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Design, synthesis, inhibitory activity, and SAR studies of hydrophobic *p*-aminosalicylic acid derivatives as neuraminidase inhibitors

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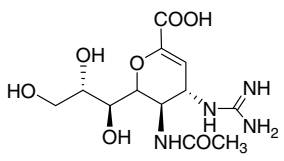
Abstract—A series of hydrophobic *p*-aminosalicylic acid derivatives containing a lipophilic side chain at C-2 and an amino or guanidine at C-5 were synthesized and evaluated for their ability to inhibit neuraminidase (NA) of influenza A virus (H3N2). All compounds were synthesized in good yields starting from commercially available *p*-aminosalicylic acid (PAS) using a suitable synthetic strategy. These compounds showed potent inhibitory activity against influenza A NA. Within this series, six compounds, **11**, **12**, **13e**, **16e**, **17c**, and **18e**, have the good potency (IC_{50} = 0.032–0.049 μM), which are compared to Oseltamivir (IC_{50} = 0.021 μM) and could be used as lead compounds in the future.

1. Introduction

Influenza is worldwide one of the deadliest infectious diseases that can affect millions of people every year.[1] Vaccines against influenza virus are ineffective due to the rapid emergence of mutant viral antigens. The M2 protein ion channel blockers are only effective on type A influenza with undesirable side effects and rapidly generated resistant mutants.[2] Because effective and safe anti-influenza therapeutics are lacking, developing effective anti-influenza agents become a high-priority and attractive area in drug discovery.

In recent years, virology studies of influenza virus illustrated the replication mechanism of the virus and some molecular targets have been identified for drug intervention such as hemagglutinin (HA), neuraminidase (sialidase, NA), M2 protein, and endonuclease.[3] Among those potential targets, NA appears to be an attractive target for drug development. As a glycoprotein in viral surface, NA is essential for viral replication due to its ability to catalyze removal of terminal SA linked to glycoproteins and glycolipids. Scientific research showed that NA is not only crucial in the release of virion progeny away from infected cells,[4] but also important in the movement of the virus through mucus of respiratory tract and reducing the propensity of the virus particles to aggregate. Despite the homology identity of NA in different strains is only about 30%, the catalytic site of NA in all influenza A and B virus is completely conserved.[5] Mutations of these conserved residues generally result in enzyme inactivation, suggesting that the virus may not easily escape NA-targeted drug therapy. Now, two NA inhibitors, Zanamivir (**1**) and Oseltamivir (**2**), have been confirmed as effective and safe for the treatment of influenza and approved by FDA.[6]



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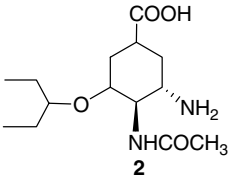

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It is reported that NA exists as tetramer consisting of four spherical subunits in the influenza virus, and a hydrophobic region is located in the central.[7] According to the X-ray crystal structure of the NA and the inhibitor, Wang et al.[8] proposed an 'airplane' model of the

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NA active site as illustrated in Figure 1 to summarize the basic structural requirements of a potent NA inhibitor. Each monomeric subunit has an active site cavity lined with ten conserved residues and four water molecules. The active site of NA has four main binding sites. The positively charged site 1 consists of Arg118, Arg292, and Arg371 and interacts with the carboxylate. The negatively charged site 2 consists of Glu119, Glu227, and Asp151 and interacts with the amino or guanidine. The small hydrophobic pocket consists of Ile222 and Trp178 (site 3) accommodates the acetyl group, and site 4, consisting of Glu276 and Glu277, binds to the hydrophobic side chain.

According to the studies on NA active site and SAR of published NA inhibitors, inhibition of NA is mainly determined by the relative positions of substituents of the central ring. And all the reported molecules have shown the importance of all four substituents (carboxylate, glycerol or hydrophobic side chain, acetamido, amino or guanidine) for the activity on NA A and B. For example, Sudbeck et al. reported a series of tri-substituted benzoic acid analogs which contain a benzene ring to replace the pyranose ring in Zanamivir (**2**).[9] In the inhibition assay, one compound (BANA 113, **3**) appeared to be the strongest inhibitor with an IC_{50} of 10 μM. The crystal structure of BANA113 complexed with NA shows that the guanidine group extends to reach a small pocket between residues Glu119 and Glu227. And the carboxylate group forms an electrostatic interaction with three arginine triad in the active site. Opposite the Arg pocket, the methyl group of the *N*-acetylamino fits into a hydrophobic pocket lined with residues Trp178 and Ile222.

During our previous work, the 4-hydroxy-L-proline has been used to prepare a series of pyrrolidine derivatives as NA inhibitors.[10] In our ongoing work, we wanted to use benzene ring to replace pyrrolidine ring and studied the substituted benzoic acid derivatives. We first screened all the benzoic acid derivatives in our compound library including not only the target compounds and intermediates we synthesized before, but also some commercially available compounds. The pharmacological result showed that *p*-aminosalicylic acid (PAS), one antibacterial agent, exhibited modest activity against influenza virus A (H_3N_2) NA (IC_{50} = 0.27 μM) and could be used as lead compound in future.

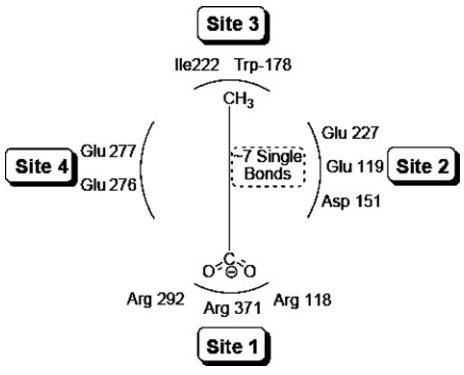
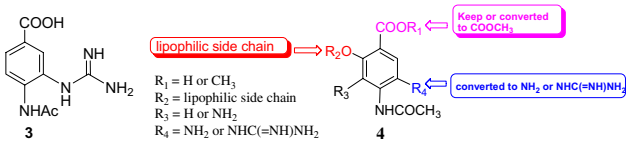


Figure 1. 'Airplane' model of NA active site (Ref. 8).

