™ F96 Microwell™ white polystyrene plates with a final volume of 100 μl per well. Each well contained 34 μM CAIR dissolved in 25 mM Tris pH 8 buffer. The enzymatic reaction was initiated with the addition of 104 ng of N^5-CAIR mutase. After 10 minutes, the reaction was stopped by the addition of 300 μM $ZnCl_2$ along with 30 μM isatin-fluorescein (I-F, compound **4**). The plate was incubated at room temperature for 1 hour and the fluorescence was measured every minute using a BioTek Synergy 2 multimode microplate reader with excitation and emission wavelength filters of 485 nm/528 nm respectively.

Vmax and Km Determination.: The UV CAIR decarboxylation assay was run as follows. Into a quartz cuvette with a final volume of 1 ml, 25 mM Tris pH 8.0 buffer was added followed by CAIR (5.7–120 μM). The background absorbance at 260 nm was taken using a Varian Cary 1 Bio UV-Visible spectrophotometer. The reaction was initiated by the addition of 100 ng of N^5-CAIR mutase. The absorbance was measured at 5-minute intervals for 60 minutes and the concentration of AIR at each time point was calculated from the absorbance using the extinction coefficient of ε_{260}= 8,930 M^{-1} cm^{-1}. This was repeated for each concentration of CAIR used. The fluorescence assay was run as described above except that the concentration of CAIR was varied (5.7–120 μM). The reaction was initiated by the addition of 100 ng of N^5-CAIR mutase, and the plate sealed with a rubber septum. Enzymatic assays were stopped by the addition of 300 μM $ZnCl_2$ at times ranging from 5–60 minutes. To each reaction, 20 μM I-F (**4**) was added, and the reaction incubated for 60 minutes. The fluorescence value at 60 minutes was measured as described above and the concentration of AIR was determined using a standard curve. For both sets of data (UV and fluorescence), a plot of [AIR] versus time was generated at each concentration of CAIR used. The resulting progress curve was fitted to the equation below to generate the initial velocity. In this equation, v_0 represents the initial velocity and n is the rate of change for the non-linear portions of the plot. Plots of v_0 versus CAIR were generated and fitted to the Michaelis-Menten equation to obtain Vmax and Km values.

$$[AIR] = \frac{v_0}{n}(1 - e^{-nt}) \quad (1)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

[1]. CDC, U.S. Department of Health and Human Services, CDC 2019.

[2]. World Health Organization: Geneva, Switzerland 2019.

[3]. Dheman N, Mahoney N, Cox EM, Farley JJ, Amini T, Lanthier ML, Clin Infect Dis 2021, 73, e4444–e4450. [PubMed: 32584952]

[4]. McFarland WC, Stocker BA, Microb Pathog 1987, 3, 129–141. [PubMed: 2849016]

[5]. Samant S, Lee H, Ghassemi M, Chen J, Cook JL, Mankin AS, Neyfakh AA, PLoS Pathog 2008, 4, e37. [PubMed: 18282099]

[6]. Kappock TJ, Ealick SE, Stubbe J, Curr Opin Chem Biol 2000, 4, 567–572. [PubMed: 11006546]

[7]. Firestine SM, Misialek S, Toffaletti DL, Klem TJ, Perfect JR, Davisson VJ, Arch Biochem Biophys 1998, 351, 123–134. [PubMed: 9500840]

[8]. Streeter CC, Lin Q, Firestine SM, Biochemistry 2019, 58, 2260–2268. [PubMed: 30964980]

[9]. Firestine SM, Wu W, Youn H, Davisson VJ, Bioorg Med Chem 2009, 17, 794–803. [PubMed: 19095456]

[10]. Dewal MB, Firestine SM, Biochemistry 2013, 52, 6559–6567. [PubMed: 23899325]

[11]. Fawaz MV, Wayne State University 2012.

[12]. Firestine SM, Purdue University 1995.

