Supporting Information

Selective and Sensitive Detection and Quantification of Arylamine N-Acetyltransferase 2 by a Ratiometric Fluorescence Probe

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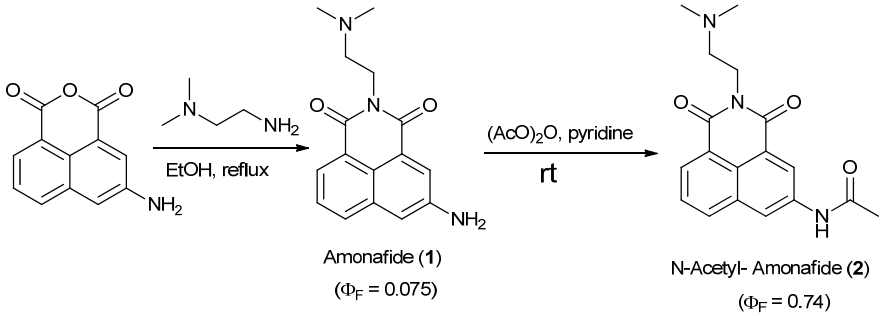
1. Materials and general methods.

Silica gel P60 (Qingdao) was used for column chromatography. All chemicals were purchased from Sigma-Aldrich or Aldrich and were used as received. ^{1}H-NMR spectra were collected in $CDCl_3$ (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC on a Bruker AV-400 spectrometer at NMR Facility of East China University of Science and Technology (ECUST). All chemical shifts are reported in the standard δ notation of parts per million using the peak of residual proton signals of $CDCl_3$ as an internal reference. Mass spectral analyses were carried out at the Analysis and Test Center of East China University of Science and Technology (ECUST).

2. Synthesis



Scheme S1 Synthesis of amonafide (**1**) and metabolite product *N*-acetyl-amonafide (**2**)

5-amino-2-[2-(dimethylamino)ethyl]-1H-benzo[de]isoquinoline-1,3(2H)-dione (amonafide) (1).

A suspension of 5.0 g of 3-amino-1,8-naphthalic anhydride (23.5 mmol) and 2.17 g of 1,1-dimethylethylenediamine (24.7 mmol; 1.05 eq.) were dissolved in 20 mL ethanol and then was heated to reflux for about 2.5 h. The solution was slowly cooled and at room temperature the precipitate was observed and then the solids were isolated by vacuum filtration and washed initially with 5 mL of ethanol and then with 10 mL of *n*-hexane in 2 portions. The resulting brown solid was dried in a vacuum oven to a constant weight. 5.50 g (19.5 mmol) of compound **1** was obtained with a yield of 83.0%. m.p. 294-295 ^{o}C (lit.[1]: 295-297 ^{o}C). ^{1}H NMR ($CDCl_3$): δ = 2.30 (6H, s), 2.49 (2H, t, *J* = 7.2Hz), 4.07 (2H, s) 4.25 (2H, t, *J* = 7.2 Hz), 7.22 (1H, d, *J* = 2.1 Hz), 7.53 (1H, t, *J* = 7.8 Hz), 7.85 (1H, d, *J* = 2.1 Hz), 7.94 (1H, d, *J* = 7.8 Hz), 8.24 (1H,d, *J* = 7.8 Hz). ^{13}C NMR ($CDCl_3$): δ =37.4, 45.3, 56.4, 111.6, 120.4, 121.6, 121.6, 122.4, 125.3, 126.8, 131.4, 133.4, 147.8, 163.4 and 163.4. EI-MS: 58 (100); 71 (31) and 283 (6). HR-MS (EI+) Calcd for $([M])^{+}$,283.1321; found, 283.1324 (5.9); 71.0722 (34); 58.0631 (100)

2-[2-(dimethylamino)ethyl]-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-5-yl-aceta mide (2)

2.83 g of amonafide (1) (10 mmol) was added to 4 mL of a pyridine/acetic anhydride mixture (1:1 v/v). The reaction mixture was stirred for 24 hours at room temperature and then evaporated to dryness under vacuum. The residual yellow oil was dissolved in chloroform (50 mL), and the solution was washed with water (2 × 50mL), the organic layer was collected and dried over anhydrous sodium sulfate and evaporated. The solid that remained was recrystallized from ethyl acetate to give pale yellow crystals.[1] The yield was 80% (2.60 g). m.p. 220-221 ^{o}C (lit.[1]: 221-223 ^{o}C).^{1}H NMR ($CDCl_3$): δ = 2.27 (3H, s), 2.53 (6H, s), 2.92 (2H, t, *J* = 6.0Hz), 4.38 (2H, t, *J* = 6.0 Hz), 7.65 (1H, t, *J* = 8.0 Hz), 7.90 (1H, d, *J* = 8.0 Hz), 7.94 (1H, s), 8.35 (1H, d, *J* = 7.8 Hz), 8.54 (1H, s), 8.59 (1H, s). ^{13}C NMR ($CDCl_3$): δ = 24.7, 37.7, 46.1, 58.3,

