

## Development of highly sensitive fluorescent assays for fatty acid amide hydrolase

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### Abstract

Fatty acid amide hydrolase (FAAH) is a pharmaceutical target whose inhibition may lead to valuable therapeutics. Sensitive substrates for high-throughput assays are crucial for the rapid-screening FAAH inhibitors. Here we describe the development of novel and highly sensitive fluorescent assays for FAAH based on substituted aminopyridines. Examining the relationship between the structure and the fluorescence of substituted aminopyridines suggested that a methoxy group in the *para* position relative to the amino group in aminopyridines greatly increased the fluorescence (i.e., quantum yields approach unity). These novel fluorescent reporters had a high Stokes' shift of 94 nm, and their fluorescence in buffer systems increased with pH values from neutral to basic. Fluorescent substrates with these reporters displayed a very low fluorescent background and high aqueous solubility. Most importantly, fluorescent assays for FAAH based on these substrates were at least 25 times more sensitive than assays using related compounds with published colorimetric or fluorescent reporters. This property results in shorter assay times and decreased protein concentrations in the assays. Such sensitive assays will facilitate distinguishing the relative potency of powerful inhibitors of FAAH. When these fluorescent substrates were applied to human liver microsomes, results suggested that there was at least one amide hydrolase in addition to FAAH that could hydrolyze long-chain fatty acid amides. These results show that these fluorescent substrates are very valuable tools in FAAH activity assays including screening inhibitors by high-throughput assays instead of using the costly and labor-intensive radioactive ligands. Potential applications of novel fluorescent reporters are discussed.

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Fatty acid amide hydrolase (FAAH)<sup>1</sup> is a mammalian integral membrane enzyme that plays critical roles in regulating the levels of endogenous signaling lipids such as the cannabinoid anandamide, sleeping-inducing substance oleamide, anorexigenic compound *N*-oleoylethanolamide, and antiinflammatory agent *N*-palmitoylethanolamide [1–5]. Inhibition of FAAH is associated with treatments of multiple pathological disorders such as pain, anxiety,

inflammation, and other disorders [6–15]. Therefore, compounds that inhibit this enzyme are of medical and therapeutic significance.

To meet increasing demands for identifying novel inhibitors from large libraries of chemical compounds, simple, highly sensitive, and specific assays, which are suitable for a high-throughput format such as colorimetric or fluorescent assays in 96- or 384-well plates or alternative formats, are crucial for screening. Most assays for FAAH activity are commonly associated with the use of radiolabeled substrates or chromatographic techniques, which are costly, labor-intensive, and incompatible with high-throughput format or have other limitations [16–24]. Recently, two assays that are suitable for high-throughput formats such as 96 or 384-well plate assays have been

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<sup>1</sup> Abbreviations used: FAAH, fatty acid amide hydrolase; BSA, bovine serum albumin; RFU, relative fluorescent unit; DMSO, dimethyl sulfoxide.

reported [25,26]. One is a coupled colorimetric assay, i.e., it utilizes the ability of FAAH to hydrolyze oleamide and measures the resultant production of ammonia by a NADH/NAD<sup>+</sup>-coupled enzyme [25]. Its application is limited by its sensitivity and the complexity of using a second enzyme (e.g., L-glutamate dehydrogenase). The other method is a fluorescent assay using the substrate arachidonyl 7-amino-4-methylcoumarin amide [26]. The application of this assay is also limited by the sensitivity of the assay, the poor aqueous solubility, and the instability of the substrate.

It is well known that 2-, 3-, and 4-aminopyridines are fluorescent compounds [27,28]. Among these aminopyridines, 2- and 3-substituted isomers show relatively stronger fluorescence than 4-substituted isomers. Therefore, both 2- and 3-isomers have been used as fluorescent reporters of hydrolytic activities of multiple enzymes such as nucleotide pyrophosphatase [29], transferases [30,31], and hyaluronidase [32]. However, the fluorescent strength (or quantum yield) of these aminopyridines is relatively low, compared with 7-aminocoumarin, and greatly varies with the change of pH values in media [27] due to protonization or deprotonization of both nitrogen atoms. Generally, strong fluorescence for aminopyridines is shown in acidic or neutral aqueous media, but weak fluorescence is shown in basic aqueous media. These properties severely limit the applications of simple aminopyridines in most enzymatic assays such as those for amide hydrolases and aminopeptidases, which show maximal hydrolytic activities in basic aqueous media. Extensive attention has been paid to investigating the fluorescence of more complicated compounds containing one or multiple pyridine rings [33,34]. However, there is little work on the use of simple aminopyridines as fluorescent reporters.

Here we describe an investigation of fluorescence of substituted 3- or 5-aminopyridines. Based on this investigation, a simple, novel, highly sensitive, and continuous fluorescent assay for FAAH activity has been developed. This sensitive assay is based on measuring the strong fluorescence of a substituted 3- or 5-aminopyridine after the amide hydrolysis. These reporters may be particularly valuable with enzymes where the size of the catalytic site is limited. The tools developed in this study will be useful for studying the hydrolytic activity of numerous amide hydrolases including FAAH and other enzymes.

## Materials and methods

### Chemicals and enzymes

Fluorescent reporters including 3-amino-4-methylpyridine (**2**), 3-amino-6-methylpyridine (**3**), 3-amino-2-fluoropyridine (**4**), 5-amino-2-fluoropyridine (**5**), 5-amino-2-methoxypyridine (**6**), 3-amino-2-methoxypyridine (**7**), and 3-amino-6-methoxy-2-picoline (**8**) were purchased from Lancaster Synthesis, Inc. (**2**; Windham, NH, USA), and AB Chem., Inc. (**3**; Quebec, Canada), Matrix Scientific (**4**; Columbia, SC, USA), Beta Pharma, Inc. (**5**; New Haven,

CT, USA), Asychem (**6**, **7**, **8**; Durham, NC, USA), respectively. 3-Aminopyridine (**1**) and 7-amino-4-trifluoromethylcoumarin (**9**) were purchased from Sigma–Aldrich (Saint Louis, MI, USA). Pooled human liver S9 and microsomes were purchased from BD Biosciences (San Jose, CA, USA). Rabbit anti-FAAH polyclonal antibody, peroxidase-conjugated anti-rabbit IgG, and ECL detection kit were purchased from Cayman Chemical (Ann Arbor, MI, USA), Sigma Chemical (Saint Louis, MI, USA), and GE Healthcare Bio-Sciences (Piscataway, NJ, USA), respectively. Structural identification and purity are based on <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, GC/MS and LC/MS analysis. All substrates showed as single spots on TLC with no fluorescence at 254 or 280 nm. Data are presented in [Supplemental information](#).