[13]. Kim A, Wolf NM, Zhu T, Johnson ME, Deng J, Cook JL, Fung LW, Bioorg Med Chem 2015, 23, 1492–1499. [PubMed: 25737087]

[14]. Meyer E, Leonard NJ, Bhat B, Stubbe J, Smith JM, Biochemistry 1992, 31, 5022–5032. [PubMed: 1534690]

[15]. Lei H, Jones C, Zhu T, Patel K, Wolf NM, Fung LW, Lee H, Johnson ME, Bioorg Med Chem 2016, 24, 596–605. [PubMed: 26740153]

[16]. Firestine SM, Davisson VJ, Biochemistry 1994, 33, 11917–11926. [PubMed: 7918410]

[17]. Sullivan KL, Huma LC, Mullins EA, Johnson ME, Kappock TJ, Anal Biochem 2014, 452, 43–45. [PubMed: 24525042]

[18]. Meyer E, Kappock TJ, Osuji C, Stubbe J, Biochemistry 1999, 38, 3012–3018. [PubMed: 10074353]

[19]. Bratton AC, Marshall EK, J Biol Chem 1939, 128, 537–550.

[20]. Zhang JH, Chung TD, Oldenburg KR, J Biomol Screen 1999, 4, 67–73. [PubMed: 10838414]

[21]. Hoskins AA, Morar M, Kappock TJ, Mathews II, Zaugg JB, Barder TE, Peng P, Okamoto A, Ealick SE, Stubbe J, Biochemistry 2007, 46, 2842–2855. [PubMed: 17298082]

[22]. Martin MM, Lindqvist L, Journal of Luminescence 1975, 10, 381–390.

[23]. Lakowicz JR, Malicka J, D'Auria S, Gryczynski I, Anal Biochem 2003, 320, 13–20. [PubMed: 12895465]

[24]. Chen H, Shi D, J Comb Chem 2010, 12, 571–576. [PubMed: 20515044]

[25]. Yang J, Wu L, Fang D, Cao J, Ji S, Chinese Science Bulletin 2013, 58, 2944–2949.

[26]. Subhendu Naskar e. a., Journal of Chemical Research 2009.

[27]. Chen H, Shi D, Tetrahedron 2011, 67, 5686–5692.

[28]. Snavely FA, Un S, The Journal of Organic Chemistry 1981, 46, 2764–2766.

[29]. Ibrahim S, Mohamed E, Rahman RA, Halim AA, Journal of The Chemical Society of Pakistan 2011, 9, 523.

[30]. Firestine SM, Poon SW, Mueller EJ, Stubbe J, Davisson VJ, Biochemistry 1994, 33, 11927–11934. [PubMed: 7918411]

[31]. Franciskovich JBH, D. K.; Klimkowski VJ; Marquart AL; Maters JJ; Mendel D; Ratz AM; Smiyh GF; Wiley MR; and Yee YK, 2009.

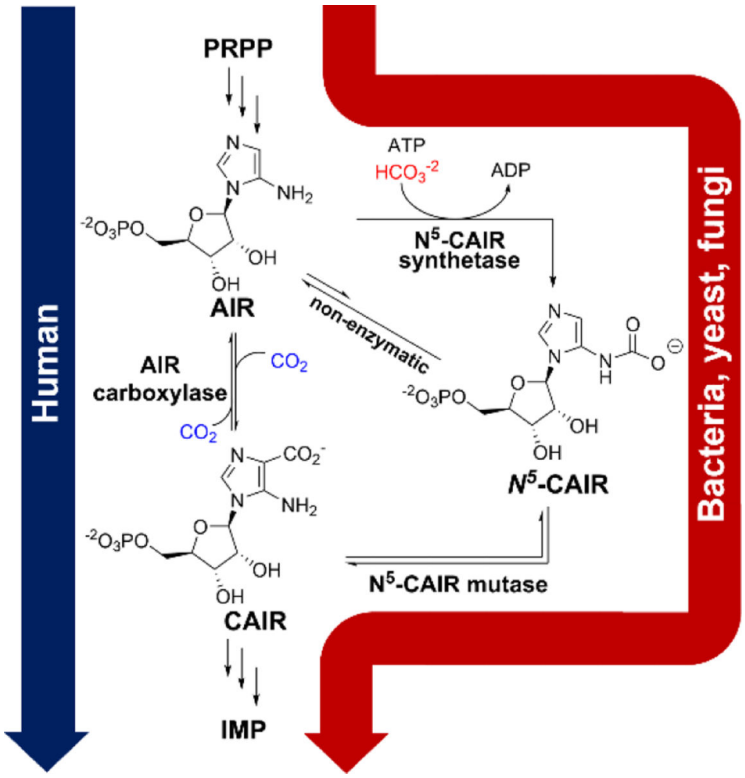


Figure 1. Divergence in the de novo purine biosynthesis pathway.

