JOC The Journal of Organic Chemistry

Note

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J. Org. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.joc.9b02857 • Publication Date (Web): 18 Dec 2019

Synthesis of Small Fluorescent Molecules and Evaluation of Photophysical Properties

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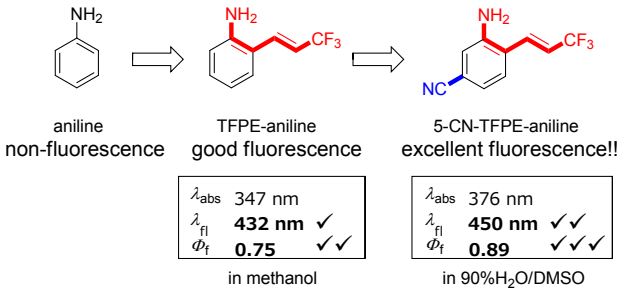
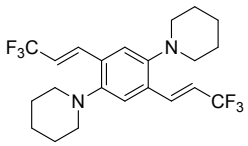
GRAPHICAL ABSTRACT

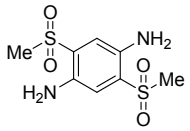


A series of aniline-based fluorophores were newly synthesized. To increase their fluorescence quantum yields, it was particularly important to substitute 3,3,3-trifluoroprop-1-enyl (TFPE) groups next to the amino group to benefit from an extended π-electron delocalization. Among these, 5-CN-2-TFPE-aniline was found to behave as an excellent fluorophore with a reasonable fluorescence quantum yield of 0.89 even in aqueous solution. L-Alanine peptide, a non-fluorescent analogue of 5-CN-2-TFPE-aniline, was synthesized and successfully employed as an enzyme probe to detect aminopeptidase N activity.

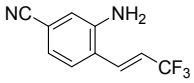
Recent progress in medical and life science technology have accelerated intensively with the advent of useful and well-designed fluorescent probes.[1] Fluorescein and Rhodamine, polycyclic arenes with extended aromaticity, are used as convenient fluorophores to elaborate functionalized fluorescent probes for a variety of specific targets such as proteins[2], enzymes[3], receptors[4], and ions[5]. These polycyclic fluorophores can emit visible fluorescent radiation in the range of 400 to 700 nm, useful for identification and visualization of targets. However, fluorophores comprised of relatively small and simple molecules have been developed recently.[6] They are composed of only one benzene ring which has electron-withdrawing and -donating substituents, a so-called 'push-pull' system. Two typical compounds, bis(alkenyl)benzene and 2,5-bis(methylsulfonyl)-1,4-diaminobenzene, are shown in Figure 1. Compared with the large polycyclic type, small fluorophores have obvious benefits particularly when they are used in a fluorescent probe for enzymes with high substrate specificity. Such specific enzymes usually recognize small differences in substrate structure and catalyze the reaction only when the substrate is compatible with the enzyme requirements.



Bis(alkenyl)benzene



2,5-Bis(methylsulfonyl)-
1,4-diaminobenzene



2-TFPE-5-CN-aniline (**3f**)

Figure 1. Fluorescent compounds with a push-pull system based on a donor-π-conjugated-acceptor structure.

We set out to produce small fluorophores for use in fluorescent probes for specific enzymes. We have already described several fluorescent compounds based on aniline moiety substituted by 3,3,3-trifluoroprop-1-enyl (TFPE) groups. Among them, 2,4-disubstituted aniline (2,4-TFPE-aniline) gave fluorescence quantum yields (Φ_f) of 0.27 at 461nm even in an aqueous solution (water:DMSO=9:1). These fluorescence properties in aqueous solution enabled 2,4-TFPE-aniline to be used as the fluorophore in a fluorogenic probe for dipeptidyl peptidase-4 (DPP-4)[7].

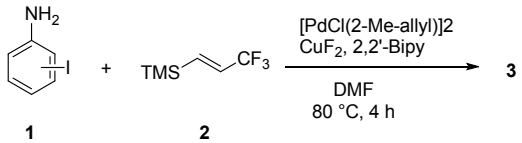
In the course of our work on synthesizing small and high fluorescent compounds based on 2-TFPE-aniline moiety, we found that 5-CN substituted for 2-TFPE-aniline (2-TFPE-5-

CN-aniline) exhibited excellent fluorescent performance even in aqueous solution. In this paper, we describe synthesis, fluorescent properties and preliminary results of 2-TFPE-5-CN-aniline used as a fluorogenic probe for aminopeptidase N.