### General synthetic procedures of substrate preparation

To an ice-cooled solution containing a fluorescent reporter (substituted aminopyridine or 7-amino-4-trifluoromethylcoumarin, 100 mg) and dry CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was slowly added the appropriate acid chloride (1.05 eqv) and 4-methylmorpholine (1.05 eqv). The reaction was warmed slowly to room temperature and stirred overnight. The reaction mixture was washed with saturated sodium bicarbonate solution and extracted three times with ethyl acetate (3 X 20 mL). The combined organic phase was dried by magnesium sulfate. A crude solid product was obtained after filtration and evaporation under reduced pressure. The crude product was chromatographed on a gradient of hexane and ethyl acetate. Abbreviations of substrates ([Fig. 1](#)) are expressed by referring to the acid moiety followed by an abbreviation for the reporter such as *N*-(6-methoxypyridin-3-yl) acetamide (Acetyl-MP), *N*-(6-methoxy-2-methylpyridin-3-yl) acetamide (Acetyl-MMP), *N*-(4-(trifluoromethyl)-2-oxo-2H-chromen-7-yl) acetamide (Acetyl-TFMCoumarin), *N*-(6-methoxypyridin-3-yl) octanamide (Octanoyl-MP), *N*-(6-methoxy-2-methylpyridin-3-yl) octanamide (Octanoyl-MMP), *N*-(4-(trifluoromethyl)-2-oxo-2H-chromen-7-yl) octanamide (Octanoyl-TFMCoumarin), and *N*-(*p*-nitrophenyl) octanamide (Octanoyl-*p*-NP). Spectral characterization of these substrates is referred to the accompanying [Supplemental information](#).

(5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(6-methoxypyridin-3-yl) icoso-5,8,11,14-tetraenamide (Arachidonyl-MP)

Arachidonic acid (200 mg, 0.65 mmol) was added to an ice-cooled CH<sub>2</sub>Cl<sub>2</sub> solution (10 mL) containing triethylamine (0.65 mmol) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (139 mg, 0.73 mmol) under nitrogen protection. After 20 min reaction under an ice-cooled bath, a dichloromethane solution (2 mL) containing 5-amino-2-methoxypyridine (65 mg, 0.52 mmol) was added to the reaction. The reaction was stirred 3 h under the ice-cooled bath. The reaction mixture was washed twice with

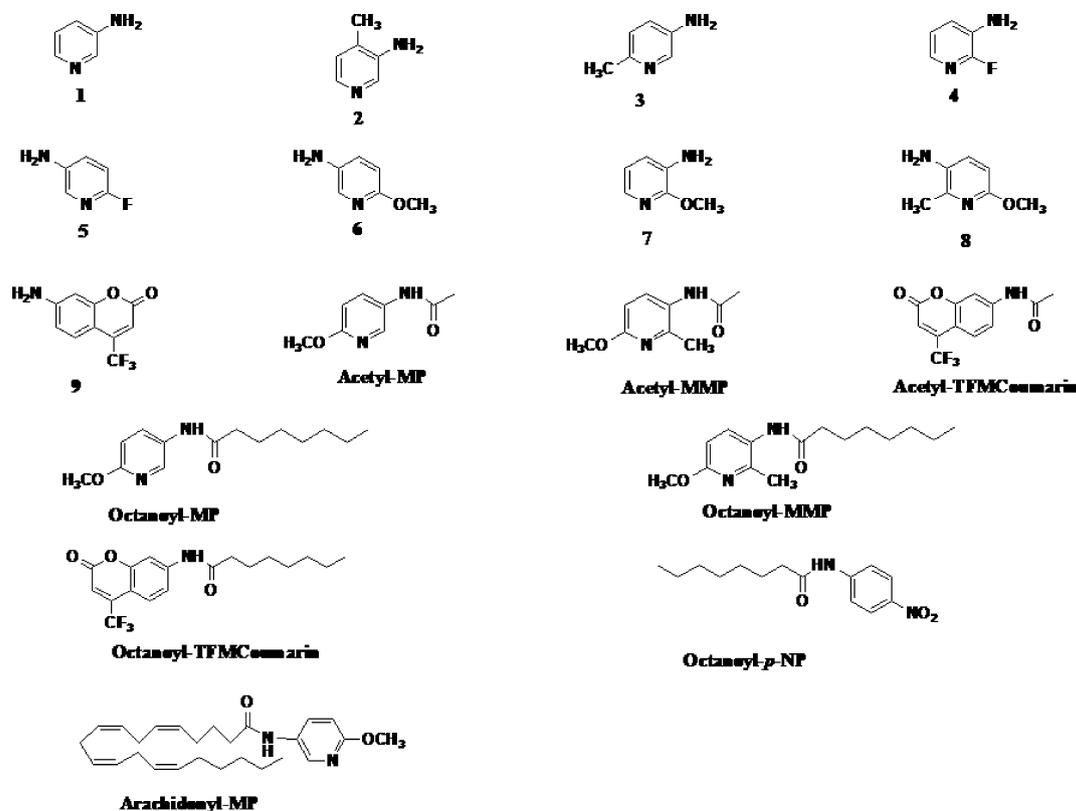


Fig. 1. Structures of fluorescent reporters and substrates used in this study.