The de novo pathway converts phosphoribosyl pyrophosphate (PRPP) into inosine monophosphate (IMP) in 10-steps in humans (blue arrow) or 11-steps in microbes (red arrow). Humans directly convert AIR into CAIR via the enzyme AIR carboxylase. Microbes synthesize CAIR via the intermediate N^5-CAIR. N^5-CAIR is chemically unstable and will decarboxylate back to AIR at a rate that is dependent on pH, temperature, and CO_2 concentrations. N^5-CAIR is synthesized by the enzyme N^5-CAIR synthetase and is converted into CAIR by the enzyme N^5-CAIR mutase. N^5-CAIR mutase and AIR carboxylase are structurally and evolutionarily related and both are reversible enzymes.

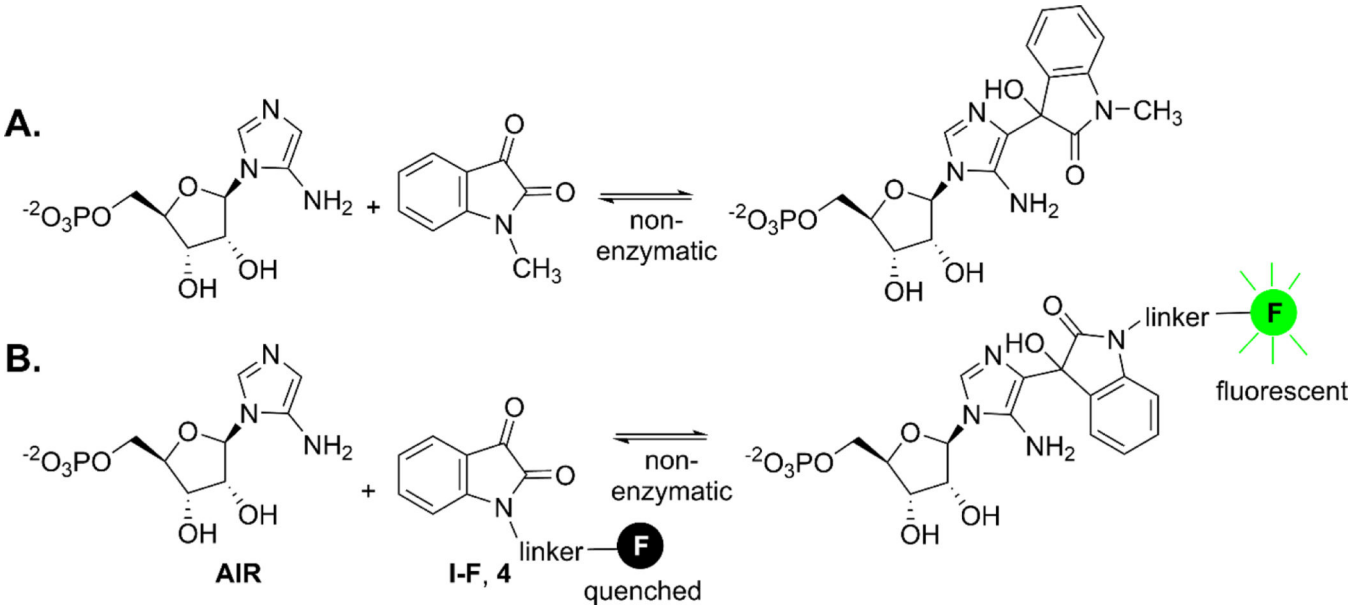


Figure 2. Reaction of isatin with AIR.

A. The non-enzymatic, reversible reaction of isatin with AIR occurs by the attack of C4 of AIR onto the C3 carbonyl group of N-methylisatin. Deprotonation of the resulting tetrahedral intermediate results in rearomatization of AIR to generate the product shown. B. Attack of AIR onto the labeled isatin (**I-F**, **4**) converts it from a weakly compound into a highly fluorescent one.