Considering the SAR of lead compound (BANA 113, **3**), we designed and synthesized several novel aromatic inhibitors (**4**) of NA from commercially available PAS. In order to improve the affinity of lead compounds, we optimized the structure of PAS with the following chemical modification: (i) C-1 carboxylic acid was kept or converted to other derivatives such as methyl ester or hydroxymate; (ii) C-2 hydroxy group in aromatic ring was changed to various phenolic ether in order to increase the hydrophobic interaction with site 4; (iii) hydrogen at C-3 position was kept or replaced with amino group; (iv) amino group at C-4 position was acetylated; and (v) hydrogen at C-5 position was converted to free nitro group or amino group or guanido group.

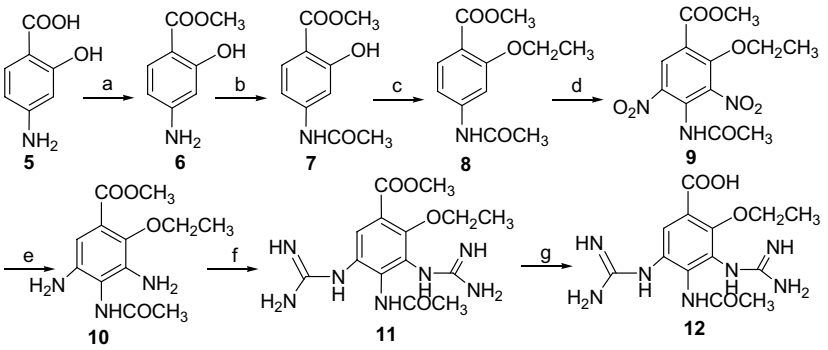


2. Chemistry

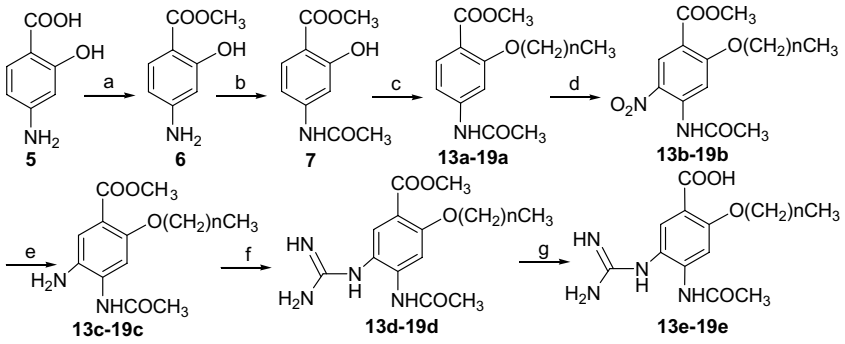
The synthesis of *p*-aminosalicylic acid derivatives possessing NA inhibitory activities is described in Schemes 1 and 2. Benzoic acids **12** and **13e–19e** were synthesized from commercially available PAS (**5**). The methyl ester **6** was prepared to avoid side-reactions of the carboxylate group.[11] Then **6** underwent selective acetylation on the 4-amino group using acetic anhydride to provide amide **7**.[12] Selective alkylation of compound **7** with various alkylogen in the presence of K_2CO_3 or NaH to give intermediate **8** or **13a–19a**, respectively.[13,14] The compound **8** or **13a–19a**, on nitration with fuming HNO_3 and glacial acetic acid at 0 °C gave nitro derivatives **9** or **13b–19b**.[15] Reduction of the nitro groups of **9** and **13b–19b** proceeded without problem using transfer hydrogenation, following the procedure described by Singh et al.[16] Whereas the synthesis of**11** and **13d–19d** was accomplished in 60% yield by reaction of **10** and **13c–19c** with cyanamide and HCl.[17] Hydrolysis of the methyl esters **11** and **13d–19d** with NaOH/H_2O yielded the target compounds **12** and **13e–19e**.[18]

3. Results and discussion

All the target compounds were evaluated for in vitro neuraminidase inhibitory activity. Preliminary result showed that 33 compounds displayed inhibitory activities with IC_{50} value from 0.032 to 9.26 μM (Table 1). Compound **12** with two guanidine groups at C-3 and C-5 and ethyl as hydrophobic side chain showed the best inhibitory activity (IC_{50} = 0.032 μM). The other five compounds containing guanidine (**11**, **13e**, **14e**, **16e**, and **18e**) exhibited good activities (0.036–0.049 μM). Generally, the compound with guanidine at C-5 and carboxyl group at C-1 showed better activities.



Scheme 1. Reagents and conditions: (a) MeOH, concd H_2SO_4, △; (b) Ac_2O, acetone; (c) $BrCH_2CH_3$, K_2CO_3, acetone, △; (d) fuming HNO_3; (e) 10% $Pd/CaCO_3$, H_2NNH_2, EtOH; (f) H_2NCN, concd HCl, EtOAC, △; (g) 1—NaOH; 2—HAc.



Scheme 2. Reagents and conditions: (a) MeOH, concd H_2SO_4, △; (b) Ac_2O, acetone; (c) $Br(CH_2)_nCH_3$, NaH, DMF, △; (d) fuming HNO_3; (e) 10% $Pd/CaCO_3$, H_2NNH_2, EtOH; (f) H_2NCN, concd HCl, EtOAC, △; (g) 1—NaOH; 2—HAc.

In summary, our studies have discovered a new series of *p*-aminosalicylic acid derivatives that have potent NA inhibitory activity. The binding of compound **12** in the active site of NA is shown in Figure 2, and we found that the carboxylate makes tight salt-bridge interactions with an arginine triad consisting of Arg118, Arg292, and Arg371, and the lipophilic side chain binds to the hydrophobic pocket (site 4) formed by Glu277 and Glu276, whereas the guanidine group binds to the negatively charged site 2 created by Glu227, Glu119, and Asp151. The carbonyl of the *N*-acetyl group hydrogen bonds to Arg152 and the methyl group occupies a hydrophobic pocket created by Trp178 and Ile222. Meanwhile, we found that another guanidine interacts electrostatically with Asn294, Asn347, and Cly348. Compared to other research, we reported a more convenient and economical method of the synthesis of *p*-aminosalicylic acid NA inhibitors. Compared to the other research, *p*-aminosalicylic acid we used appeared to be an ideal starting material because of its low cost and commercial abundance.