1 M sodium bicarbonate solution (30 mL) and saturated NaCl solution (30 mL). The combined organic phase was dried under magnesium sulfate for 1 h. After filtration and evaporation, it gave rise to an oily product, which was chromatographed with a gradient of hexane and ethyl acetate. Slight yellowish oil (46 mg) was given (17% yield). **Caution:** For all procedures described here, aluminum foil was used to protect solution from being exposed to light. Spectral characterization of this substrate is referred to the accompanying [Supplemental information](#).

#### Preparation of recombinant human FAAH

Transgenic expression of the human FAAH in a baculovirus expression system was performed by following a method described previously [35]. The cDNA encoding the human FAAH (GenBank Accession No. [NM\\_001441](#)) was amplified by PCR using a human liver cDNA library (Invitrogen, Carlsbad, CA, USA) as a template. The primer pair was 5'-AGATCTATGGTGCAGTACGAGCTGTGGCC-3' and 5'-GAATTCTCAGGATGACTGCTTTTCAGGGGT-3'. *Bg*III and *Eco*RI endonuclease sites (underlines) were incorporated just upstream of the start codon and downstream of the stop codon of the coding sequence of FAAH, respectively. The PCR products were cloned into a pCR2.1 vector (Invitrogen) and the nucleotide sequence was verified by DNA sequencing (DBS DNA Sequencing Facility, University of California at Davis). The cDNA fragments were then excised and directionally ligated to the *Bg*III and *Eco*RI sites

of a baculovirus transfer vector pAcUW21 [36]. Recombinant baculoviruses harboring the human FAAH gene, Ac-hFAAH, were generated by cotransfection of cells derived from *Spodoptera frugiperda* (Sf21 cells) with the recombinant transfer vector plasmid and *Bsu*36I-cleaved BacPAK6 viral DNAs (Clontech Laboratories, Mountain view, CA, USA), as previously described [37]. Cells from *Trichoplusia ni* (High Five cells) ( $1 \times 10^6$  cells/mL) were inoculated with a high titer of Ac-hFAAH. At 72 h postinfection, the infected cells were harvested by centrifugation at 2000g for 20 min at 4°C and suspended in 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1 mM EDTA, 1 μM pepstatin, 100 μM leupeptin, and 0.1 mg/mL aprotinin. The cell suspension was then homogenized using a Polytron homogenizer and centrifuged at 10,000g for 20 min at 4°C to collect the supernatant. The microsomal fraction was then isolated by ultracentrifugation of the supernatant at 100,000g for 60 min at 4°C. The pellet was resuspended in 20 mM Tris-HCl (pH 8.0) containing 10% (w/v) glycerol and 1% (w/v) Triton X-100 and stored at -80°C until use. Protein concentration was measured by BCA assay using BSA as a standard in this study. Partial characterization of FAAH is described in the accompanying [Supplemental information](#).

#### Molar extinction coefficient

The molar extinction coefficients are determined according to Beer's law,  $A_\lambda = \epsilon \cdot C \cdot L$ . In this equation,  $A_\lambda$  is the

absorbance at wavelength ( $\lambda$  nm),  $\epsilon$  is the molar extinction coefficient,  $L$  is the width of the sample cuvette, and  $C$  is the concentration of the solute. In this study, a series of concentrations falling between 10 and 50  $\mu\text{M}$  (final concentration) for each fluorescent reporter was used for determination of the molar extinction coefficient. Assays were performed in a quartz cuvette (1-cm optical passage). To 990  $\mu\text{L}$  freshly distilled  $\text{H}_2\text{O}$  was added 10  $\mu\text{L}$  stock solution of a reporter in ethanol (1, 2, 3, 4, or 5 mM). Freshly distilled water was used to avoid a buildup of dissolved  $\text{CO}_2$ , altering the pH. The resulting mixture was degassed by sonication with an ultrasonic cleaner Model 750 (VWR International, Wester, PA, USA) for 10 s at a power level of 9. Measurements were performed with a Spectra Max M2 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at a wavelength 290 nm. A slope (or extinction coefficient) was obtained by using an average absorbance of triplicate samples for each concentration.

#### Maximal excitation and emission wavelengths

A stock solution in ethanol of each compound (10  $\mu\text{L}$ , 5 mM) was added into 990  $\mu\text{L}$  of sodium phosphate buffer (0.1 M, pH 8.0, final substrate concentration 50  $\mu\text{M}$ ) in a 1-cm quartz cuvette. The quartz cuvette was scanned from 200 to 500 nm to determine an absorption spectrum with a Spectra Max M2 spectrophotometer. Maximal excitation and emission wavelengths were obtained from scanning from 250 to 600 nm and from 360 to 600 nm by a 2-nm interval at 30°C, respectively.

#### Quantum yield

The fluorescence quantum yield,  $\phi_f$ , was calculated according to the equation [38]

$$\phi_F = \phi_{\text{std}} * (I/I_{\text{std}}) * (\text{OD}_{\text{std}}/\text{OD}) * (n/n_{\text{std}})^2,$$

where  $\phi_F$  is the quantum yield of the tested fluorescent reporter,  $\phi_{\text{std}}$  is the fluorescence quantum yield of the standard,  $I$  and  $I_{\text{std}}$  are the integrated emission intensities of the sample and the standard, respectively,  $\text{OD}$  and  $\text{OD}_{\text{std}}$  are the absorbance of the sample and standard, respectively, at the desired wavelength  $\lambda_{\text{ex}}$ , and  $n$  and  $n_{\text{std}}$  are the indexes of refraction of the sample and standard solutions, respectively.

In this study, 3-aminopyridine in distilled water at the maximal excitation wavelength 290 nm was used as a standard ( $\phi_{\text{std}} = 0.32$ ,  $\epsilon_{\text{max}} = 2800 \text{ M}^{-1} \text{ cm}^{-1}$ , [27]). To minimize inner filter effects, the optical densities of samples at the excitation wavelength ( $\lambda = 290 \text{ nm}$ ) were kept in the range of 0.095–0.13 (less than 0.150–0.165, [27]). The final concentration of each tested sample in a quartz cuvette was 10  $\mu\text{M}$  with 1% ethanol in distilled water, same as that for the standard. The integrated area of fluorescent intensity for the standard or the samples was based on the area falling between 360 and 500 nm. The difference of the index of

refraction between the standard and the sample was neglected because (1) all measurements were performed in the same quartz cuvette and spectrofluorimetry; (2) the final concentrations of the sample and the standard were the same, and (3) the structures of the sample and the standard were very similar—only one pyridine ring. The quantum yields presented in Table 1 were based on calculation of the average of OD and integrated fluorescent intensities of triplicates. The standard deviation for each quantum yield was less than  $\pm 5\%$ .