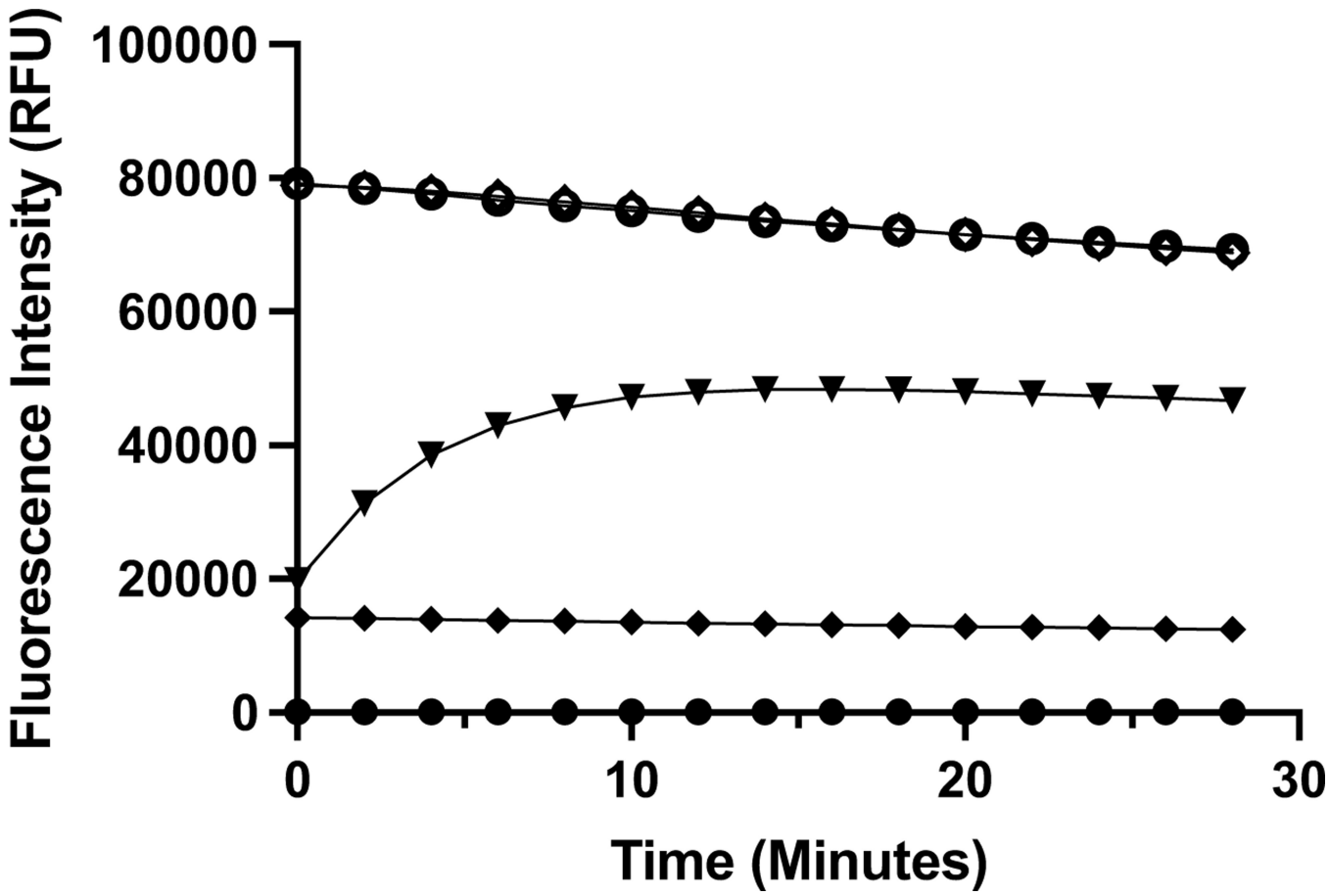


Figure 3. Change in fluorescence upon reaction of AIR with I-F.

• AIR in buffer without I-F; ◆ 10 μM I-F in buffer without AIR; ▼ excess AIR + 10 μM I-F; ○ 10 μM protected I-F in buffer without AIR;◊ excess AIR + protected I-F. Error bars are not shown due to minimal error between replicates. Overlapping data points for “protected I-F” and “excess AIR + protected I-F” are shown.