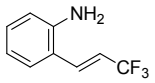
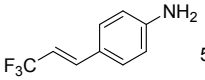
It has been reported that benzene derivatives with a push-pull system have the propensity to become fluorescent compounds, despite the structure of a less extended π-system compared with polycyclic fluorophores. In 2009, Shimizu et al. reported a rigorous study in which non-fluorescent phenylenediamines became fluorescent through multiple substitutions of electron-withdrawing TFPE groups. This pioneering study prompted us to examine how many substitutions are necessary to provide the phenylenediamines with good fluorescence. For this purpose, two compounds with the simplest substitution pattern, 2-TFPE-aniline (**3a**) and 4-TFPE-aniline (**3b**), were prepared to determine their fluorescent responses. For the synthesis, they were obtained in moderate yields based on our previous report using the Hiyama cross-coupling reaction of corresponding aryl iodide with (*E*)-3,3,3-trimetyl(3,3,3-trifluoro-1-propenyl)silane (**2**).[8] Their photophysical properties, fluorescence

emissions, molar absorption coefficients (ε) and quantum yields (Φ_f), were measured as shown in Table 1.

Table 1. Synthesis and photophysical properties of 3a and 3b.



1 **2**

		λ_{abs} (nm)[b]	ε ($M^{-1}cm^{-1}$)[b]	λ_{fl} (nm)[b]	ϕ_f[b]
	**3a** 68%[a]	347	5154	423	0.77
	**3b** 54%[a]	337	6999	374	0.01

[a] Isolated yield

[b] Measured in THF (1.0×10^{-5} M)

Drastic changes in the fluorescence behavior of **3a** and **3b** were observed. The quantum yield (Φ_f) of *para*-isomer **3a** was 0.11, actually a non-fluorescent compound, whereas that of *ortho*-isomer 3b was 0.77, a highly emissive compound.

To explain these differences, theoretical calculations of **3a** and **b** were conducted by the Gaussian 16 package with DFT to give HOMO and LUMO orbitals with energy levels as

shown in Figure 2. Compared with **3b**, **3a** indicated slightly expanded HOMO and LUMO orbitals, resulting from the interaction between π orbital and σ* orbital of C-F bond, namely π-σ* conjugation in HOMO and π*-σ* integration in LUMO. In contrast, we could find no significant difference in HOMO-LUMO energy gaps of both compounds, which suggested that both compounds could be excited via a similar process.

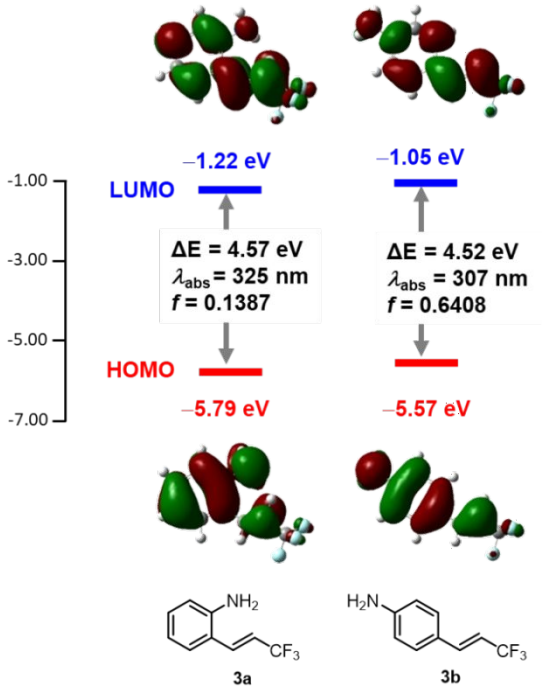


Figure 2. Frontier molecular orbitals and energy levels of 3a and 3b calculated at the RB3LYP/6-31G(d,p) level of theory. Excitation energies calculated by TD-DFT calculations at the UB3LYP/6-31+G(d,p) level of theory. The *f* values refer to the oscillator strength.

We reasoned that the distinction between both compounds would be in the relaxation and radiative process after both compounds were excited. To examine the radiative process in detail, fluorescence lifetime (τ_s), radiative rate constant (k_f), and nonradiative rate constant (k_{nr}) were measured for **3a** and **b**, as shown in Table 2.[9]

Table 2. Difference of fluorescence lifetimes, radiative and nonradiative rate constants between 3a and 3b.