#### Dependence of relative fluorescent intensity on the pH values

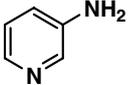
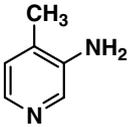
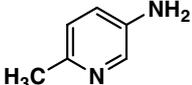
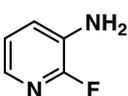
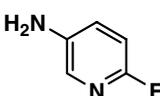
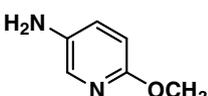
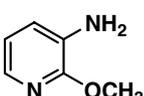
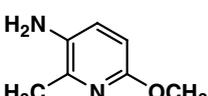
To determine whether protonization/deprotonation will affect the relative fluorescent intensities of the reporters, we used 5-amino-2-methoxypyridine (**6**) as an example. A stock solution of **6** in ethanol (5  $\mu\text{L}$ , 5 mM) was added into 995  $\mu\text{L}$  0.1 M sodium phosphate buffer (from pH 6.0 to pH 8.0) or 0.1 M Tris-HCl buffer (pH 9.0) in a quartz cuvette (final substrate concentration 25  $\mu\text{M}$ ). Relative fluorescent unit/intensity (RFU) for emission spectra was recorded by a 2-nm interval at room temperature using fixed excitation wavelength ( $\lambda = 302 \text{ nm}$ ). The RFU of each pH value was an average of triplicates and the standard deviation for each point was less than 1%. Similarly, relative fluorescent unit/intensity RFU for excitation spectra was recorded by a 2-nm interval at room temperature using fixed emission wavelength ( $\lambda = 396 \text{ nm}$ ) in 0.1 M, pH 8.0, phosphate buffer.

The quenching effect of proteins on the fluorescence is an important factor determining the assay sensitivity. This effect was determined by using 5-amino-2-methoxypyridine and BSA as an example. The detailed experimental procedure is available in the [Supplemental information](#).

#### Comparison of aqueous solubility of substrates

The aqueous solubilities of the aminopyridine derivatives (e.g., Octanoyl-MP, Octanoyl-MMP) and the 7-amino-coumarin derivative (e.g., Octanoyl-TFMCoumarin) were determined in clear 96-well styrene flat-bottomed microtiter plates with a Spectra Max M2 spectrophotometer. Different concentrations of the substrates [(Octanoyl-MP, Octanoyl-MMP, and Octanoyl-TFMCoumarin); final concentration: 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 200  $\mu\text{M}$  in 200  $\mu\text{L}$  0.1 M, pH 8.0, sodium phosphate buffer (1% ethanol)] were used and the absorbance was recorded at wavelength 800 nm at 37°C. The measurements were performed in three replicates for each concentration. The wells were read at a wavelength where the substrate has little or no known absorbance. When a solution becomes saturated by the substrate and micelles or particulates form, the absorbance reading will increase dramatically due to light scattering. Based on this phenomenon, the estimated substrate solubility is determined by the point where the

Table 1  
Optical properties of different substituted aminopyridines

| Compound No. | Structure   | $\epsilon_{\max}$ ( $M^{-1}cm^{-1}$ ) <sup>a</sup> | Ex (nm) <sup>b</sup> | Em (nm) <sup>b</sup> | $\Phi_F$ <sup>c</sup> |
|--------------|---|--|----------------------|----------------------|-----------------------|
| 1            |    | 2890 (2800) <sup>d</sup>                           | 286                  | 378                  | 0.32 <sup>d</sup>     |
| 2            |    | 2580   | 280                  | 376                  | 0.53                  |
| 3            |    | 2700   | 290                  | 384                  | 0.62                  |
| 4            |    | 3670   | 282                  | 374                  | 0.04                  |
| 5            |    | 2360   | 292                  | 384                  | 0.72                  |
| 6            |    | 2340   | 302                  | 396                  | 0.95                  |
| 7            |   | 4670   | 284                  | 378                  | 0.27                  |
| 8            |  | 4130   | 304                  | 392                  | 0.97                  |

<sup>a</sup> Molar extinction coefficients were determined in H<sub>2</sub>O containing 1% ethanol at wavelength 290 nm and 30°C, and the final concentrations of the solute were 10, 20, 30, 40, and 50  $\mu$ M.

<sup>b</sup> Ex and Em were obtained from scanning from 250 to 600 nm and from 360 to 600 nm by a 2-nm interval at 30 °C, respectively, and 50  $\mu$ M final concentration for each reporter in 0.1 M phosphate buffer. The standard deviation for each datum was  $\pm 2$  nm.

<sup>c</sup>  $\Phi_F$ : standard deviations were under  $\pm 5\%$ .

<sup>d</sup> Data are from [27].

substrate concentration elicits the absorbance skewing from the horizontal line.

#### Comparison of the lowest detection limits of protein concentrations of FAAH toward colorimetric and fluorescent substrates

In general, all assays were performed in 96-well styrene flat-bottomed microtiter plates with Spectra Max M2 spectrophotometer at 37°C for 10 min. The total volume of each well was 201  $\mu$ L consisting of 0.1 M, pH 8.0, sodium phosphate buffer (190  $\mu$ L) containing 1% glycerol and 0.1% Triton X-100, stock or diluted protein solution (10  $\mu$ L), and the substrate solution (1  $\mu$ L) in ethanol (final concentration 200  $\mu$ M for Octanoyl-MP and Octanoyl-MMP) or in DMSO (final concentration 25  $\mu$ M for Octanoyl-TFMCoumarin and Octanoyl-*p*-NP, and 50  $\mu$ M for