120.9, 121.9, 122.6, 122.8, 124.2, 127.4, 129.5, 132.1, 133.7, 136.8, 163.5, 164.4 and 169.2. ESI-MS (+):326 (M+H, 100). HR-MS (EI+) Calcd for $([M])^+$, 324.1426; found, 324.1464(2); 310.1288(100); 212.0579 (78)

3. Determination of quantum yield

The quantum yield of sensor Amonafide and *N*-acetyl-Amonafide were determined according to the literature.[2]

$$\phi_1 = \frac{\phi_B I_1 A_B \lambda_{exB} \eta_1}{I_B A_1 \lambda_{ex1} \eta_B}$$

Where Φ is quantum yield; I is integrated area under the corrected emission spectra; A is absorbance at the excitation wavelength; λ_{ex} is the excitation wavelength; η is the refractive index of the solution; the subscripts 1 and B refer to the unknown and the standard, respectively. We chose Quinine sulfate with 0.1 M H_2SO_4 as standard,[2] which has the quantum yield of 0.58. The quantum yields of Amonafide and *N*-acetyl-Amonafide were calculated as 0.075 and 0.74, respectively.

4. Spectroscopic materials and methods.

Double distilled water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 0.05 M Tris-HCl buffer (containing 1% DMSO unless special instructions) (pH = 7.4 at 37 ºC). All pH measurements were made with a Sartorius basic pH-Meter PB-10. Absorption spectra were recorded using a Varian Cary100 Bio UV-Visible spectrophotometer. Fluorescence spectra were recorded using a Varian Cary Eclipse scanning spectrofluorometer equipped with a Xenon flash lamp. Samples for absorption and fluorescence measurements were contained in 1 cm×1 cm quartz cuvettes (3.5 mL volume) or 40 µL Fluor Micro Cell (Varian Cary FLR). All cell images were taken Olympus IX51 with Xenon lamp and Olympus digital camera.

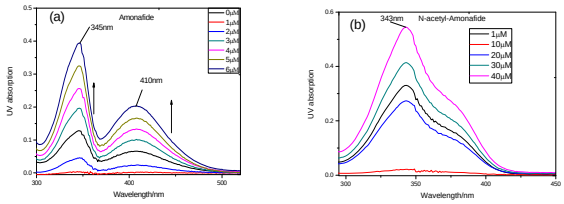


Fig. S1 The UV (panels a and b) absorption spectra and fluorescent emission spectra (panels c and d) of amonafide (panels a and c) and N-acetyl-amonafide (panels b and d) in Tris-HCl solution (0.05 M Tris-HCl, pH = 7.4, with 1% DMSO).

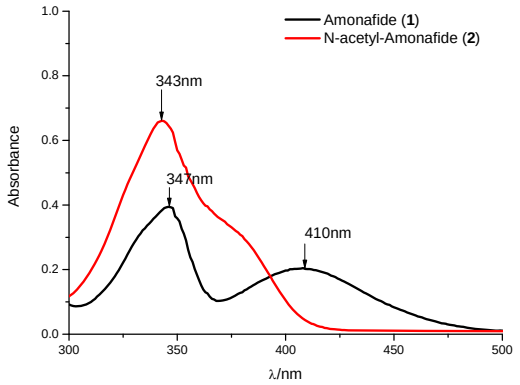


Fig. S2 Absorbance spectra of amonafide (**1**) (black line), N-acetyl-amonafide (**2**) (red line)

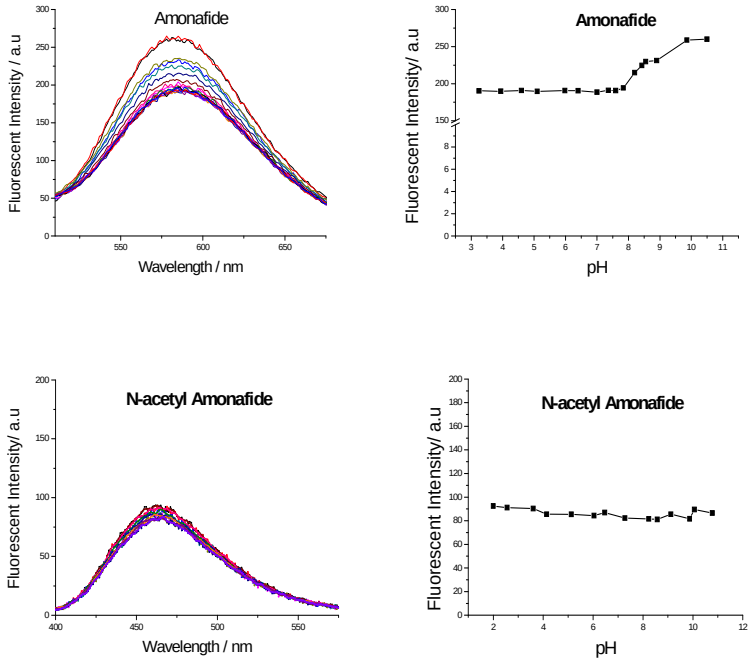
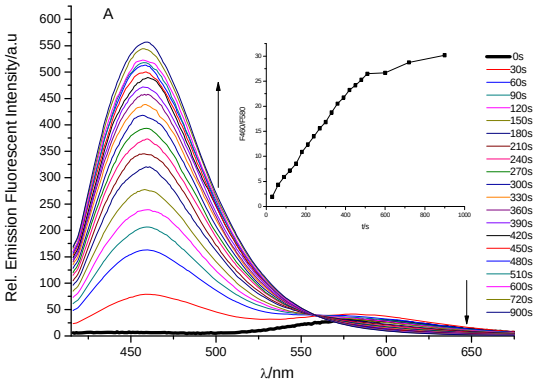