4. SAR studies

4.1. Dataset and molecular modeling

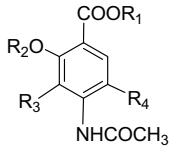
We used Sybyl 7.0 program to carry out the SAR studies of these *p*-aminosalicylic acid derivatives. The CoMFA studies were carried out with the QSAR model of Sybyl. The test set consisted of compounds **7**, **13a**, **15b**, **17c**, and **18e**, considering the last three compounds (**19a**, **19b**, **19c**) all had a long side chain R_2 for CoMFA, so the other 25 compounds composed of the training set. The IC_{50} values were converted into pIC_{50} according to the formula: $pIC_{50} = -\lg IC_{50}$.

Based on the docking results, the template molecule **12** was taken and the rest of the molecules were aligned to it using the benzoic acid as scaffold by DATABASE ALIGNMENT method in the Sybyl.

The steric and electrostatic CoMFA fields were calculated at each lattice intersection of a regularly spaced grid of 2.0 Å in all three dimensions within defined region. An sp^3 carbon atom with +1.00 charge was used as a probe atom. The steric and electrostatic fields were truncated at +30.00 kal mol^{-1}, and the electrostatic fields were ignored at the lattice points with maximal steric interactions.

PLS (partial least square) method was used to linearly correlate the CoMFA fields to the inhibitory activity values. The cross-validation analysis was performed using the leave one out (LOO) method in which one compound is removed from the dataset and its activity is predicted using the model derived from the rest of the dataset. The cross-validated q^2(0.526) that resulted in optimum number of components ($n = 7$) and lowest standard error of prediction were considered for further

Table 1. The structure and in vitro inhibitory activities of compounds against NA



Compound	R_1	R_2	R_3	R_4	IC_{50} (μM)	pIC_{50}^{pre}	pIC_{50}^{pre}	Res.
7	CH_3	H	H	H	0.99	6.00	6.23	−0.22
8	CH_3	CH_3CH_2	H	H	0.22	6.66	6.70	−0.04
9	CH_3	CH_3CH_2	NO_2	NO_2	2.15	5.67	5.69	−0.02
10	CH_3	CH_3CH_2	NH_2	NH_2	0.31	6.51	6.57	−0.06
11	CH_3	CH_3CH_2	$N{=}C(NH_2)_2$	$N{=}C(NH_2)_2$	0.036	7.44	7.49	−0.05
12	H	CH_3CH_2	$N{=}C(NH_2)_2$	$N{=}C(NH_2)_2$	0.032	7.49	7.46	0.03
13a	CH_3	$(CH_3)_2CH$	H	H	5.32	5.27	5.87	−0.60
13b	CH_3	$(CH_3)_2CH$	H	NO_2	0.57	6.24	6.26	−0.01
13c	CH_3	$(CH_3)_2CH$	H	NH_2	0.23	6.64	6.46	0.18
13d	CH_3	$(CH_3)_2CH$	H	$N{=}C(NH_2)_2$	0.12	6.92	7.00	−0.08
13e	H	$(CH_3)_2CH$	H	$N{=}C(NH_2)_2$	0.049	7.31	7.18	0.13
14a	CH_3	$CH_3CH_2CH_2$	H	H	9.26	5.03	5.13	−0.10
14b	CH_3	$CH_3CH_2CH_2$	H	NO_2	3.57	5.45	5.48	−0.03
14c	CH_3	$CH_3CH_2CH_2$	H	NH_2	1.26	5.90	5.61	0.29
14e	H	$CH_3CH_2CH_2$	H	$N{=}C(NH_2)_2$	0.72	6.14	6.37	−0.23
15a	CH_3	$CH_3CH_2(CH_3)CH$	H	H	2.97	5.53	5.52	0.00
15b	CH_3	$CH_3CH_2(CH_3)CH$	H	NO_2	1.59	5.80	5.87	−0.08
15c	CH_3	$CH_3CH_2(CH_3)CH$	H	NH_2	0.95	6.02	6.24	−0.22
15d	CH_3	$CH_3CH_2(CH_3)CH$	H	$N{=}C(NH_2)_2$	0.74	6.13	6.11	0.02
16a	CH_3	$CH_3(CH_2)_3$	H	H	0.23	6.64	6.52	0.12
16b	CH_3	$CH_3(CH_2)_3$	H	NO_2	0.14	6.85	6.84	0.02
16c	CH_3	$CH_3(CH_2)_3$	H	NH_2	0.07	7.15	7.12	0.04
16e	H	$CH_3(CH_2)_3$	H	$N{=}C(NH_2)_2$	0.04	7.40	7.57	−0.18
17a	CH_3		H	H	0.49	6.31	6.13	0.18
17b	CH_3		H	NO_2	0.33	6.48	6.68	−0.20
17c	CH_3		H	NH_2	0.038	7.42	6.74	0.68
18a	CH_3	$(CH_3)_2CH(CH_2)_2$	H	H	3.81	5.42	5.53	−0.11
18b	CH_3	$(CH_3)_2CH(CH_2)_2$	H	NO_2	2.96	5.53	5.63	−0.10
18c	CH_3	$(CH_3)_2CH(CH_2)_2$	H	NH_2	0.97	6.01	5.97	0.04
18e	H	$(CH_3)_2CH(CH_2)_2$	H	$N{=}C(NH_2)_2$	0.041	7.39	7.25	0.14
19a	CH_3	$CH_3(CH_2)_{15}$	H	H	2.69	5.57	6.05	−0.48
19b	CH_3	$CH_3(CH_2)_{15}$	H	NO_2	1.54	5.81	6.40	−0.58
19c	CH_3	$CH_3(CH_2)_{15}$	H	NH_2	0.59	6.23	6.72	−0.49
2					0.021			

analysis. We have evaluated different filter value σ and at least selected σ as 2.00 kal mol^{-1} to speed up the analysis and reduce noise.