	τ_s (ns)[a]	k_f (10^9 s^{-1})[b]	k_{nr} (10^9 s^{-1})[c]
3a	7.32	0.077	0.060
3b	0.23	0.043	4.304

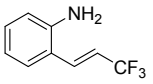
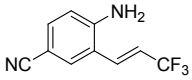
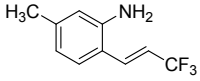
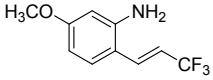
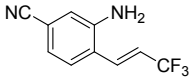
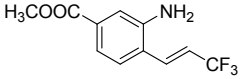
[a] measured in THF (1×10^{-5} M) using a single-photon-counting method [b] Radiative rate constant ($k_f = \Phi_f / \tau_s$). [c] Non-radiative rate constant ($k_{nr} = (1 - \Phi_f) / \tau_s$).

From the results summarized in Table 2, the ratio of k_f and k_{nr} in **3b** was almost 1 to 100, which suggests that once **3b** was excited, almost all the excited compounds would get down to the ground state through nonradiative processes without fluorescence radiation. In contrast, **3a** had the same order of k_f and k_{nr} (0.077 and 0.060, respectively), so that almost half of **3a,** once excited, would relieve the energy with fluorescence radiation through the radiative process. This led to the fluorescent properties of **3a**, indicating the importance of

ortho substitution of the TFPE group. With these results in hand, other 2-TFPE-aniline analogues were prepared for screening to explore potential and useful fluorophores.

As shown in Table 3, several analogues (**3c-g)** were obtained in moderate yields by running the above-mentioned reaction with corresponding iodo anilines. Table 3 and Figure 3 summarize absorption maximum at the longest wavelength (λ_{abs}), molar absorption coefficients (ε), fluorescence maximum wavelength (λ_{fl}) and quantum yields (Φ_f) for these analogues, which were measured in THF and water:DMSO (9:1) solution.

Table 3. Structures and photophysical properties of 3a and 3c-g.

Compounds	λ_{abs} (nm)[b]	ε ($M^{-1}cm^{-1}$)[b]	λ_{abs} (nm)[c]	ε ($M^{-1}cm^{-1}$)[c]	λ_{fl} (nm)[d]	Φ_f[d]	λ_{fl} (nm)[e]	Φ_f[e]
 **3a**	347	5150	325	4650	427	0.77	452	0.32
 **3c** 77%[a]	348	7520	334	3200	409	0.47	429	0.53
 **3d** 42%[a]	346	5200	324	4340	423	0.62	451	0.12
 **3e** 44%[a]	339	6500	321	5230	410	0.32	434	0.40
 **3f** 63%[a]	367	6740	346	4220	439	0.82	451	0.89
 **3g** 65%[a]	372	5500	349	4410	447	0.74	463	0.82

[a]Isolated yields. [b]Observed absorption maximum at the longest wavelengths in THF and corresponding εs. [c]Observed absorption maximum at the longest wavelengths in H_2O:DMSO = 9:1 and corresponding εs. [d]Fluorescence maximum peak wavelengths in THF, excitation at λ = 350 nm for **3a**, 330 nm for **3c**, 345 nm for **3d**, 345 nm for **3e**, 360 nm for **3f**, 370 nm for **3g** and corresponding Φ_fs. [e]Fluorescence maximum peak wavelengths in H_2O:DMSO = 9:1, excitation at λ = 220 nm for **3a**, 290 nm for **3c**, 330 nm for **3d**, 350 nm for **3e**, 370 nm for **3f**, 350 nm for **3g** and corresponding Φ_fs.

The fluorescence behavior of **3c** compared with that of **3a** showed blue shift in λ_{fl} and decrease of Φ_f due to the substitution of electron-withdrawing cyano groups at position 4, resulting in unexpected behavior for fluorophore exploration. However, substitution of an electron-donating group at position 5 in **3e** also led to a negative effect in fluorescence performance, while a more limited electron-donating substitution in **3d**, appeared to give a better result than **3e**, which suggested that an electronegative substitution at position 5 would produce an improvement in fluorescence behavior of **3a**. As anticipated, **3f** and **3g**,

from the cyano and methoxycarbonyl groups, respectively, exhibited excellent fluorescence compared to other derivatives especially in Φ_f. In particular, **3f** exhibited a high Φ_f of 0.82 and it should be noted that the Φ_f of **3f** did not decrease even in an aqueous solution of water:DMSO (9:1), which would be expected to keep the good fluorescence in an *in vitro* assay experiment to become a fluorophore with small size and simple structure.