Fig. S3. The pH titration of compounds Amonafide and N-acetyl-amonafide

5. N-acetyltransferase assay.

NAT2 and *NAT1* activity experiments were performed in 40 µL Fluor Micro Cell and employed recombinant forms of human *NAT2* (2.5 mg/mL in Tris-HCl buffer solution, pH = 7.5, Sigma-Aldrich) and *NAT1* (2.5 mg/mL in Tris-HCl buffer solution, pH = 7.5, BD Biosciences) expressed from infected BTI-TN-5B1-4 insect cells. Stock solutions of Amonafide and *N*-acetyl-amonafide were prepared in pure DMSO (0 to 1 mM) and diluted in enzyme assay buffer (0.05 mM acetyl CoA, Tris-HCl, pH 7.4) to a final concentration containing 1% DMSO. Amonafide was added to a final protein concentration of 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL and 1 µg/mL enzyme per 40 µL well at 37 ºC, and fluorescence measurements were taken at 30 second intervals from 0 to 15 min on a Varian Cary Eclipse scanning spectrofluorometer with excitation at 350 nm and emission intensity collected from 415 nm to 675 nm.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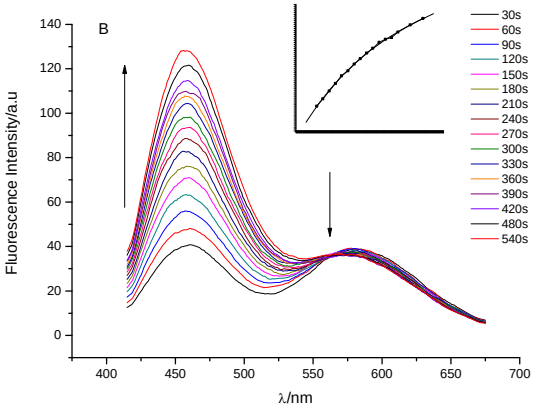
720s
900s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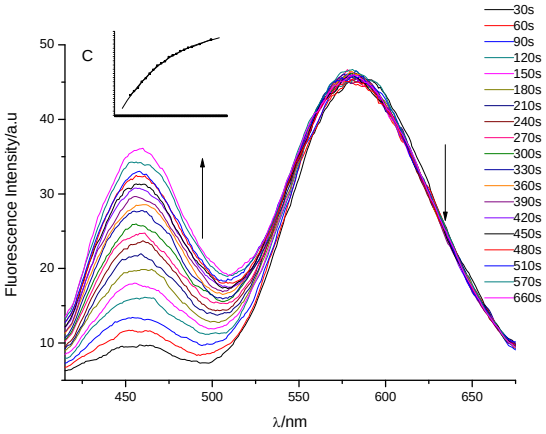
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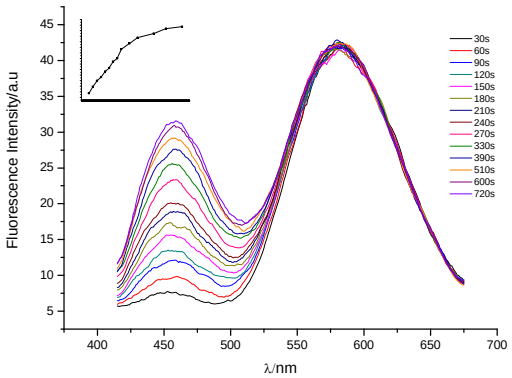
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λ/nm
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510s
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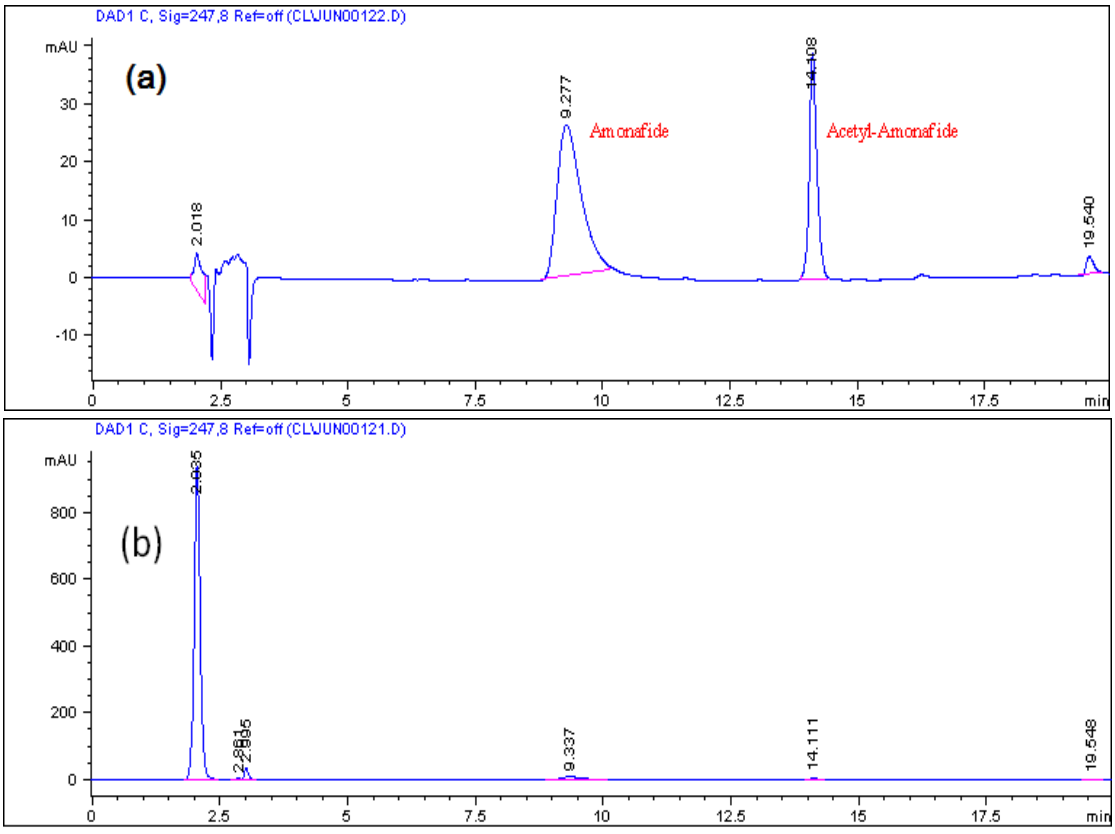
720s