4.2. Results and discussion

From the docking results and the actual results, we can both obtain the conclusions: the order of increasing activity is R_4: $-N{=}C(NH_2)_2 > -NH_2 > -NO_2 > -H$. The LOO cross-validated q^2 of the CoMFA model is 0.528, and the noncross-validated r^2 for the model established by the study is 0.971. The value of the variance ratio F ($n_1 = 7$, $n_2 = 17$) is 81.631 and standard error of the estimate (SEE) is 0.147. The contribution of electrostatic and steric is 68.2% and 31.8%, respectively.

From Figure 3(b) we can find that the CoMFA model can predict compounds **7**, **15b**, **18e** well, but not very well to **13a** and **17c** in the test set. The poor predictability may be caused by the sample size being more lower when the actual $pIC_{50} < 5.2$ (including 1 compounds) than it >5.2(including 24 compounds). From Table 1 we can see that for R_4, the activity of compound with $-NH_2$ is 2- to 3-fold than that of compound with $-NO_2$ while they have the same R_2 and R_3 except **17b** and **17c** (the activity of **17c** is nearly 10-fold than **17b**), and it maybe the reason for the poor predictability of **17c**. We also try to use the model to predict the last three compounds with very long R_2 side chain. The points of the three compounds are all in ±0.5log unit in Figure 3(b), which shows that large capacity change of R_2 has little influence on the activity. What is more, from the

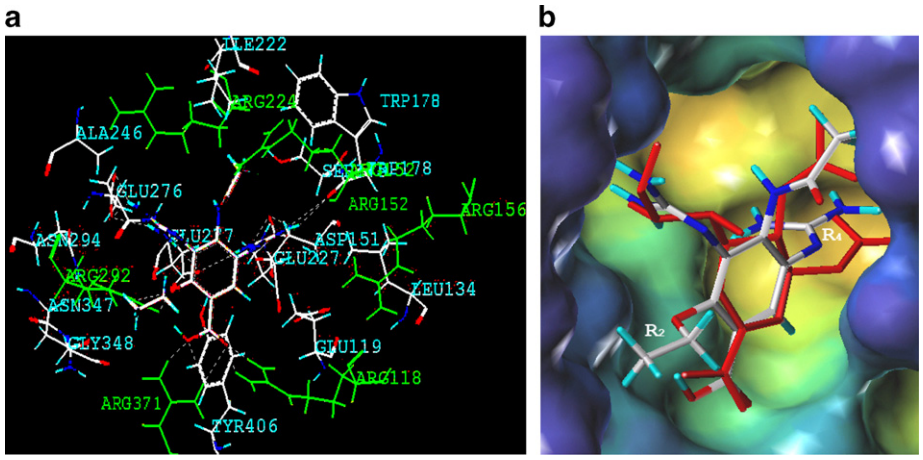


Figure 2. FlexX docked result. Compound **12** in the active site of NA (PDB ID:1nnc).[19] (a) Compound **12** reacting with the amino acids of the active pocket of NA. (b) Comparing the docking orientation of **12** to the ligand-SA (showed in red color) in the complex.

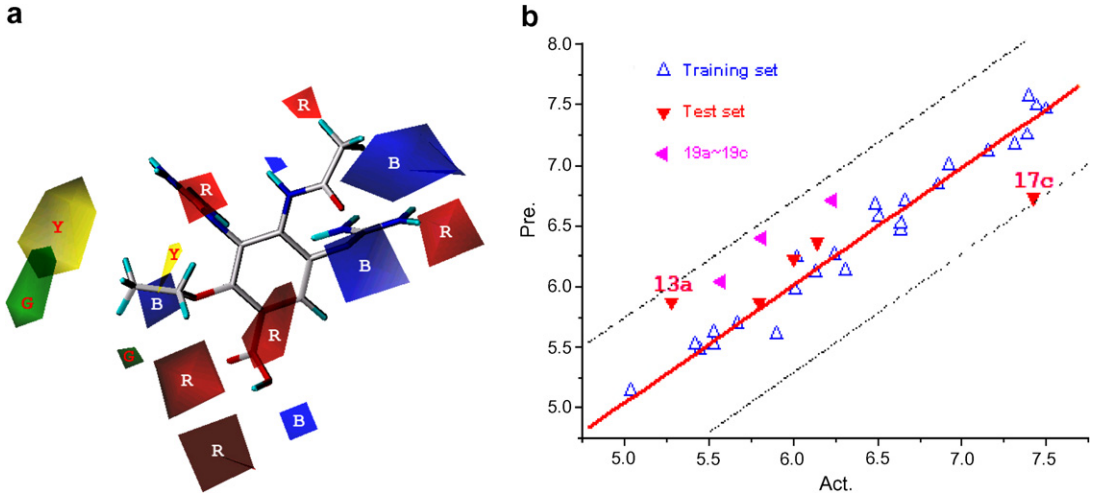


Figure 3. (a) The most active molecule **12** is shown in the background. Red (R) color represents the negative charge region, blue (B) is the positive charge region, green (G) is the more bulky region, yellow (Y) is the less bulky region. (b) The predictability of the CoMFA model.

docking result we find that R_2 is to stretch out of the active pocket of NA in Figure 2(b), which can explain why it cannot show distinguished influence to activity.

5. Conclusions

We have described the synthesis and properties of a series of *p*-aminosalicylic acid derivatives as influenza NA inhibitors. All of the compounds were shown to possess potent influenza NA inhibitory activity, and the most potent compound of the series is compound **12** (IC_{50} = 0.032 μM), which in addition to good enzyme inhibitory activity, displays potent anti-viral activity in vitro. We reported a more convenient and economical method of the synthesis of *p*-aminosalicylic acid NA inhibitors. Compared to other research, *p*-aminosalicylic acid we used appeared to be an ideal starting material because of its low cost and commercial abundance. Establishing a consistent binding mode was critical to predictive structure-based drug design and discovering potent compounds in the nanomolar range that would potentially be useful for anti-viral therapy. The compounds we have got all showed potent NA inhibitory activity, and this finding could be used to design further influenza NA inhibitors. The properly substituted *p*-aminosalicylic acids provide an attractive structural template for developing potent inhibitors of influenza NA.