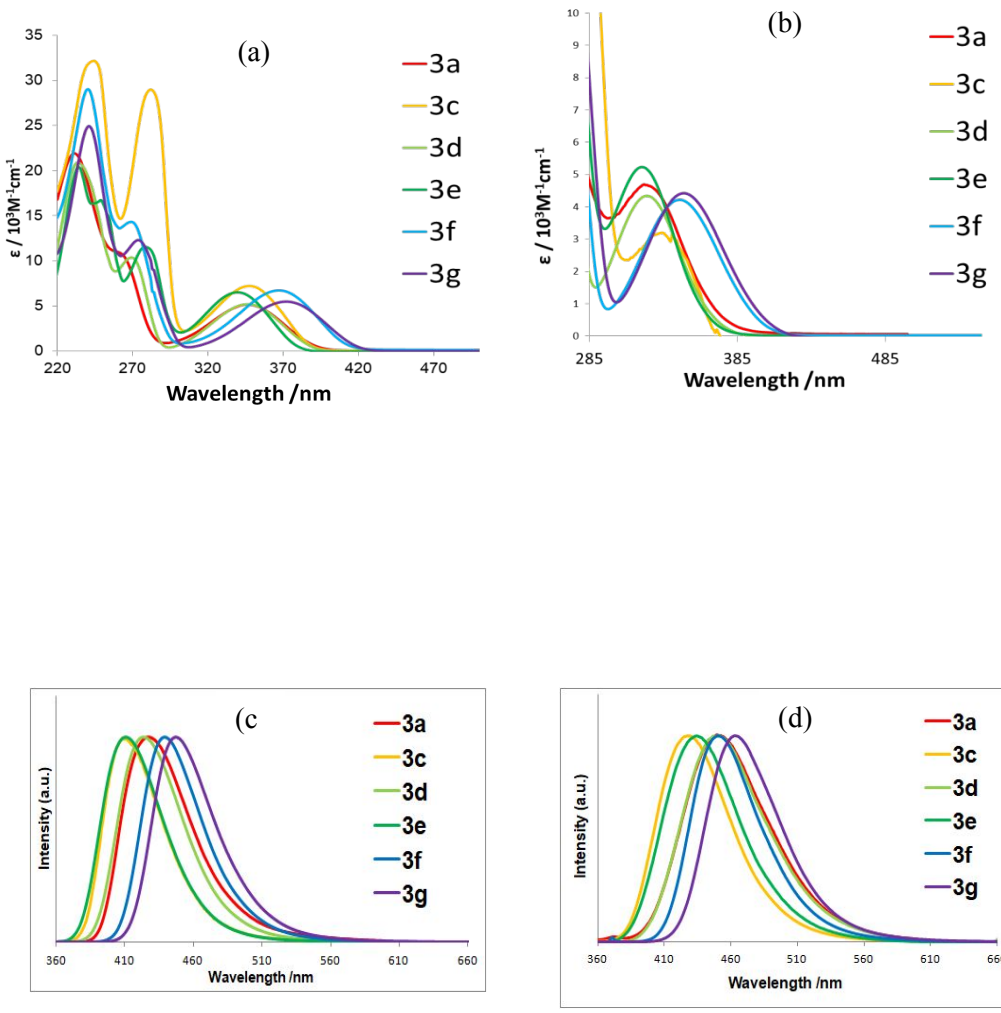
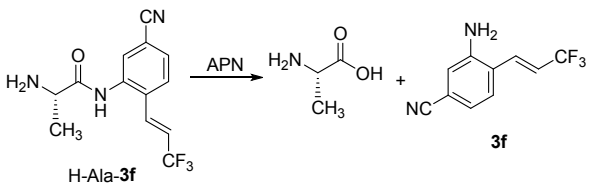


Figure 3. Photophysical properties of 3a and **3c-g.** (a) Electronic absorption spectra in THF; (b) Electronic absorption spectra in aqueous solution H_2O:DMSO = 9:1; (c) Fluorescence spectra in THF; (d) Fluorescence spectra in aqueous solution H_2O:DMSO = 9:1.