Fig. S4 Direct fluorometric detection of *NAT2* activity with Amonafide. Figure (A) shows the fluorescence response of Amonafide at the concentration of 10 μM to *NAT2* cytosol at the concentration of 5 μg/mL. Figure (B) (C) and (D) show the fluorescence response of Amonafide at the concentration of 10 μM to *NAT2* cytosol at the concentration of 2.5, 1.25 and 1 μg/mL respectively. Enzyme reactions were performed at 25 °C in assay buffer (100 mM Tris-HCl, pH 7.4 with 1% DMSO) with *NAT2* at a final protein concentration of 50 μg/mL. Fluorescence excitation was provided at 360 nm, and traces are shown after 0, 30 s, 60 s, 90 s, 120 s….after adding Amonafide to *NAT2*.

6. HPLC for N-acetyltransferase 2 metabolism assay

HPLC was performed on a ZoRBAX RX-C18 column (Analytical 4.6×250mm 5-micrm, Agilent) with a HP 1100 system. The reaction solutions were analyzed by HPLC after filtering through a filter (MILLEX@GP, 0.22μm, Carrigtwohill, Co Cork). The HPLC solvents employed were 15% acetonitrile and 85% buffer (acetic acid and ammonium acetate pH=6.0). HPLC conditions were as follows: solvent A: solvent B = 0:100 (0 min)-100:0 (20 min), flow rate 2 mL/min, detection by UV (280 nm).

The reaction solution of Amonafide (10 μM) and Acetyl CoA (1 mM), NAT2 (5μg/mL) which was incubated with or without irreversible inhibitor Iodoacetamide (10 μM, J&K Chemica) for 20 min. After reaction, the solution was filtered through filter.