6. Experimental

6.1. Neuraminidase inhibition assay

All compounds were evaluated for in vitro inhibitory actions using the method reported by Guanhua Du.[20,21] The strain A (Yuefang 72-243 A) influenza virus, which was donated by Chinese Centers for Disease Control, was used as source of NA. The NA was obtained by the method described by Laver.[22] The assay employed a spectrofluorometric technique that uses the compound 2′-(4-methylumbelliferyl)-α-D-acetylneuraminic acid (MUNANA) as substrate. And cleavage of this substrate by NA produces a fluorescent product, which

can emit an emission wavelength of 460 nm with an excitation wavelength of 355 nm. The intensity of fluorescence can reflect the activity of NA sensitively.

In the enzyme reaction system, there were 30 μL of the enzyme in 33 mmol/L MES buffer (pH 3.5), 10 μL of 4 mmol/L $CaCl_2$, 20 μL of 20 μmol/L MUNANA, and 30 μL water in a 96-well microplate. The terminal volume was 100 μL. After 10 min at 37 °C, 150 μL of 14 mmol/L NaOH in 83% ethanol was added to 0.1 mL of the reaction mixture to terminate the reaction. The intensity of the fluorescence was quantitated in Fluostar Galaxy (excitation, 360 nm; emission, 450 nm), and substrate blanks were subtracted from the sample readings. The IC_{50} was calculated by plotting percent inhibition versus the inhibitor concentration, and determination of each point was performed in duplicate.

6.2. Chemistry: General procedures

All reactions except those in aqueous media were carried out by standard techniques for the exclusion of moisture. All reactions were monitored by thin-layer chromatography on 0.25 mm silica gel plates (60GF-254) and visualized with UV light, or iodine vapor. ^{1}H NMR spectra were determined on a Brucker Avace 300 spectrometer using TMS as an internal standard. ESI-MS were determined on an API 4000 spectrometer. Melting points were obtained on an electrothermal melting point apparatus and are uncorrected. Anhydrous reactions were carried out in over-dried glassware under a nitrogen atmosphere.

6.2.1. Methyl 4-amino-2-hydroxybenzoate (6). A mixture of *p*-aminosalicylic acid (**5**, 3.06 g, 20 mmol) and concentrated H_2SO_4 (0.5 mL) in anhydrous methanol (50 mL) was stirred at reflux for 12 h. The solvent was evaporated in vacuo and the resulting residue was neutralized with saturated $NaHCO_3$ solution to pH 7–8. The aqueous layer was extracted with EtOAc (3× 50 mL). The organic layer was washed with saturated $NaHCO_3$ (3× 15 mL) and water (15 mL), dried with $NaSO_4$, and evaporated to give **6** (2.91 g, 87%) as a white solid: mp 125–126 °C, ESI-MS *m*/*z* 168.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.78 (s, 3H); 6.15 (br, 2H); 5.99 (d, J = 2.1 Hz, 1H); 6.12 (dd, J = 8.1 Hz, 2.1 Hz, 1H); 7.45 (d, J = 8.4 Hz, 1H); 10.77 (s, 1 H).

6.2.2. Methyl 4-(acetylamino)-2-hydroxybenzoate (7). To a solution of compound **6** (1.67 g, 10 mmol) in anhydrous acetone (25 mL), a solution of acetic anhydride (1.05 mL) in acetone (5 mL) was added dropwise with magnetic stirring. After reaction for 5 h, the solvent was evaporated in vacuo, and the solid residue was washed with water. The isolated product was recrystrallized from methanol/water (2:1) to provide **7** (1.67 g, 80%) as a white solid: mp 153–154 °C, ESI-MS *m*/*z* 210.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.07 (s, 3H); 3.86 (s, 3H); 7.06 (dd, J = 8.7 Hz, 1.8Hz, 1H); 7.38 (d, J = 1.8 Hz, 1H); 7.71 (d, J = 8.7 Hz, 1H); 10.24 (s, 1H); 10.62 (s, 1H).

6.2.3. Methyl 4-(acetylamino)-2-ethoxybenzoate (8). A suspension of compound **7** (1.88 g, 9 mmol), bromoethane (1.09 g, 0.75 mL, 10 mmol) and K_2CO_3 (1.50 g, 10.8 mmol) in 30 mL anhydrous acetone was stirred at reflux for 8 h. Bromoethane (1.09 g, 0.75 mL, 10 mmol) and K_2CO_3 (1.50 g, 10.8 mmol) were added and the reaction continued for another 6 h at reflux. The reaction mixture was evaporated in vacuo to dryness. The crude material was suspended in 50 mL of 2 N NaOH and the product extracted into EtOAc (3× 50 mL). The combined organic layers were washed with water (3× 15 mL), dried with Na_2SO_4 and evaporated to give **8** (1.71 g, 80%) as a white solid: mp 131–132 °C, ESI-MS *m*/*z* 238.3 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.34 (t, J = 6.9 Hz, 3H); 2.07 (s, 3H); 3.74 (s, 3H); 4.02 (q, J = 6.9 Hz, 1H); 7.17 (dd, J = 8.7 Hz, 1.8 Hz, 1H); 7.47 (d, J = 1.8 Hz, 1H); 7.65 (d, J = 8.4 Hz, 1H); 10.20 (s, 1H).

6.2.3.1. Methyl 4-(acetylamino)-2-isopropoxybenzoate (13a). To a suspension of 70% NaH (0.8 g) in 5 mL dry DMF was added a solution of compound **7** (3.48 g, 16 mmol) in 7 mL of DMF followed by *iso*-propyl bromide (2.46 g, 1.88 mL, 20 mmol) in 5 mL DMF. The reaction mixture was heated at 55 °C for 8 h. The reaction mixture was cooled to room temperature and then partitioned between water and EtOAc. The combined organic layers were washed with water, dried with Na_2SO_4 and concentrated to give a solid **13a** (3.25 g, 81%): mp 118–119 °C, ESI-MS *m*/*z* 252.5 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.29 (d, J = 6.0 Hz, 6H); 2.06 (s, 3H); 3.73 (s, 3H); 4.49 (m, 1H); 7.16 (dd, J = 8.4 Hz, 1.8 Hz, 1H); 7.49 (d, J = 1.8 Hz, 1H); 7.63 (d, J = 8.4 Hz, 1H); 10.18 (s, 1H).