Next, we proceeded to explore the utility of **3f** in the use of fluorescent probes for appropriate enzymes. Aminopeptidase N (APN), an important peptidase existing in many animals, was chosen for this study.[10] There have been many reports concerning the fluorescent probe for APN. In most cases, the fluorescent off/on switching was controlled by the peptide bond formation (switch off) and cleavage (switch on).[11] According to these reports, our fluorescent probe for APN was designed as H-Ala-**3f** which was prepared by condensation of **3f** and L-alanine. Fortunately, H-Ala-**3f** was nonfluorescent because the newly bound amide carbonyl significantly inhibited the aryl nitrogen to donate electrons to the benzene ring. This broke the push-pull system leading to a loss of the fluorescence character based on **3f**. When H-Ala-**3f** was added to the reaction mixture with different

amounts of APN (10, 5, and 1 ng), the enzymatic reaction proceeded smoothly, whereas no reaction occurred without APN (blank condition) as shown in Figure 4. The reaction progress was monitored by measuring fluorescence intensity of **3f** liberated by enzymatic hydrolysis of H-Ala-**3f**. Thanks to the simple structure of **3f**, the fluorescence of H-Ala-**3f** was so weak that the reaction could be monitored clearly even in a very low concentration of APN (0.1 nM) without interference of background fluorescence of H-Ala-**3f**. From these results, K_m, K_{cat} and K_{cat}/K_m were calculated to be 138 μM, 2590 min^{-1} and 19 $min^{-1}\cdot min^{-1}$, respectively. To compare the performance of H-Ala-**3f** as probe, Ala-**MCA**, a commonly used fluorescent probe for aminopeptidase, was employed in the same reaction to give K_{cat}/K_m of 27 $min^{-1}\cdot min^{-1}$. The result obtained by Ala-**MCA** was found to be comparable to that of H-Ala-**3f**, suggesting that H-Ala-**3f** could be a useful probe for APN based on the newly prepared fluorophore **3f**.



H-Ala-**3f** **3f**

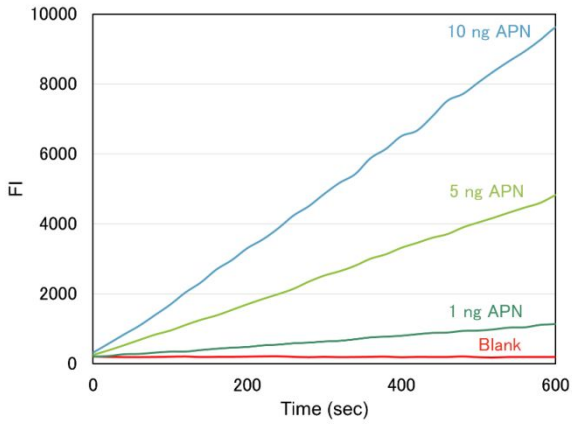


Figure 4. Response of H-Ala-3f against enzyme reaction of APN.

CONCLUSION

In conclusion, we found that 2-TFPE-aniline **3a**, a structurally simple and small compound, showed emissive properties with a fluorescence quantum yield (Φ_f) of 0.77. Further derivatization of **3a** afforded **3f** that showed high Φ_f even in an aqueous solution. We are continuing to explore the reason of 3f to show excellent fluorescence even in an aqueous solution. The utility of **3f** was confirmed by using the peptide derivative H-Ala-**3f** as a fluorescent probe for APN. Even in the low concentration of APN, the enzymatic reaction was monitored clearly by tracing the fluorescence resulting from **3f,** released by the reaction

progress. Further application of **3f** to fluorescent probes for enzymes with severe substrate specificity is under investigation.

EXPERIMENTAL SECTION

Measurement. All experiments were carried out under an argon atmosphere in flame-dried glassware using standard inert techniques for introducing reagents and solvents, unless otherwise noted. *N,N*-Dimethylformamide (DMF) was distilled over calcium hydride and stored in a bottle with activated molecular sieves (4Å). All commercially available materials were used as received without further purification. ^{1}H NMR, ^{13}C NMR and ^{19}F NMR spectra were measured on a JEOL ECZS 400S (^{1}H: 400MHz, ^{13}C: 100MHz, ^{19}F: 376 MHz). Chemical shifts of ^{1}H NMR and ^{13}C NMR are reported in parts per million from tetramethylsilane (TMS), used as an internal standard at 0 ppm. Chemical shifts of ^{19}F NMR are reported in parts per million from tricholofluoromethane ($CFCl_3$), used as an internal standard at 0 ppm. All dates are reported as follows: chemical shifts, relative integration value, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet), and coupling constants (Hz). High-resolution mass spectroscopy (HRMS) experiments were performed with a double-focusing mass spectrometer with EI. Melting points were measured on Yanaco melting point apparatus MP-500V without correction. Fluorescence life times were measured using HAMAMATSU Quantaurus-Tau compact fluorescence life time spectrometer

C11367-01. Fluorescence intensity of **3f** corresponding to APN activity was measured using Multi plate reader SH-9000 (Corona electric, Japan).