Arachidonyl-MP). The lowest detection limits of microsomal fatty acid amide hydrolase by substrates were based on signals which were determined by the fact that the signals at 10 min after initiation of the kinetic measurements were equal to or over three times those of the noise or baseline. The colorimetric substrate (Octanoyl-*p*-NP) was evaluated in clear 96-well microtiter plates using an absorbance wavelength at 382 nm. The fluorescent substrates containing 5-amino-2-methoxypyridine (e.g., Octanoyl-MP, Arachidonyl-MP) were measured at excitation wavelength 302 nm, emission wavelength 396 nm, and auto cutoff wavelength 325 nm. Similarly, the fluorescent substrates containing 5-amino-2-methoxy-6-methyl pyridine (e.g., Octanoyl-MMP) were measured at an excitation wavelength 304 nm, emission wavelength 392 nm, and autocutoff wavelength 325 nm. In addition, excitation wavelength 366 nm, emission wavelength 496 nm, and

autocutoff wavelength 495 nm were used for the substrate (e.g., Octanoyl-TFM-Coumarin) containing 7-amino-4-trifluoromethyl coumarin.

#### *Comparison of hydrolytic rates of different substrates by FAAH*

To further compare the hydrolytic ratios of different substrates by FAAH, we incubated microsomal FAAH with different substrates at 37 °C for 90 min and recorded the hydrolysis using a 2-min interval. Assays were performed by the following procedures: a substrate solution (1  $\mu$ L 10 mM in DMSO) was added into the protein-containing buffer (200  $\mu$ L). This buffer consisted of 0.1 M, pH 7.4, phosphate buffer (or 0.1 M, pH 9.0, Tris-HCl buffer), 1% glycerol, 0.1% Triton X-100, 2.5% DMSO, and FAAH microsome (0.67  $\mu$ g for Octanoyl-MP, Octanoyl-MMP, and Arachidonyl-MP; 100  $\mu$ g for Octanoyl-TFM-Coumarin). The final concentration of the all examined substrates was 50  $\mu$ M. The excitation and emission wavelengths of measurements were followed exactly as described above.

#### *Kinetic study*

The kinetic assays were also performed using the method described above for the sensitivity assay. Briefly, the total volume (201  $\mu$ L) contained 0.1 M, pH 8.0, sodium phosphate buffer (190  $\mu$ L) with 1% glycerol and 0.1% Triton X-100, protein solution (10  $\mu$ L), and substrate solution (1  $\mu$ L). The protein content was 0.67  $\mu$ g per well (201  $\mu$ L). The concentrations for Octanoyl-MP in DMSO were 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78  $\mu$ M. Similarly, the concentrations of Arachidonyl-MP in DMSO were 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, and 0.39  $\mu$ M. Four replicates were used for each concentration. Reported kinetic data ( $K_m$  and  $V_{max}$ ) were analyzed by the software Sigmaplot-Enzymatic Kinetics 2001 using the Michaelis-Menten equation.

## **Results and discussion**

#### *Optical properties*

The molar extinction coefficient for absorption, fluorescence spectral profiles, and quantum yield of a fluorophore are the most important indexes determining its fluorescent intensity in the enzymatic reaction media. Due to deprotonization or protonization, aminopyridines (e.g., 2-aminopyridine) can display from over one (1.20) to almost zero (0.05) quantum yields in the different aqueous media [28]. Distilled water with 1% ethanol (dissolving the aminopyridines) was chosen as a medium for measuring the indexes described above. Results with distilled water as a medium (Table 1) indicated that these indexes varied greatly with the substituted group and position on the 3- or 5-aminopyridine. For example, the methoxy group located between

the nitrogen on the pyridine ring and the carbon with an amino group of 3-aminopyridine (e.g., compound 7, Fig. 1) displayed a higher molar extinction coefficient but a much lower quantum yield than compound 6 with the same methoxy group and a different position (Fig. 1). The compounds 6 and 8 possessed quantum yields of almost unity (for efficient laser dyes it approaches unity) and redshifted excitation and emission wavelengths compared with those of other aminopyridines. However, the Stokes' shift for all aminopyridines examined was almost the same. These interesting results provided us the impetus for further investigating their applications as fluorescent reporters of enzymatic activities.

#### *Dependence of relative fluorescent intensity on the pH values*

It is wellknown that hydroxide ions are fluorescent quenchers for aminopyridines and  $\alpha$ - or  $\beta$ -naphthylamine [28,39]. This effect dramatically decreases in fluorescent intensity at maximal emission wavelength or quantum yields of aminopyridines from acid media to basic media. For example, the quantum yield of 3-aminopyridine drops from 1.07 in 0.1 N H<sub>2</sub>SO<sub>4</sub> to 0.32 in water or to 0.03 in 1 N NaOH [28]. This may be one of the major limitations to the application of aminopyridines as fluorescent reporters of enzymatic activities.

To determine whether hydroxide ions are also fluorescent quenchers of simple substituted aminopyridines, we investigated the dependence on pH values using sodium phosphate buffer (pH 6.0–8.0) and Tris-HCl buffer (pH 9.0), in which the concentrations of hydroxide ions in buffer increase with the buffer pH values. Results with 5-amino-2-methoxypyridine (6) as an example (Fig. 2) indicated that (1) there was no redshift for either excitation or emission wavelength with an increase of pH from 6.0 to 9.0, (2) the relative fluorescent intensity of either excitation (only pH 8.0 is shown for clarity) or emission increased with an increase of pH value, (3) the relative fluorescent intensity of Octanoyl-MP emission at the maximal excitation wavelength of 6 was very low (less than 200 RFU), and (4) the profiles of excitation and emission wavelengths were completely separated. Similar properties were observed with compound 8 (data not shown here). It seems that hydroxide ions in these buffer systems do not strongly quench the fluorescence of 6 as it does on compound 1.

#### *Effects of protein amount on the relative fluorescent intensity*

The quenching effect of proteins on the fluorescence of the reporters was studied. It is a common phenomenon that proteins quench the fluorescence of reporters. Data suggested that this effect is stronger for substituted aminopyridines than for coumarins. However, this quenching should not affect the sensitivity of fluorescent assays based on the substituted aminopyridines as fluorescent reporters. The detailed information on protein quenching is available in the Supplemental information.