6.2.3.2. Methyl 4-(acetylamino)-2-propoxybenzoate (14a). Mp 93–94 °C, ESI-MS *m*/*z* 252.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.01 (t, J = 7.5 Hz, 3H); 1.75 (m, 2H); 2.07 (s, 3H); 3.74 (s, 3H); 3.92 (t, J = 6.3 Hz, 2H); 7.16 (dd, J = 8.4 Hz, 1.8 Hz, 1H); 7.47 (d, J = 1.5 Hz, 1H); 7.66 (d, J = 8.4 Hz, 1H); 10.20 (s, 1H).

6.2.3.3. Methyl 4-(acetylamino)-2-sec-butoxybenzoate (15a). Mp 142–143 °C, ESI-MS *m*/*z* 266.5 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.95 (t, J = 7.5 Hz, 3H); 1.26 (d, J = 6.0 Hz, 3H); 1.64 (m, 2 H); 2.07 (s, 3H); 3.73 (s, 3H); 4.30 (m, 1H); 7.06 (dd, J = 8.7 Hz, 2.1 Hz, 1H); 7.38 (d, J = 1.8 Hz, 1H); 7.72 (d, J = 8.7 Hz, 1H); 10.62 (s, 1H).

6.2.3.4. Methyl 4-(acetylamino)-2-butoxybenzoate (16a). Mp 73–74 °C, ESI-MS *m*/*z* 266.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.93 (t, J = 7.5 Hz, 3H); 1.50 (m, 2H); 1.73 (m, 2H); 2.06 (s, 3H); 3.73 (s, 3H); 3.96 (t, J = 6.3 Hz, 2H); 7.16 (dd, J = 8.7 Hz, 1.8 Hz, 1H); 7.47 (d, J = 1.8 Hz, 1H); 7.65 (d, J = 8.7 Hz, 1H); 10.20 (s 1H).

6.2.3.5. Methyl 4-(acetylamino)-2-(3-methylbutoxy) benzoate (17a). Mp 100–101 °C, ESI-MS *m*/*z* 278.5 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.59-1.75 (m, 8H); 2.07 (s, 3H); 3.73 (s, 3H); 4.75 (m, 1H); 7.16 (dd, J = 8.4 Hz, 2.1 Hz, 1H); 7.48 (d, J = 2.1 Hz, 1H); 7.63 (d, J = 8.4 Hz, 1H); 10.18 (s, 1H).

6.2.3.6. Methyl 4-(acetylamino)-2-(cyclopentyloxy) benzoate (18a). Mp 72–74 °C, ESI-MS *m/z* 280.5 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.93 (d, J = 6.6 Hz, 6H); 1.63 (m, 2H); 1.82 (m, 1H); 2.07 (s, 3H); 3.73 (s, 3H); 3.99 (t, J = 6.3 Hz, 2H); 7.17 (dd, J = 8.4 Hz, 2.1 Hz, 1H); 7.48 (d, J = 1.8 Hz, 1H); 7.65 (d, J = 8.4 Hz, 1H); 10.20 (s, 1 H).

6.2.3.7. Methyl 4-(acetylamino)-2-(hexadecyloxy)benzoate (19a). Mp 62–63 °C, ESI-MS *m/z* 434.8 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.85 (t, J = 6.3 Hz, 3H); 1.23 (m, 24H); 1.44 (m, 2H); 1.72 (m, 2H); 2.06 (s, 3H); 3.73 (s, 3H); 3.95 (t, J = 6.3 Hz, 2H); 7.16 (dd, J = 8.4 Hz, 2.1 Hz, 1H); 7.46 (d, J = 2.1 Hz, 1H); 7.65 (d, J = 8.4 Hz, 1H); 10.19 (s, 1H).

6.2.4. Methyl 4-(acetylamino)-2-ethoxy-3,5-dinitrobenzoate (9). To fuming nitric acid (50 mL) cooled in an ice bath was added slowly compound **8** (4.74 g, 20 mmol). After the reaction mixture was stirred at 0–5 °C for 45 min and at room temperature for an additional 45 min, it was poured into ice water (100 mL). The light yellow solid obtained was collected by filtration, washed with water and dried under vacuo to furnish 4.25 g of crude product. The solid was recrystallized from EtOAc to provide compound **9** (4.25 g, 65%) as a light yellow solid: mp 85–86 °C, ESI-MS *m/z* 328.3 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.27 (t, J = 7.2 Hz, 3H); 2.05 (s, 3H); 3.94 (s, 3H); 4.31 (q, J = 7.2 Hz, 2H); 8.85 (s, 1H); 10.78 (s, 1 H).

6.2.4.1. Methyl 4-(acetylamino)-2-isopropoxy-5-nitrobenzoate (13b). Mp 125–126 °C, ESI-MS *m/z* 297.5 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.30 (d, J = 6.3 Hz, 6H); 2.17 (s, 3H); 3.87 (s, 3H); 4.72 (m, 1H); 7.77 (s, 1H); 8.45 (s, 1H); 10.43 (s, 1H).

6.2.4.2. Methyl 4-(acetylamino)-5-nitro-2-propoxybenzoate (14b). Mp 102–103 °C, ESI-MS *m/z* 297.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.02 (t, J = 7.5 Hz, 3 H); 1.78 (m, 2H); 2.11 (s, 3H); 3.75 (s, 3H); 3.93 (t, J = 6.3 Hz, 2H); 7.67 (s, 1H); 8.46 (s, 1H); 10.44 (s, 1 H).

6.2.4.3. Methyl 4-(acetylamino)-2-sec-butoxy-5-nitrobenzoate (15b). Mp 141–142 °C, ESI-MS *m/z* 311.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.91 (t, J = 7.5 Hz, 3H); 1.07 (d, J = 5.7 Hz, 3H); 1.34 (m, 2H); 2.03 (s, 3H); 3.75 (s, 3H); 3.91 (m, 1H); 7.77 (s, 1H); 8.47 (s, 1H); 10.62 (s, 1H).