Typical procedure for Hiyama cross-coupling reaction to obtain 3

In a glovebox purged with argon gas, iodoaniline **1** (219 mg, 1.0 mmol), (2-methylallyl)palladium(II) (39 mg, 0.1mmol), CuF_2 (204 mg, 2.0 mmol), and 2,2'-bipyridyl (312 mg, 2.0 mmol) were placed in a flask. To the flask were added anhydrous DMF (6.0 mL) and **2** (336 mg, 2.0 mmol), and the mixture was stirred at 80 °C using a silicon oil bath. After the reaction mixture was stirred for 4 h, it was poured into ice water. The mixture was extracted with CH_2Cl_2, and the organic layer was dried over anhydrous $MgSO_4$. After filtration of solids, the solvent was removed in vacuo, and the residue was purified by silica gel column chromatography to give product **3**.

(*E*)-2-(3,3,3-Trifluoroprop-1-enyl)aniline (3a)

The title product was purified by column chromatography and was obtained in 68% yield (127 mg). A colorless solid; mp 48-49 °C (recrystallized from AcOEt and hexane); ^{1}H-NMR ($CDCl_3$) δ: 3.81 (2H, br s), 6.13 (1H, dq, *J* = 16.0, 6.4 Hz), 6.72 (1H, dd, *J* = 8.0, 0.8 Hz),

6.84-6.76 (1H, m), 7.21-7.14 (1H, m), 7.25 (1H, dq, *J* = 16.0, 2.0 Hz), 7.29 (1H, dd, *J* = 7.6, 1.2 Hz); $^{13}C\{^{1}H\}$ NMR ($CDCl_3$) δ: 116.6 (q, *J* = 33.4 Hz), 116.8, 119.2, 119.4, 123.6 (q, *J* = 267.4 Hz), 127.9, 130.9, 133.3 (q, *J* = 6.7 Hz), 144.8; ^{19}F-NMR ($CDCl_3$) δ: -63.03 (3F, dd, *J* = 6.4, 1.9 Hz); MS m/z 187 (M^+), 148, 118, 91; HRMS calcd for $C_9H_8F_3N$ 187.0609 (M^+), found 187.0603.

(*E*)-4-(3,3,3-Trifluoroprop-1-enyl)aniline (3b)

The title product was purified by column chromatography and was obtained in 54% yield (101 mg). A colorless solid; mp 53-54 °C (recrystallized from AcOEt and hexane); ^{1}H-NMR ($CDCl_3$) δ: 3.88 (2H, br s), 5.98 (1H, dq, *J* = 16.0, 6.8 Hz), 6.66 (2H, d, *J* = 8.4 Hz), 7.02 (1H, dq, *J* = 2.0, 16.0 Hz), 7.26 (2H, d, *J* = 8.4 Hz); $^{13}C\{^{1}H\}$ NMR ($CDCl_3$) δ: 111.52 (q, *J* = 33.5 Hz), 114.90, 123.65, 124.21 (q, *J* = 266.4 Hz), 129.08, 137.42 (q, *J* = 6.7 Hz), 148.19; ^{19}F-NMR ($CDCl_3$) δ: -62.34 (3F, dd, *J* = 6.4, 2.3 Hz); MS m/z 187 (M^+), 168, 118; HRMS calcd for $C_9H_8F_3N$ 187.0609 (M^+), found 187.0603.

(*E*)-4-Amino-3-(3,3,3-trifluoroprop-1-enyl)benzonitrile (3c)

The title product was purified by column chromatography and was obtained in 77% yield (163 mg). A colorless solid; mp 105-106 °C (recrystallized from AcOEt and hexane); ^{1}H-

NMR ($CDCl_3$) δ: 4.36 (2H, br s), 6.17 (1H, dq, *J* = 16.0, 6.4 Hz), 6.73 (1H, d, *J* = 8.0 Hz), 7.13 (1H, dq, *J* = 16.0, 2.0 Hz), 7.42 (1H, dd, *J* = 8.8, 2.0 Hz), 7.55 (1H, d, *J* = 2.0 Hz); $^{13}C\{^1H\}$ NMR ($CDCl_3$) δ: 101.4, 116.3, 119.1, 119.2, 119.4 (q, *J* = 33.6 Hz), 123.0 (q, *J* = 267.8 Hz), 131.5 (q, *J* = 6.7 Hz), 132.4, 134.2, 148.3; ^{19}F-NMR ($CDCl_3$) δ: -63.56 (3F, dd, *J* = 6.4, 1.9 Hz); MS *m/z* 212 (M^+), 173, 143, 116; HRMS calcd for $C_{10}H_7F_3N_2$ 212.0561 (M^+), found 212.0556.