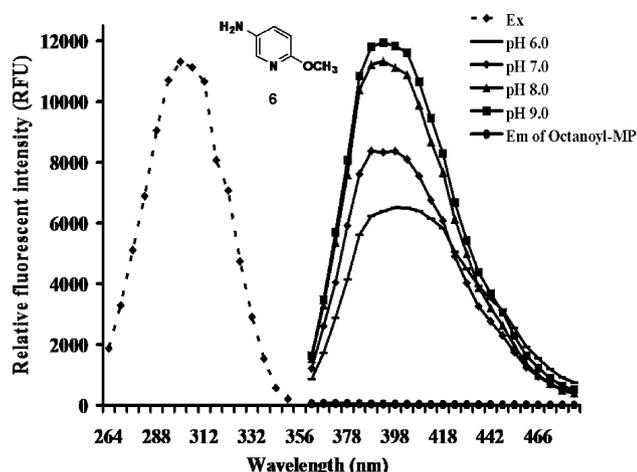


Fig. 2. Fluorescent spectra of 5-amino-2-methoxypyridine (**6**) and its amide derivative (Octanoyl-MP). The dashed curve on the left side is the excitation spectrum of 5-amino-2-methoxypyridine (**6**) from 250 to 350 nm. This spectrum was determined by using 25  $\mu\text{M}$  **6** in 0.1 M, pH 8.0, sodium phosphate buffer containing 1% ethanol at a fixed emission wavelength (396 nm) and room temperature. The five curves from top to bottom on the right side are curves of the emission spectra of the reporter **6** at pH 9, 8, 7, and 6 and *N*-(6-methoxypyridin-3-yl) octanamide (Octanoyl-MP) at pH 8, respectively. These five curves were determined at 25  $\mu\text{M}$  final concentration of **6** or Octanoyl-MP in 0.1 M sodium phosphate buffer (pH 6–8) or Tris–HCl (pH 9.0) in 1-cm cuvette at a fixed excitation wavelength (302 nm) and room temperature.

#### Comparison of aqueous solubility of substrates

Substrate solubility in aqueous media is one of the important components when one considers a kinetic study. Although this may be easily solved by adding more cosolvent or detergents, some of the activities may be sacrificed or enzyme properties altered. Based on the catalytic properties displayed by FAAH for the panel of *p*-nitroaniline substrates, the length of acyl chain (C6 to C9) displayed a relatively strong enzyme–substrate interaction [40]. Thus, we chose compound C8 for comparison of substrates with

*p*-nitroaniline, 7-amino-4-trifluoromethylcoumarin (**9**), and aminopyridines. With the same acid moiety, comparison of the aqueous solubility of pyridine substrates (e.g., Octanoyl-MP, Octanoyl-MMP) to a substrate with 7-amino-4-trifluoromethylcoumarin (**9**) suggested that pyridine substrates have much higher solubility in 0.1 M, pH 8.0, phosphate buffer (between 150 and 200  $\mu\text{M}$ ) than Octanoyl-TFMCoumarin (between 3.10 and 6.25  $\mu\text{M}$ ). This may partially contribute to the high substrate selectivity of FAAH described next. Partial characterization of FAAH suggested that expression of human FAAH was successful, and the FAAH protein was approximately 0.5 to 1% of the total microsomal proteins (A more detailed discussion of the characterization of FAAH protein is in Supplemental information).

#### Comparison of the lowest detection limits of microsomal FAAH with different substrates

As described above, protein quenching and background noise resulting from some ingredients in proteins are two factors that contribute to the sensitivity of these assays. These aminopyridines have high molar extinction coefficients and quantum yields. However, another major factor determining sensitivity of assays of hydrolytic activities is the selectivity of hydrolases toward their substrates. For high-throughput assays, it is the ability to detect the lowest possible enzyme concentration with acceptable signal to noise ratio that determines practical sensitivity. The lowest detection limit of FAAH proteins for a particular substrate was determined from an assay with a signal after a 10 min kinetic measurement that was equal to three times that of the background noise. Results (Table 2) indicated that (1) the background noise or baselines varied with substrates, higher background for Octanoyl-MP and Octanoyl-MMP than for Octanoyl-TFMCoumarin and Arachidonyl-MP [may result from higher substrate concentration, i.e.,

Table 2

Comparison of the lowest detection limits of microsomal FAAH toward the colorimetric substrate (Octanoyl-*p*-NP) and fluorescent substrates (Octanoyl-MP, Octanoyl-MMP, Octanoyl-TFMCoumarin, and Arachidonyl-MP)<sup>a</sup>

| Substrate              | Protein ( $\mu\text{g}/200 \mu\text{L}$ ) | Signal (OD or RFU) | Average (OD or RFU)    | Standard deviation |      |
|------------------------|---|--------------------|------------------------|--------------------|------|
| Octanoyl-MP            | 0.11                                      | Baseline           | 310, 290, 296, 314     | 302                | 11   |
|                        |   | Signal             | 1083, 1126, 1069, 1086 | 1091*              | 24   |
| Octanoyl-MMP           | 0.67                                      | Baseline           | 215, 161, 199, 201     | 194                | 23   |
|                        |   | Signal             | 760, 697, 694, 760     | 728*               | 38   |
| Octanoyl-TFMCoumarin   | 100                                       | Baseline           | 134, 130, 124, 139     | 132                | 6    |
|                        |   | Signal             | 154, 150, 157, 182     | 161                | 15   |
| Octanoyl- <i>p</i> -NP | 100                                       | Baseline           | 0.15, 0.16, 0.16, 0.16 | 0.16               | 0.01 |
|                        |   | Signal             | 0.16, 0.17, 0.18, 0.19 | 0.18               | 0.01 |
| Arachidonyl-MP         | 0.5                                       | Baseline           | 140, 140, 164, 140     | 148                | 11   |
|                        |   | Signal             | 566, 590, 534, 545     | 559*               | 24   |