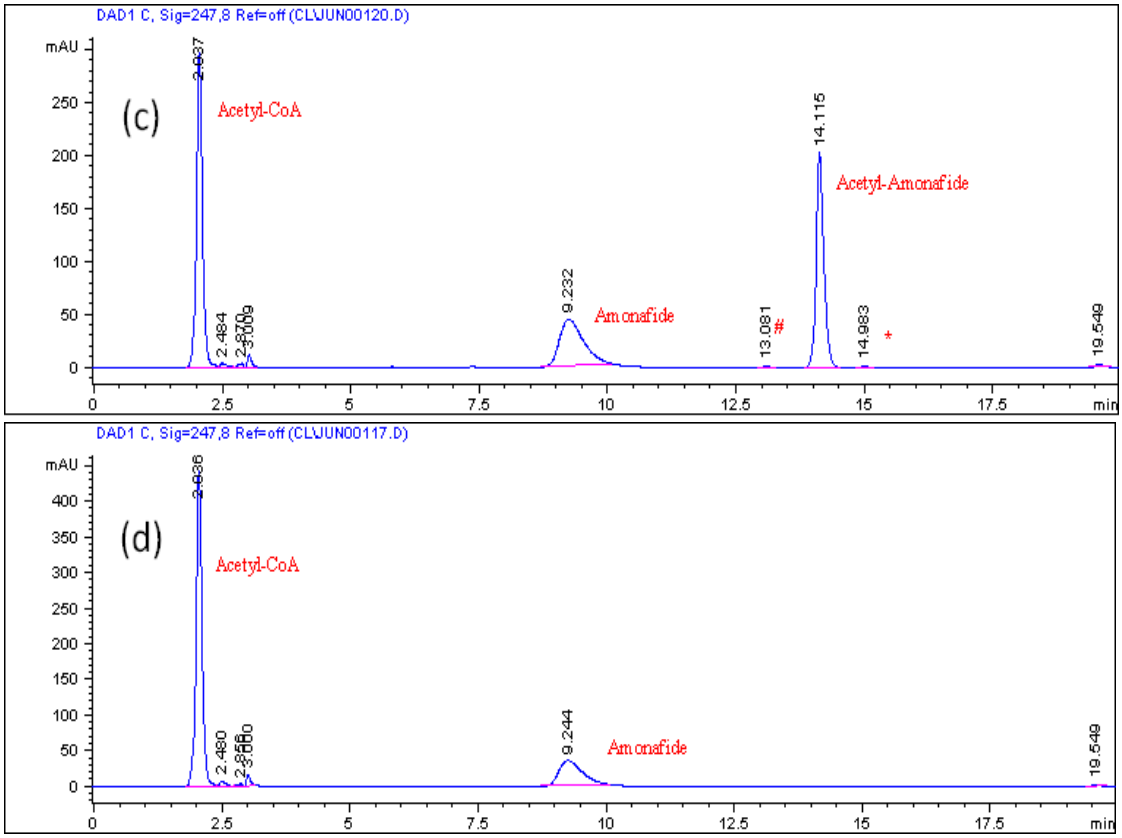


Fig. S5 HPLC profiles (detected by UV at 280 nm) of a) Amonafide (10 μM) and N-acetyl-Amonafide 7(10 μM) or b) Acetyl CoA (1 mM) as standard sample. Amonafide (10 μM) and Acetyl CoA (1 mM) upon treatment with NAT 2 for 20 min. c) NTA 2 solution (5 μg/ mL) with Acetyl CoA (1mM) and Amonafide (10 μM) without irreversible inhibitor Iodoacetamide[1]. d) NTA 2 solution (5 μg/ mL) with Acetyl CoA (1mM) and Amonafide (10 μM) with irreversible inhibitor