(*E*)-5-Methyl-2-(3,3,3-trifluoroprop-1-enyl)aniline (3d)

The title product was purified by column chromatography and was obtained in 42% yield (84 mg). A colorless solid; mp 55-56 °C (recrystallized from AcOEt and hexane); ^1H-NMR ($CDCl_3$) δ: 2.27 (3H, s), 3.77 (2H, br s), 6.08 (1H, dq, *J* = 16.0, 6.4 Hz), 6.54 (1H, s), 6.62 (1H, d, *J* = 8.0 Hz), 7.19 (1H, d, *J* = 8.0 Hz), 7.21 (1H, dq, *J* = 16.0, 2.0 Hz); $^{13}C\{^1H\}$ NMR ($CDCl_3$) δ: 21.4, 115.4 (q, *J* = 32.9 Hz), 116.7, 117.4, 120.4, 123.8 (q, *J* = 267.4 Hz), 127.9, 133.2 (q, *J* = 6.7 Hz), 141.4, 144.8; ^{19}F-NMR ($CDCl_3$) δ: -62.81 (3F, dd, *J* = 6.8, 2.3 Hz); MS *m/z* 201 (M^+), 180, 162, 132; HRMS calcd for $C_{10}H_{10}F_3N$ 201.0765 (M^+), found 201.0768.

(*E*)-5-Methoxy-2-(3,3,3-trifluoroprop-1-enyl)aniline (3e)

The title product was purified by column chromatography and was obtained in 44% yield (96 mg). A colorless solid; mp 49-50°C (recrystallized from AcOEt and hexane); ^{1}H-NMR ($CDCl_3$) δ: 3.78 (3H, s), 3.85 (2H, br s), 6.00 (1H, dq, *J* = 16.0, 6.4 Hz), 6.23 (1H, d, *J* = 2.4 Hz), 6.38 (1H, dd, *J* = 8.8, 2.4 Hz), 7.16 (1H, dq, *J* = 16.0, 2.4 Hz), 7.23 (1H, d, *J* = 8.0 Hz); ^{13}C{^{1}H} NMR ($CDCl_3$) δ: 55.2, 101.5, 105.8, 112.5, 114.0 (q, *J* = 33.3 Hz), 124.0 (q, *J* = 267.1 Hz), 129.4, 132.8 (q, *J* = 6.7 Hz), 146.4, 162.0; ^{19}F-NMR ($CDCl_3$) δ: -62.52 (3F, dd, *J* = 6.8, 1.9 Hz); MS *m/z* 217 (M^+), 178, 148; HRMS calcd for $C_{10}H_{10}F_3NO$ 217.0714 (M^+), found 217.0716.

(*E*)-3-Amino-4-(3,3,3-trifluoroprop-1-enyl)benzonitrile (3f)

The title product was purified by column chromatography and was obtained in 63% yield (134 mg). A colorless solid; mp 144-145 °C (recrystallized from AcOEt and hexane); ^{1}H-NMR ($CDCl_3$) δ: 4.03 (2H, br s), 6.22 (1H, dq, *J* = 16.0, 6.4 Hz), 6.98 (1H, d, *J* = 1.6 Hz), 7.05 (1H, dd, *J* = 8.0, 1.6 Hz), 7.20 (1H, dq, *J* = 16.0, 2.0 Hz), 7.35 (1H, d, *J* = 8.0 Hz); ^{13}C{^{1}H} NMR ($CDCl_3$) δ: 114.0, 118.5, 119.4, 119.9 (q, *J* = 33.6 Hz), 122.2, 122.5 (q, *J* = 268.3 Hz), 123.4, 128.7, 131.9 (q, *J* = 6.7 Hz), 145.0; ^{19}F-NMR ($CDCl_3$) δ: -63.62 (3F, dd, *J*

= 6.4, 2.3 Hz); MS *m/z* 212 (M^+), 173, 143, 116; HRMS calcd for $C_{10}H_7F_3N_2$ 212.0561 (M^+), found 212.0564.