6.2.4.4. Methyl 4-(acetylamino)-2-butoxy-5-nitrobenzoate (16b). Mp 84–85 °C, ESI-MS *m/z* 311.9 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.94 (t, J = 7.5 Hz, 3H); 1.52 (m, 2H); 1.75 (m, 2H); 2.18 (s, 3H); 3.81 (s, 3H); 4.13 (t, J = 6.3 Hz, 2H); 7.81 (s, 1H); 8.42 (s, 1H); 10.50 (s, 1H).

6.2.4.5. Methyl 4-(acetylamino)-2-(3-methylbutoxy)-5-nitrobenzoate (17b). Mp 112–113 °C, ESI-MS *m/z* 323.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ ^{1}H NMR (300 MHz, Solvent) δ ppm 1.65–1.87 (m, 8H); 2.03 (s, 3H); 3.87 (s, 3H); 4.51 (m, 1H); 7.79 (s, 1H); 8.47 (s, 1H); 10.44 (s, 1H).

6.2.4.6. Methyl 4-(acetylamino)-2-(cyclopentyloxy)-5-nitrobenzoate (18b). Mp 88–89 °C, ESI-MS *m/z* 325.3 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.95 (d, J = 6.3 Hz, 6H); 1.65 (m, 2H); 1.83 (m, 1H); 2.12 (s, 3H); 3.85 (s, 3H); 3.97 (t, J = 6.3 Hz, 2H); 7.76 (s, 1H); 8.43 (s, 1H); 10.42 (s, 1H).

6.2.4.7. Methyl 4-(acetylamino)-2-(hexadecyloxy)-5-nitrobenzoate (19b). Mp 68–69 °C, ESI-MS *m/z* 479.5 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.84 (t, J = 6.6 Hz, 3H); 1.23 (m, 24H); 1.45 (m, 2H); 1.76 (m, 2H); 2.17 (s, 3H); 3.81 (s, 3H); 4.12 (t, J = 6.3 Hz, 2H); 7.81 (s, 1H); 8.42 (s, 1H); 10.49 (s, 1H).

6.2.5. Methyl 4-(acetylamino)-3,5-diamino-2-ethoxybenzoate (10). To a suspension of compound 9 (3.27 g, 10 mmol) in EtOH (50 mL) was added catalytic quantity of 10% Pd/$CaCO_3$ and 5% HCl (5 mL). Hydrazine hydrate (80%, 1.5 mL) dissolved in EtOH (5 mL) was then added dropwise to the above mixture. The reaction mixture was stirred at room temperature for 3 h, and the Pd/$CaCO_3$ was moved by filtration and the EtOH was concentrated under vacuum to give crude product. The residue obtained was recrystallized from MeOH yielding compound **10** (1.90 g, 71%): mp 226–227 °C, ESI-MS *m/z* 268.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.33 (t, J = 6.9 Hz, 3H); 2.14 (s, 3H); 2.76 (s, 3H); 4.04 (q, J = 6.9 Hz, 2H); 6.95 (s, 1H); 7.98 (s, 2H); 9.81 (s, 2H).

6.2.5.1. Methyl 4-(acetylamino)-5-amino-2-isopropoxybenzoate (13c). Mp 248–249 °C, ESI-MS *m/z* 267.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.32 (d, J = 6.3 Hz, 6H); 2.09 (s, 3H); 3.85 (s, 3H); 4.76 (m, 1H); 7.19 (s, 1H); 7.33 (s, 1H); 9.17 (s, 2H); 9.88 (s, 1H).

6.2.5.2. Methyl 4-(acetylamino)-5-amino-2-propoxybenzoate (14c). Mp 237–238 °C, ESI-MS *m/z* 267.3 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.02 (t, J = 7.2 Hz, 3H); 1.75 (m, 2H); 2.07 (s, 3H); 3.75 (s, 3H); 3.91 (t, J = 6.3 Hz, 2H); 7.17 (s, 1H); 7.42 (s, 1H); 9.12 (s, 2H); 9.85 (s, 1H).

6.2.5.3. Methyl 4-(acetylamino)-5-amino-2-sec-butoxybenzoate (15c). Mp 245–246 °C, ESI-MS *m/z* 281.3 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.93 (t, J = 7.5 Hz, 3H); 1.09 (d, J = 6.0 Hz, 3H); 1.38 (m, 2H); 2.06 (s, 3H); 3.77 (s, 3H); 3.93 (m, 1H); 7.21 (s, 1H); 7.35 (s, 1H); 9.18 (s, 2H); 9.95 (s, 1H).

6.2.5.4. Methyl 4-(acetylamino)-5-amino-2-butoxybenzoate (16c). Mp 236–237 °C, ESI-MS *m/z* 281.8 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.97 (t, J = 7.5 Hz, 3H); 1.55 (m, 2H); 1.77 (m, 2H); 2.15 (s, 3H); 3.79 (s, 3H); 4.17 (t, J = 6.3 Hz, 2H); 6.00 (d, J = 2.1 Hz, 2H); 6.13 (s, 2H); 7.45 (d, J = 2.7 Hz, 2H); 10.78 (s, 1H).

6.2.5.5. Methyl 4-(acetylamino)-5-amino-2-(3-methylbutoxy)benzoate (17c). Mp 224–225 °C, ESI-MS *m/z* 293.5 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ ^{1}H NMR (300 MHz, Solvent) δ ppm 1.67-1.92 (m, 8H); 2.07 (s, 3H); 3.88 (s, 3H); 5.35 (m, 1H); 7.21 (s, 1H); 7.34 (s, 1H); 9.18 (s, 2H); 9.92 (s, 1H).

6.2.5.6. Methyl 4-(acetylamino)-5-amino-2-(cyclopentyloxy)benzoate (18c). Mp 201–203 °C, ESI-MS *m/z* 295.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.97 (d, J = 6.3 Hz, 6H); 1.62 (m, 2H); 1.86 (m, 1H); 2.12 (s, 3H); 3.86 (s, 3H); 3.97 (t, J = 6.6 Hz, 2H); 7.35 (s, 1H); 9.27 (s, 2H); 10.01 (s, 1H).