Iodoacetamide. The symbol '*', '#' possibly corresponds to a reaction intermediate; however the compounds could not be identified.

7. Preparation of cell culture

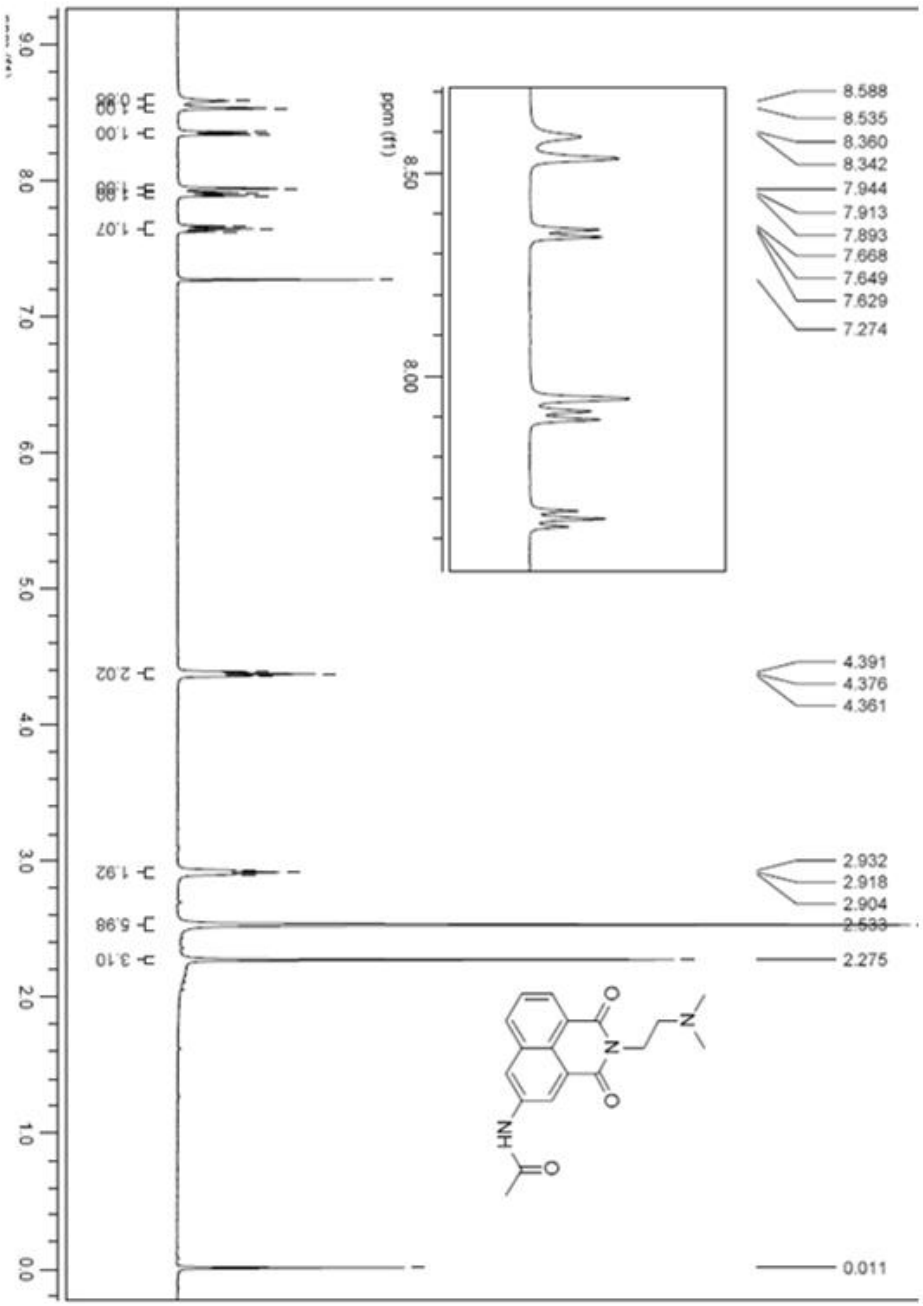
Hep G2 cells were seeded in a 6-well tissue culture dish at the Cell Culture Facility of East China University of Science and Technology (ECUST) and differentiated for 36 h in Dulbecco's Modified Eagle Medium (DMEM 1X, with phenol red, Gibco/Invitrogen) containing 10% Fetal Bovine Serum (HyClone).