(*E*)-Methyl 3-amino-4-(3,3,3-trifluoroprop-1-enyl)benzoate (3g)

The title product was purified by column chromatography and was obtained in 65% yield (159 mg). A colorless solid; mp 93-94 °C (recrystallized from AcOEt and hexane); ^{1}H-NMR ($CDCl_3$) δ: 3.91 (3H, s), 3.93 (2H, br s), 6.22 (1H, dq, *J* = 16.0, 6.4 Hz), 7.20-7.28 (1H, m), 7.34 (1H, d, *J* = 8.0 Hz), 7.40 (1H, d, *J* = 1.6 Hz), 7.44 (1H, dd, *J* = 8.0, 1.6 Hz); ^{13}C{^{1}H} NMR ($CDCl_3$) δ: 55.2, 101.5, 105.8, 112.5, 114.0 (q, *J* = 33.2 Hz), 124.0 (q, *J* = 267.4 Hz), 128.4, 129.4, 132.8 (q, *J* = 6.7 Hz), 146.4, 162.0; ^{19}F-NMR ($CDCl_3$) δ: -63.40 (3F, dd, *J* = 6.4, 2.3 Hz); MS *m/z* 245 (M^+), 214, 186, 117; HRMS calcd for $C_{11}H_{10}F_3NO_2$ 245.0664 (M^+), found 245.0662.

H-Ala-3f

To the solution of (*S*)-2-(1,3-dioxoisoindolin-2-yl)propanoic acid (219 mg, 1.0 mmol) in CH_2Cl_2 (5.0 mL) was added oxalyl dichloride (254 mg, 2.0 mmol) and one drop of DMF under the temperature of 0 °C. After stirring the mixture for 2 h at room temperature, the

solvent was removed under vacuum and the residue was dissolved with anhydrous 1,4-dioxane. To the solution was added **3f** (148 mg, 0.7 mmol) and pyridine (119 mg, 1.5 mmol) and then the mixture was stirred for 2 h at room temperature. After quenching the reaction by adding water, the mixture was extracted with chloroform, and the organic layer was dried over anhydrous $MgSO_4$. After filtration of solids, the solvent was removed in vacuo, and the residue was roughly separated by silica gel column chromatography to obtain *N*-protected Ala-**3f** fraction. After removal of the solvent of the fraction, *N*-protected Ala-**3f** clot was dissolved in ethanol (6.0 mL) and hydrazine hydrate (100 mg, 2.0 mmol) was added to the solution. After stirring at room temperature for 5 h, solid was removed by filtration and the filtrate was evaporated under vacuum, and the residue then purified by silica gel column chromatography to obtain H-Ala-**3f** in 16% (36 mg). A white solid; mp 159-162 °C; ^{1}H-NMR ($CDCl_3$) δ: 1.48 (3H, d, *J* = 7.2 Hz), 1.72 (2H, br s), 3.69 (1H, q, *J* = 7.2 Hz), 6.27 (1H, dq, *J* = 16.0, 6.0 Hz), 7.23-7.33 (1H, m), 7.43 (1H, dd, *J* = 8.0, 1.2 Hz), 7.51 (1H, d, *J* = 8.0 Hz), 8.54 (1H, d, *J* = 1.2 Hz), 10.08 (1H, br S); ^{13}C{^{1}H} NMR ($CDCl_3$) δ: 21.4, 51.2, 114.1, 118.1, 122.0 (q, *J* = 34.2 Hz), 122.6 (q, *J* = 268.3 Hz), 125.5, 127.8, 128.1, 129.0, 131.5 (q, *J* = 6.7

Hz), 136.4, 174.1; ^{19}F-NMR ($CDCl_3$) δ: -64.10 (3F, dd, *J* = 6.4, 1.9 Hz); MS *m/z* 283 (M^+), 212; HRMS calcd for $C_{13}H_{12}F_3N_3O$ 283.0932 (M^+), found 283.0930.

Enzyme reaction of H-Ala-3f with APN

Aminopeptidase N was prepared from the *Escherichia coli* XL1-Blue containing pAN14 as described previously.[12] The enzyme activity was assayed using H-Ala-**3f** as a substrate following the procedure already described.[7] The reaction mixture (total 100 μL) contained 0.1 M Tris-HCl (pH 7.0), 10-200 μM **3f**, and 0.1-10 ng of the enzyme. The reaction was initiated by the addition of the enzyme solution. Following incubation at 37 ˚C for 5 min in a 96-well plate inside a multi plate reader, the amount of **3f** liberated was determined fluorometrically. The excitation and emission wavelengths used were 340 nm and 450 nm for **3f**, respectively. K_m and k_{cat} values were calculated using Lineweaver-Burk plots.

SUPPORTING INFORMATION

Supporting information is available free of charge on the ACS Publications website, including computational data and NMR spectra for the products.

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ACKNOWLEDGMENT

This work was supported by JSPS KAKENHI grant numbers 19K05740.

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