<sup>a</sup> Assays were performed in a total volume (201  $\mu\text{L}$ ) containing 0.1 M, pH 8.0, sodium phosphate buffer with 1% glycerol and 0.1% Triton X-100 (190  $\mu\text{L}$ ), diluted protein solution (10  $\mu\text{L}$ ), and 1  $\mu\text{L}$  substrate in ethanol (final concentration 200  $\mu\text{M}$  for Octanoyl-MP and Octanoyl-MMP) or 1  $\mu\text{L}$  substrate solution in DMSO (final concentration 25  $\mu\text{M}$  for Octanoyl-TFMCoumarin, Octanoyl-*p*-NP, 50  $\mu\text{M}$  for Arachidonyl-MP) at 37  $^{\circ}\text{C}$ . Absorbance wavelength for Octanoyl-*p*-NP was 382 nm. Excitation and emission wavelengths for Octanoyl-MP and Arachidonyl-MP were 302 and 396, for Octanoyl-MMP were 304 and 392, and for Octanoyl-MMP were 366 and 496, respectively.

\* Asterisks indicate that the datum point was equal to or over three times higher than the corresponding baseline.

200  $\mu\text{M}$  (Octanoyl-MP and Octanoyl-MMP) vs 50  $\mu\text{M}$  (Octanoyl-TFMCoumarin and Arachidonyl-MP)]; (2) the lowest detection limits of FAAH proteins for Octanoyl-MP, Octanoyl-MM, and Arachidonyl-MP (0.55, 3.3, and 2.5  $\mu\text{g}$  protein/mL, respectively) were determined but were not determinable for Octanoyl-TFMCoumarin and Octanoyl-*p*-NP even at 500  $\mu\text{g}$  protein/mL in buffer system with 10% DMSO and 0.1% Triton X-100; and (3) the structure of reporters in substrates played a dramatic role in the sensitivity of assays for this hydrolase. For example, Octanoyl-TFMCoumarin with 7-amino-4-trifluoromethylcoumarin (9) was at least 900 times less sensitive than Octanoyl-MP with a 5-amino-2-methoxypyridine (6) reporter and 150 times less sensitive than Octanoyl-MMP with a 5-amino-2-methoxy-6-methylpyridine (8) reporter.

#### Comparison of rates of hydrolysis of different substrates by FAAH

Four fluorescent substrates (Octanoyl-MP, Octanoyl-MMP, Octanoyl-TFMCoumarin, and Arachidonyl-MP) were chosen for comparison with 90-min incubation at 37 °C. Results (Figs. 3A and 3B) indicated that, under the same unit protein and same buffer system, the substrate hydrolysis by FAAH followed the same order: Octanoyl-MP  $\gg$  Arachidonyl-MP > Octanoyl-MMP > Octanoyl-TFMCoumarin. This order is in agreement with the results from the lowest detection limits of proteins described above. Although it seems that there was no hydrolysis for Octanoyl-TFMCoumarin at either pH 7.4 or 9.0 (Figs. 3A and 3B), the amount of the hydrolyzed Octanoyl-TFMCoumarin was very close to that of Arachidonyl-MP during the time course except for a 150-fold difference in amount of protein (0.67  $\mu\text{g}$  proteins for Arachidonyl-MP and 100  $\mu\text{g}$  proteins for Octanoyl-TFMCoumarin, respectively). The hydrolysis of Octanoyl-MP by FAAH did not follow a straight line

during 90 min for either pH 7.4 (0.1 M phosphate buffer) or pH 9.0 (0.1 M Tris-HCl buffer), but it was a linear for the first 30 min. This assisted in the selection of the appropriate time for the following kinetic study. Although all substrates were hydrolyzed significantly faster at a higher pH value, the difference was very small (a 1.2-fold increase for Octanoyl-MP from pH 7.4 to pH 9.0).

#### Kinetic data

To further compare substrate selectivity with other substrates published in the literature, we conducted a kinetic study with Octanoyl-MP and Arachidonyl-MP. Results (Table 3) indicated that there was an eightfold difference between Octanoyl-MP and Arachidonyl-MP at  $V_{\text{max}}$  under conditions of saturating substrate concentration, very close to the results (5 times) from the experiment determining the limit of detection of the proteins' enzymatic activity (Table 2). Substrate selectivity of Octanoyl-MP was a little higher than that of Arachidonyl-MP (4.1 and 3.0 for Octanoyl-MP and Arachidonyl-MP, respectively). Although there is a difference in the percentage of FAAH protein in

Table 3  
Comparison of kinetic data of FAAH toward different substrates

| Substrates            | $K_m$<br>( $\mu\text{M}$ ) | $V_{\text{max}}$<br>( $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ) | $V_{\text{max}}/K_m$ | Selectivity ratio <sup>c</sup> |
|-----------------------|----------------------------|--|----------------------|--------------------------------|
| Octanoyl-MP           | 74.9                       | 305  | 4.1                  | 74.5                           |
| Arachidonyl-MP        | 12.5                       | 36.7   | 3.0                  | 54.5                           |
| AAMCA <sup>a</sup>    | 0.48                       | $5.8 \times 10^{-2}$   | $1.2 \times 10^{-1}$ | 2.2                            |
| Oleamide <sup>b</sup> | 104                        | 5.7  | $5.5 \times 10^{-2}$ | 1.0                            |

<sup>a</sup> Data are from [26].

<sup>b</sup> Data are from [25].

<sup>c</sup> Selectivity ratio was calculated by  $V_{\text{max}}/K_m$  of each substrate over that of oleamide ( $5.5 \times 10^{-2}$ ).