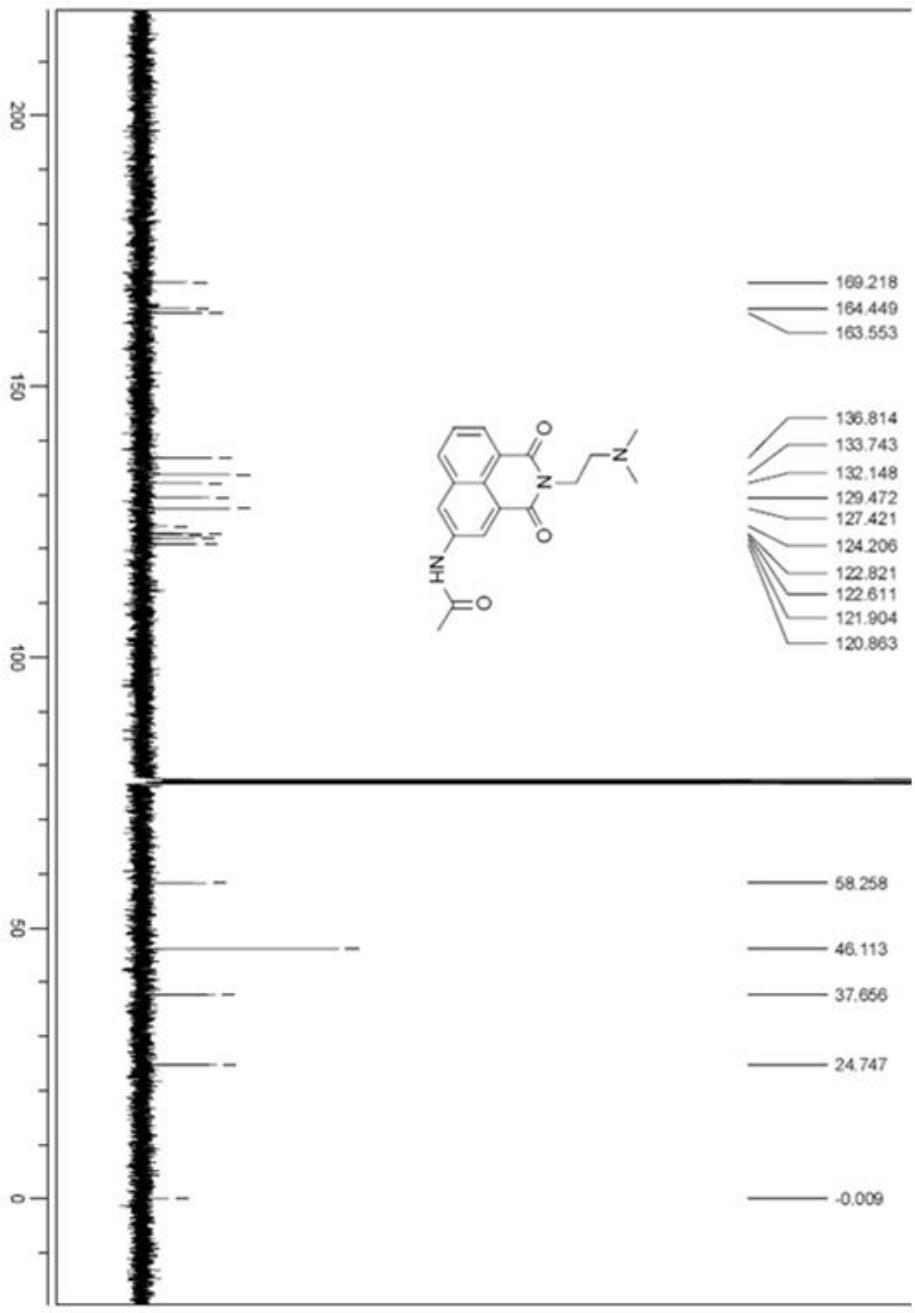
8. N-acetyltransferase live cell assay

Before the *NAT2* activity assay, the upper medium was sucked, then the cultured cell was washed with PBS buffer for three times, then washed with DMEM. Amonafide was added to cell culture medium until the final concentrations of 10 μM from 100 mM stock solutions in DMSO (final total DMSO concentration per assay 0.75% v/v) and incubated for 10, 30, 60 and 70 min at 37 ºC in a 5% CO_2 incubator. Bright field measurements on cells treated with Amonafide, or DMSO vehicle control confirm the viability of the cells throughout the experiment. After incubation with the Amonafide, the media was removed and the cells were washed twice with phosphate buffered solution (PBS, pH 7.4 at 37 ^{o}C containing 1% DMSO) to remove excess extracellular dye. Fluorescence

imaging was performed with an Olympus IX51 with Xenon lamp and Olympus digital camera. Blue emission was collected with a 430-495 nm window, and green emission was collected with a 535-600 nm window. In each wave band we set the same exposure time, 1/6 s for blue emission and 6 s for green emission.