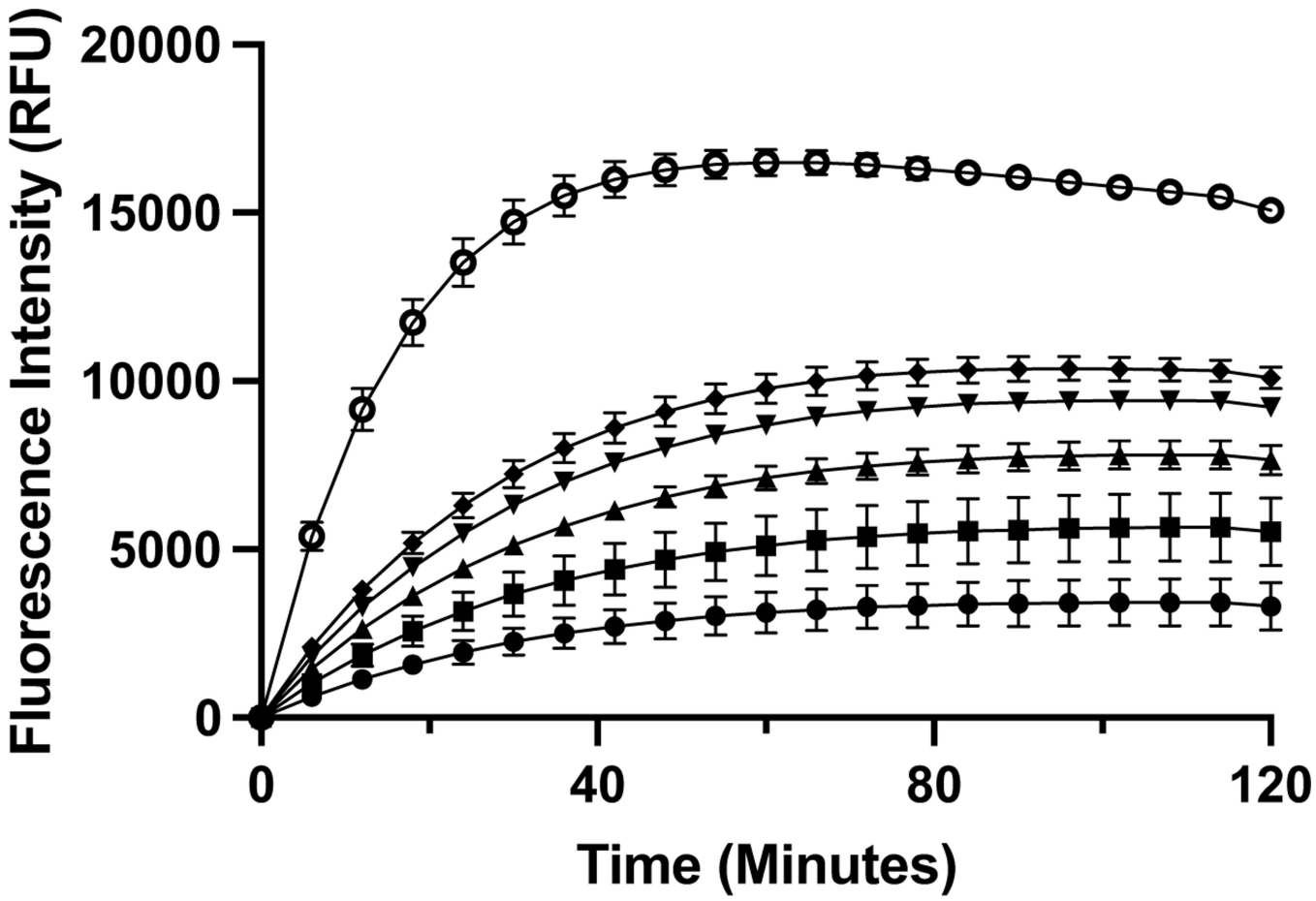


Figure 4. Dose dependence of AIR reacting with 20 μM I-F.

The concentrations of AIR tested are: • 8μM, ■ 16 μM, ▲ 24 μM, ▼ 32 μM, ◆ 40 μM, and ○ 80 μM. All data has been normalized to zero fluorescence at time zero.