6.2.5.7. Methyl 4-(acetylamino)-5-amino-2-(hexadecyloxy)benzoate (19c). Mp 171–172 °C, ESI-MS *m/z* 449.3 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.85 (t, J = 6.9 Hz, 3H); 1.24 (m, 24H); 1.52 (m, 2H); 1.83 (m, 2H); 2.09 (s, 3H); 3.87 (s, 3H); 4.52 (t, J = 6.6 Hz, 2H); 7.23 (s, 1H); 7.38 (s, 1H); 9.22 (s, 2H); 9.95 (s, 1H).

6.2.6. Methyl 4-(acetylamino)-3,5-bis(guanidino)-2-ethoxybenzoate (11). A mixture of **10** (2.67 g, 10 mmol), cyanamide (8.4 g, 200 mmol), and concd HCl (1.5 mL) in EtOAc (70 mL) was refluxed for 6 h. The reaction mixture was diluted with EtOAc (150 mL) and partitioned with K_2CO_3 solution (70 mL). The organic layer was washed with water, dried over Na_2SO_4, filtered, and the solvent was evaporated in vacuo. The residue obtained was recrystallized from MeOH yielding compound 11 (1.58 g, 45%) as a white solid: mp 242–243 °C, ESI-MS *m/z* 352.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.36 (t, J = 6.9 Hz, 3H); 3.74 (s, 3H); 3.89 (s, 3H); 4.06 (q, J = 6.9 Hz, 2H); 6.52 (s, 1H); 7.83 (br, 2H); 8.18 (br, 2H); 8.49 (br, 2H); 8.63 (s, 1H).

6.2.6.1. Methyl 4-(acetylamino)-5-(guanidino)-2-isopropoxybenzoate (13d). Mp 233–234 °C, ESI-MS *m/z* 309.5 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.85 (d, J = 6.9 Hz, 6H); 2.07 (s, 3H); 2.24 (s, 3H); 3.44 (m, 1H); 7.41 (br, 2H); 7.56 (d, J = 2.1 Hz, 1H); 7.63 (s, 1H); 7.89 (d, J = 1.6 Hz, 1H); 8.46 (s, 1H); 10.38 (s, 1H).

6.2.6.2. Methyl 4-(acetylamino)-5-(guanidino)-2-secbutoxybenzoate (15d). Mp 204–205 °C, ESI-MS *m/z* 323.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.95 (t, J = 7.5 Hz, 3H); 1.09 (d, J = 6.0 Hz, 3 H); 1.37 (m, 2H); 2.07 (s, 3H); 3.76 (s, 3H); 3.92 (m, 1H); 7.42 (br, 2H); 7.58 (d, J = 2.1 Hz, 1H); 7.66 (s, 1H); 7.91 (d, J = 1.7 Hz, 1H); 8.45 (s, 1H); 10.42 (s, 1H).

6.2.7. 4-(Acetylamino)-3,5-bis(guanidino)-2-ethoxybenzoic acid (12). The ester **11** (3.51 g, 10 mmol) was dissolved in MeOH (40 mL), and 10 mL of 2 mol/L NaOH was added over 5 min with good stirring. The reaction mixture was stirred overnight at room temperature. The pH of the resulting solution was adjusted to 7–8 with 80% acetic acid/water, and compound **12** precipitated as a white solid, which was collected by filtration and dried (2.26 g, 67%): mp 337–338 °C, ESI-MS *m/z* 338.1 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.38 (t, J = 6.9 Hz, 3H); 3.89 (s, 3H); 4.16 (q, J = 6.9 Hz, 2H); 6.57 (s, 1H); 7.86 (br, 2H); 8.23 (br, 2H); 8.52 (br, 2H); 8.76 (s, 1H).

6.2.7.1. 4-(Acetylamino)-5-(guanidino)-2-isopropoxybenzoic acid (13e). Mp 304–305 °C, ESI-MS *m/z* 295.6 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.87 (d, J = 6.9 Hz, 6H); 2.09 (s, 3H); 3.46 (m, 1H); 7.43 (br, 2H); 7.59 (d, J = 2.1 Hz, 1H); 7.67 (s, 1H); 7.92 (d, J = 1.7 Hz, 1H); 8.47 (s, 1H); 10.42 (s, 1H).

6.2.7.2. 4-(Acetylamino)-5-(guanidino)-2-propoxybenzoic acid (14e). Mp 295–296 °C, ESI-MS *m/z* 295.5 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.07 (t, J = 7.5 Hz, 3H); 1.83 (m, 2H); 2.53 (s, 3H); 3.89 (t, J = 6.3 Hz, 2H); 7.45 (br, 2H); 7.62 (d, J = 2.1 Hz, 1H); 7.75 (s, 1H); 7.97 (d, J = 1.7 Hz, 1H); 8.54 (s, 1H); 10.47 (s, 1H).

6.2.7.3. 4-(Acetylamino)-5-(guanidino)-2-butoxybenzoic acid (16e). Mp 259–260 °C, ESI-MS *m/z* 308.7 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.99 (t, J = 7.5 Hz, 3H); 1.57 (m, 2H); 1.78 (m, 2H); 2.12 (s, 3H); 4.15 (t, J = 6.3 Hz, 2H); 7.42 (br, 2H); 7.57 (d, J = 2.1 Hz, 1H); 7.65 (s, 1H); 7.91 (d, J = 1.6 Hz, 1H); 8.44 (s, 1H); 10.44 (s, 1H).

6.2.7.4. 4-(Acetylamino)-5-(guanidino)-2-(cyclopentyloxy)benzoic acid (18e). Mp 231–232 °C, ESI-MS *m/z* 323.4 (M+1); ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.98 (d, J = 6.6 Hz, 6H); 1.58 (m, 2H); 1.87 (m, 1H); 2.15 (s, 3H); 3.96 (t, J = 6.6 Hz, 2H); 7.48 (br, 2H); 7.62 (d, J = 2.1 Hz, 1H); 7.69 (s, 1H); 7.91 (d, J = 1.7 Hz, 1H); 8.48 (s, 1H); 10.38 (s, 1H).

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