9. NMR spectra

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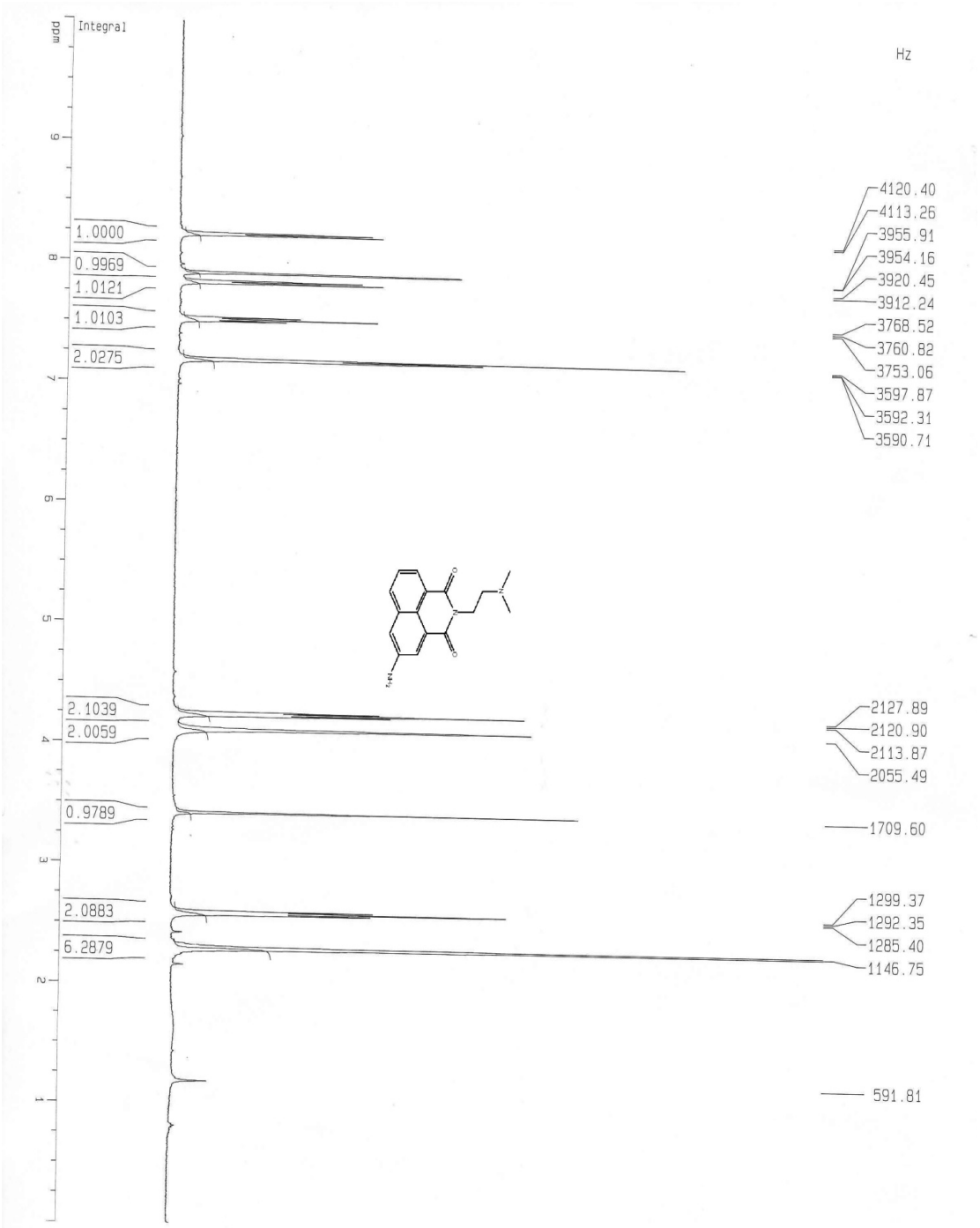
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3954.16
3920.45
3912.24
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3760.82
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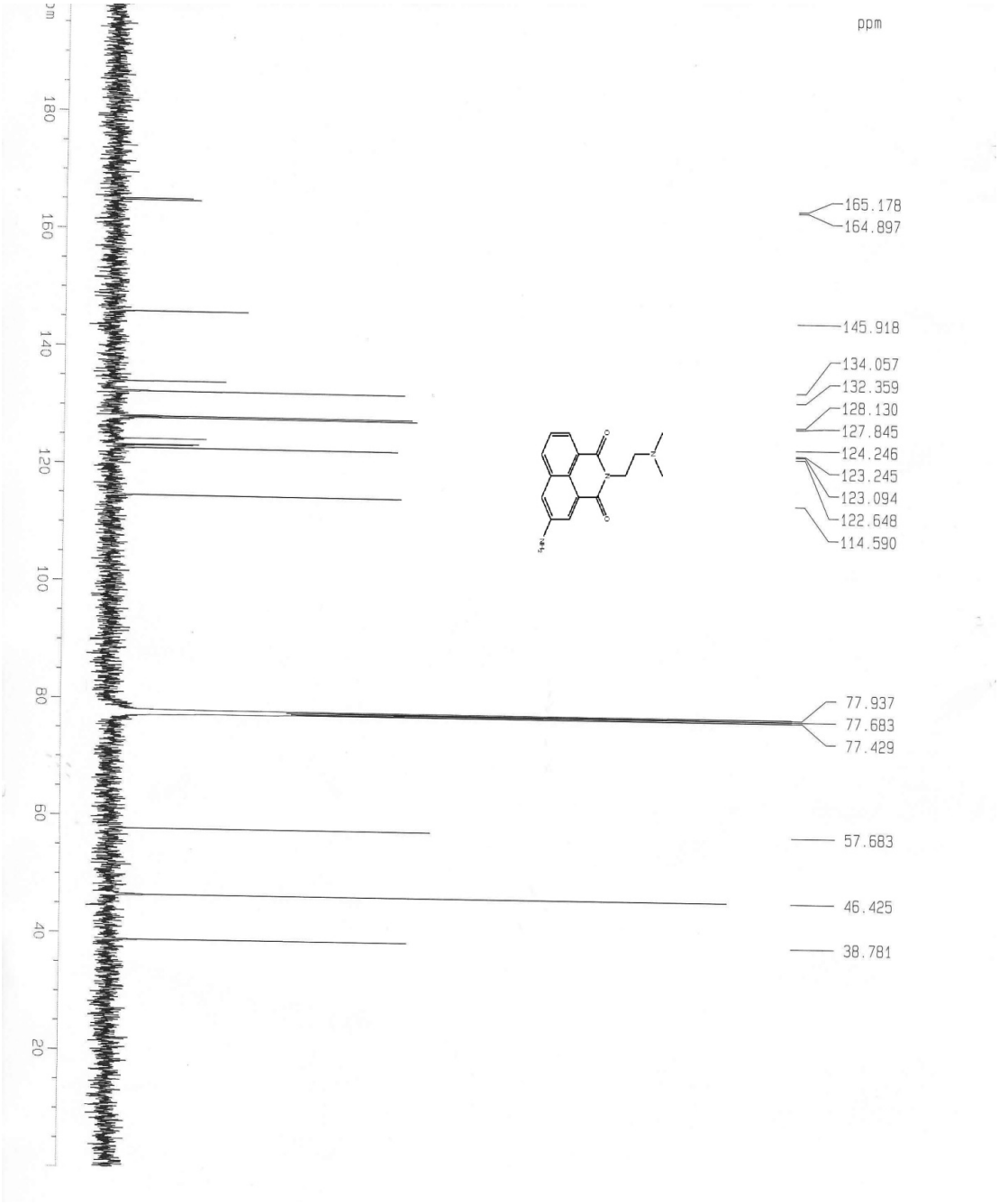

9. References

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3. H. Wang, G. M. Vath, K. J. Gleason, P. E. Hanna and C. R. Wagner, *Biochemistry*, 2004, **43**, 8234-8246.