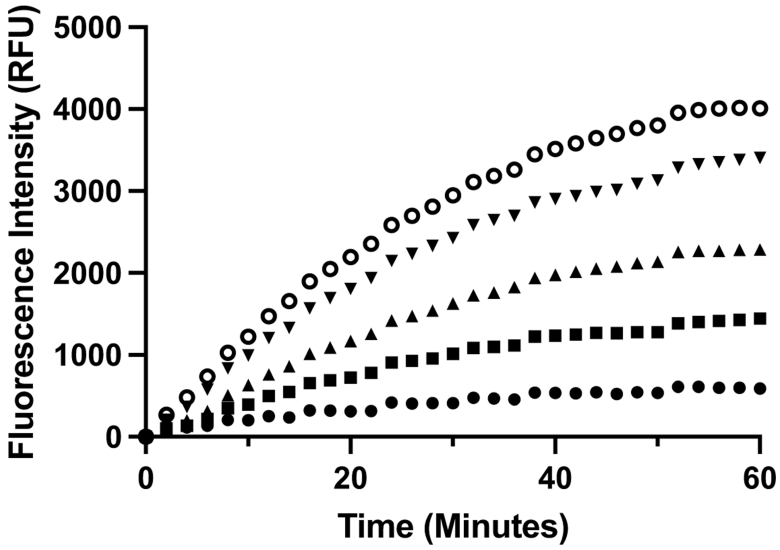


Figure 5. Aliquots of enzymatic reaction mixture were stopped with $ZnCl_2$ at varying time points, and then allowed to react with I-F for 60 minutes.

Stoppage time of each enzymatic reaction in minutes were: • 2 min ■ 8 min, ▲ 14 min, ▼ 20 min, , and ○ 26 min. All data has been normalized to zero fluorescence at time zero.

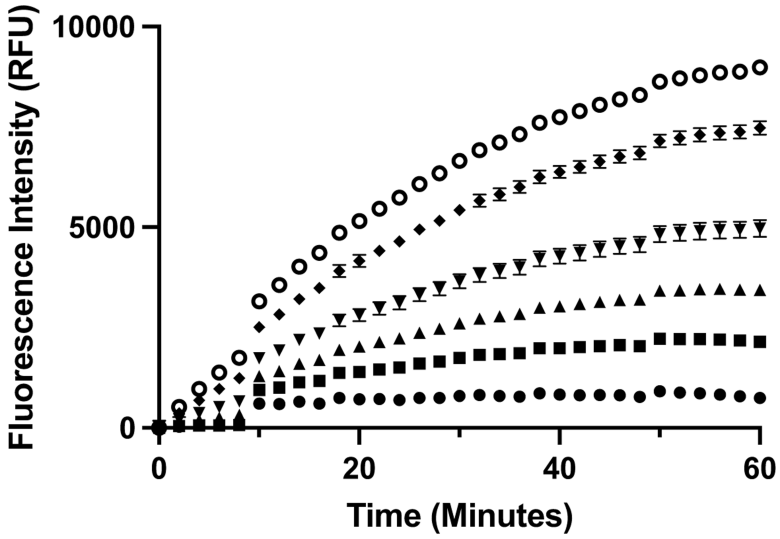


Figure 6. Varied N^5-CAIR mutase concentrations versus fluorescence.

Varied amounts of enzyme are indicated as: • 0 ng, ■ 6.4 ng, ▲ 12.8 ng, ▼ 25.5 ng, ◆ 51 ng, and ○ 102 ng. All data has been normalized to zero fluorescence at time zero.