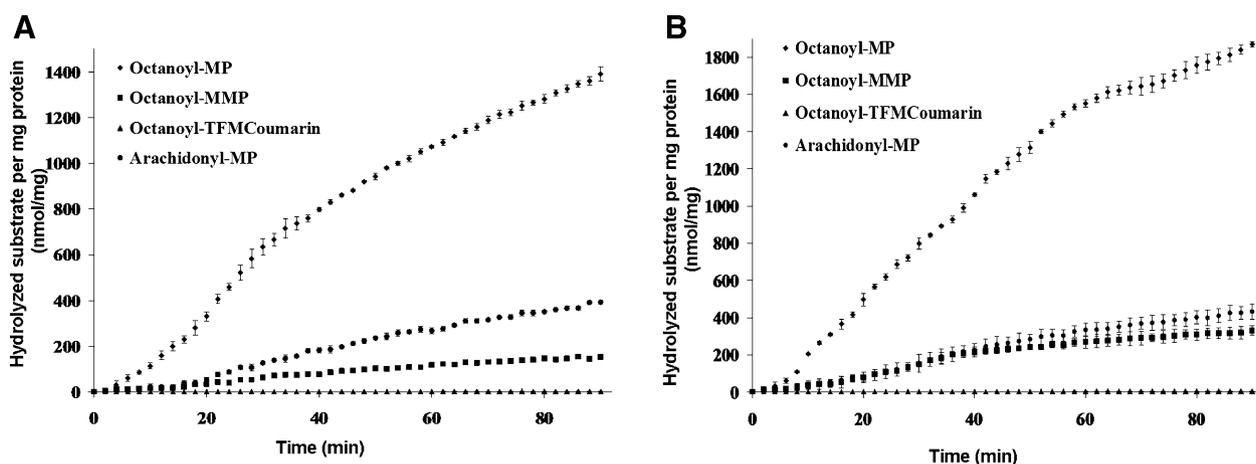


Fig. 3. Comparison of rates of hydrolysis of different substrates by FAAH. Assays were performed at 37 °C in 200  $\mu\text{L}$  0.1 M, pH 7.4, sodium phosphate buffer (A) or 0.1 M, pH 9.0, Tris-HCl buffer (B) containing 50  $\mu\text{M}$  substrate, 1% glycerol, 0.1% Triton X-100, 3% DMSO, and microsomal protein (0.67  $\mu\text{g}$  for Octanoyl-MP, Octanoyl-MMP, and Arachidonyl-MMP and 100  $\mu\text{g}$  for Octanoyl-TFMCoumarin). The assays were recorded by a 2-min interval during 90 min. The excitation and emission wavelengths (nm) for Octanoyl-MP, Octanoyl-MMP, and Arachidonyl-MMP were 302 and 396, 304 and 392, and 302 and 396, respectively. Similarly, 366 and 496 were excitation and emission wavelengths (nm) for Octanoyl-TFMCoumarin.

microsomes prepared from different laboratories and expression systems, this difference was negligible with respect to the substrate selectivity published in the literature (Table 3). Amazingly, the substrate selectivity of FAAH toward fluorescent substrates with a substituted aminopyridine as a reporter (e.g., Octanoyl-MP and Arachidonyl-MP) was over 50 times higher than that of the endogenous substrate (oleamide, Table 3) used in colorimetric assays or at least 25 times higher than that of fluorescent substrate with coumarin as a reporter (e.g., AAMCA, Table 3).

This amazing difference in sensitivity or substrate selectivity may result from a combination of multiple factors such as having higher aqueous solubility of the substrates and smaller reporters or forming electron donor or hydrogen bond interaction with the nitrogen of the pyridine ring. For example, the only difference between Octanoyl-MMP with a 5-amino-2-methoxy-6-methylpyridine (**8**) reporter and Octanoyl-MP with a 5-amino-2-methoxypyridine (**6**) reporter is one methyl group. The steric effects of the methyl group may partially block the nitrogen as electron donor or hydrogen bond, which may facilitate the hydrolysis by FAAH. This facilitation is possibly supported by the facts that (1) the sensitivity of FAAH toward Octanoyl-MMP is 6 times lower than that toward Octanoyl-MP and (2) the substrate selectivity of FAAH toward oleamide is over 50 times lower than that toward Octanoyl-MP or Arachidonyl-MP (Table 3). Of course, the effect of an increase in structural size from reporter **6** to **8** cannot be eliminated from these results. The steric effects of the reporters on the substrate selectivity are also supported by the difference between Octanoyl-MP and Octanoyl-TFMCoumarin (at least 900-fold difference, Table 2). A full explanation of the difference requires further investigation of structure–activity relationships.

Amidase activities of High Five Cells, Ac-hFAAH-infected cells, and human liver S9 microsomes were detected using these novel substrates. Results demonstrated that Arachidonyl-MP was the most selective substrate for Ac-hFAAH. They also indicated that, in addition to Ac-hFAAH, there must be another amide hydrolase that hydrolyzes long-chain fatty acid amides such as Octanoyl-MP and Octanoyl-MMP in human liver microsomes. Detailed evidence is placed in the accompanying [Supplemental information](#).

#### *Potential application of novel fluorescent reporters*

Substituted aminopyridines display high fluorescence and represent a novel class of fluorescent reporters structurally different from aminocoumarins. They possess many advantages over aminocoumarins such as higher fluorescence, better aqueous solubility, and smaller size. In addition, as described above for FAAH, the nitrogen on the pyridine ring may work as an electron donor or may contribute a hydrogen bond and thus facilitate hydrolysis of the substrates by amide hydrolases. They are potentially useful for many enzymes that hydrolyze amide or carba-

mate bonds. These enzymes are not limited to FAAH but include nucleotide pyrophosphatase, transferases, hyaluronidase, numerous aminopeptidases, and other enzymes.

#### *Conclusion*

The relationship between structure of substituted aminopyridines and fluorescence was examined in this study. Results suggested that the substituted groups on the pyridine ring dramatically affected the fluorescence quantum yields of aminopyridines. A methoxy group *para* to the amino group in these aminopyridines greatly increased fluorescence quantum yields. Based on substrate selectivity, some novel fluorescent assays using substrates with aminopyridines as reporters were at least 50 times better than those assays (either fluorescent or colorimetric assays) published in the literature. Moreover, using novel fluorescent substrates, we found that there was at least one other amide hydrolase in addition to FAAH in human liver microsomes that could hydrolyze long chain fatty acid amides. In addition, the potential application of novel reporters was discussed.

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#### **Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ab.2006.10.041](https://doi.org/10.1016/j.ab.2006.10.041).

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