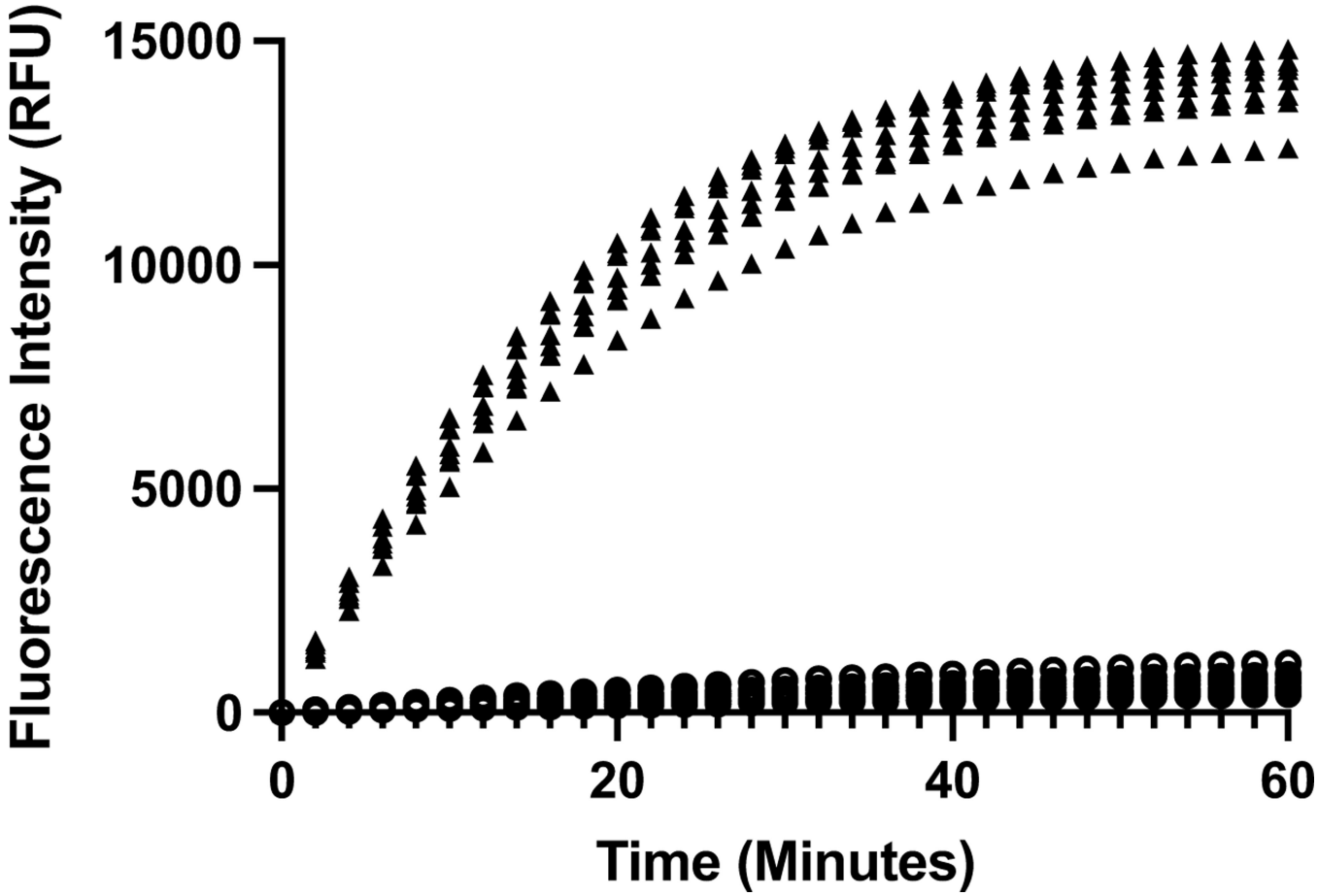


Figure 7. Reproducibility of the assay.

Replicates of 8 uninhibited (▲) N^5-CAIR mutase mediated reactions and replicates of 8 zinc inhibited (○) N^5-CAIR mutase mediated reactions. All data has been normalized to zero fluorescence at time zero.

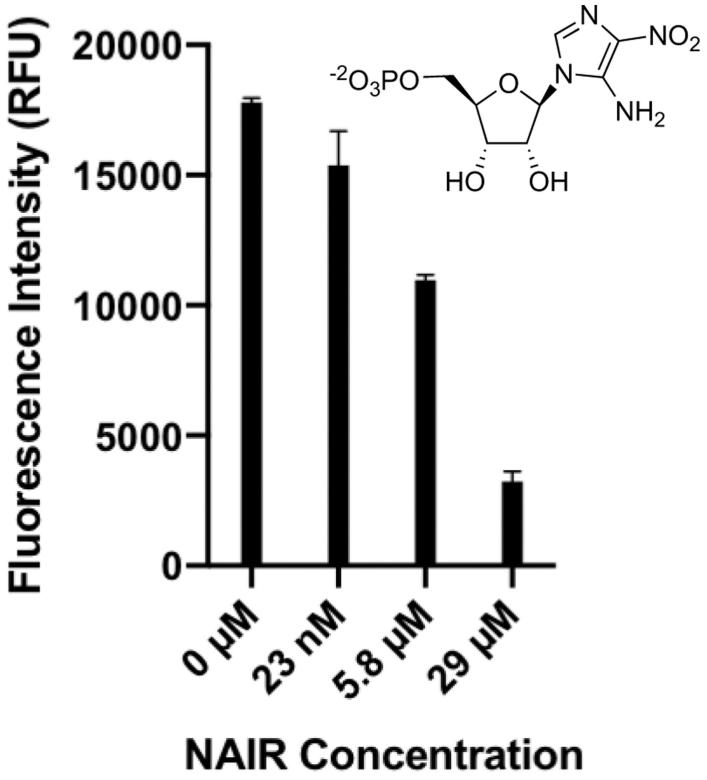
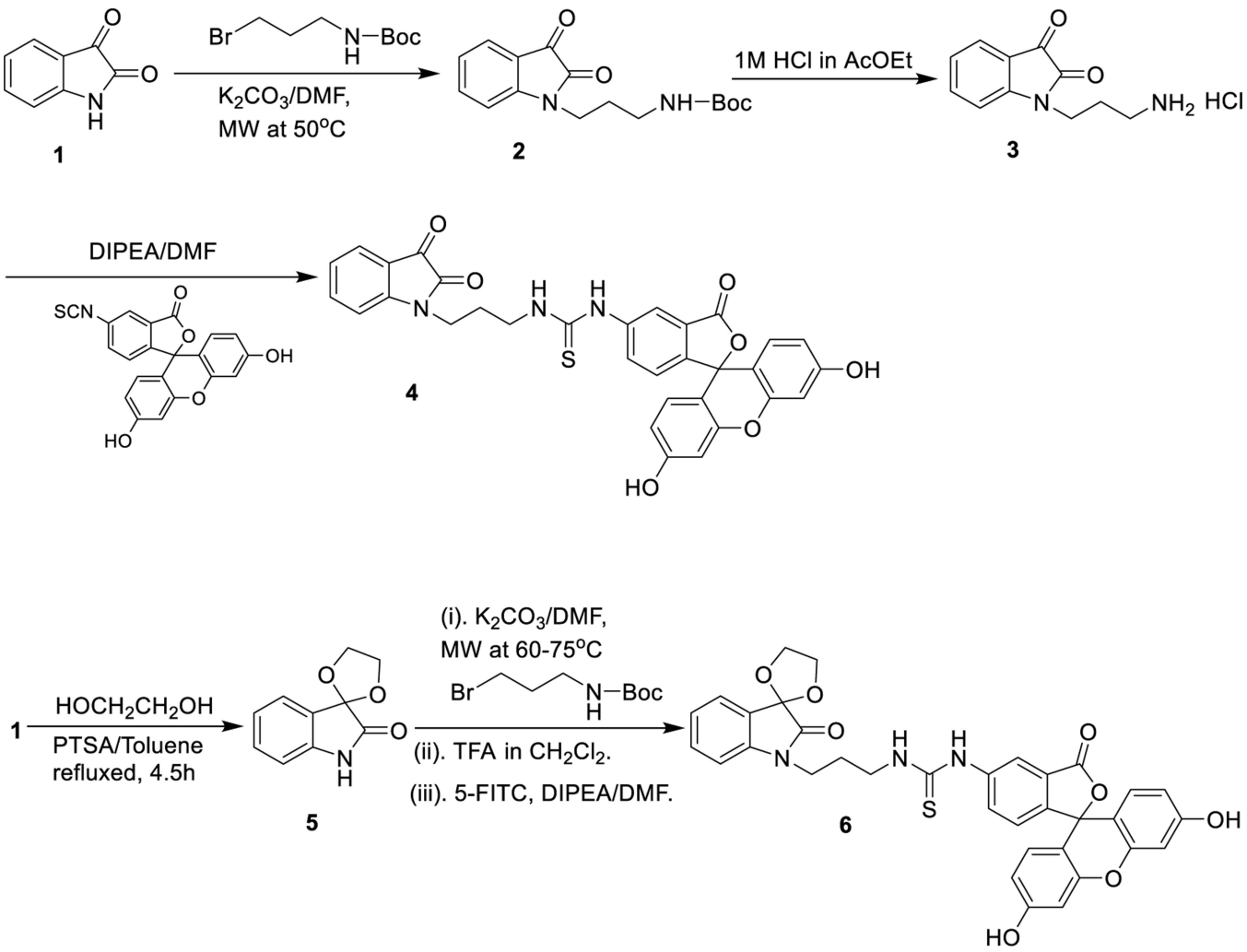


Figure 8.
Increasing concentrations of NAIR decreases the fluorescence intensity of I-F and AIR complex.



Scheme 1.

Chemical Synthesis of Isatin-Fluorescein (I-F) analogs (**4**) and (**6**).