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Synthesis and properties of novel base-discriminating fluorescent (BDF) nucleosides: a highly polarity-sensitive fluorophore for SNP typing

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Abstract—We have developed novel alkanoylpyrene-labeled BDF nucleosides, ^{AMPy}U and ^{MPy}U. These nucleosides exhibit strong fluorescence emission at long wavelength that is highly sensitive to solvent polarity. BDF probes containing ^{AMPy}U selectively emit fluorescence only when the base opposite BDF nucleoside is adenine and act as effective reporter probes for homogeneous SNP typing.

Fluorescent molecules, whose emission spectra and quantum yields are markedly sensitive to solvent polarity, are widely used as reporter probes for investigating chemical, biochemical, and biological phenomena.[1] 1-Pyrenoyl derivatives are one of such attractive fluorophores since they possess favorable photochemical properties, such as high stability, high quantum yield, and high sensitivity to solvent polarity.[2] Oligonucleotides (ODN) possessing such fluorescent molecules can be used for detecting the change in the DNA microenvironment, such as that which occurs in hybridization and conformational change, and can be used for single nucleotide polymorphisms (SNPs) typing as well.

In our continuous efforts to develop base-discriminating fluorescent (BDF) nucleosides, we devised a new homogeneous assay method that provides a clear distinction of the base on the complementary strand by fluorescence change.[3] Particularly, we have recently reported novel pyrenecarboxamide-labeled BDF nucleosides which are sensitive to solvent polarity, and indicated that the BDF probes selectively emit fluorescence only when the complementary bases are perfectly matched bases.[4] However, for easy and clearer detection of the fluorescence emission, there is still great demand for further improvement of a fluorescent probe possessing long wavelength emission. Therefore, we report herein novel BDF probes containing highly polarity-sensitive fluorescent nucleosides, 1-pyrenoyl-labeled 2′-deoxyuridine ^{AMPy}U **1**, for discrimination of adenine base on a target DNA at longer wavelength. We also synthesized other polarity-sensitive fluorescent nucleoside ^{MPy}U **2** to evaluate the effect of rigid acetylene linker on the discrimination of the targeted bases opposite BDF nucleosides.

The synthesis of a novel BDF nucleoside ^{AMPy}U **1** is outlined in Scheme 1. Pyrenecarbonyl derivative prepared from pyrene and 4-pentinoic acid was coupled with 5-iodo-2′-deoxyuridine **5** under Sonogashira conditions using $Pd(PPh_3)_4$ to afford **1**.[5] Protection of the deoxyribose 5′-hydroxy group with the 4,4′-dimethoxytrityl group afforded **6**. After conversion to phosphoramidite, **7** was incorporated into oligonucleotides by an automated DNA synthesis (Fig. 1).

The synthesis of ^{MPy}U **2** has been accomplished according to Scheme 2. Pyrenylpropyn **9** which was prepared according to the protocol of Shi Shun et al.[6] was coupled with 3′,5′-diprotected 5-iodo-2′-deoxyuridine **8** by $Pd(PPh_3)_4$ to afford **10**. Compound **10** was then hydrogenated over 10% Pd/C in methanol to give **11**, which was subsequently oxidized by MnO_2 to provide 1-pyrenoyl derivative **12**. Treatment of **12** with TBAF

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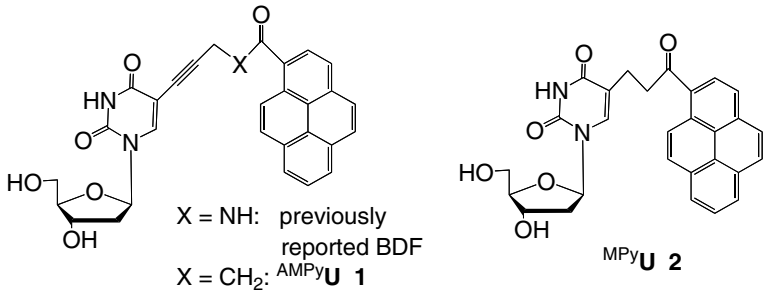
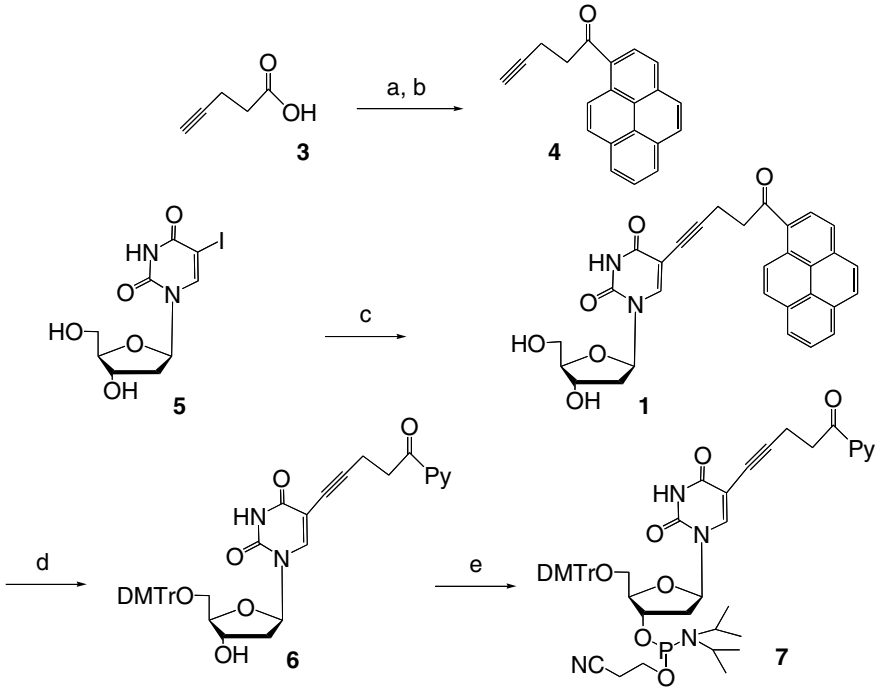


Figure 1. Structure of highly polarity sensitive fluorescent nucleosides, ^{AMPy}U and ^{MPy}U.

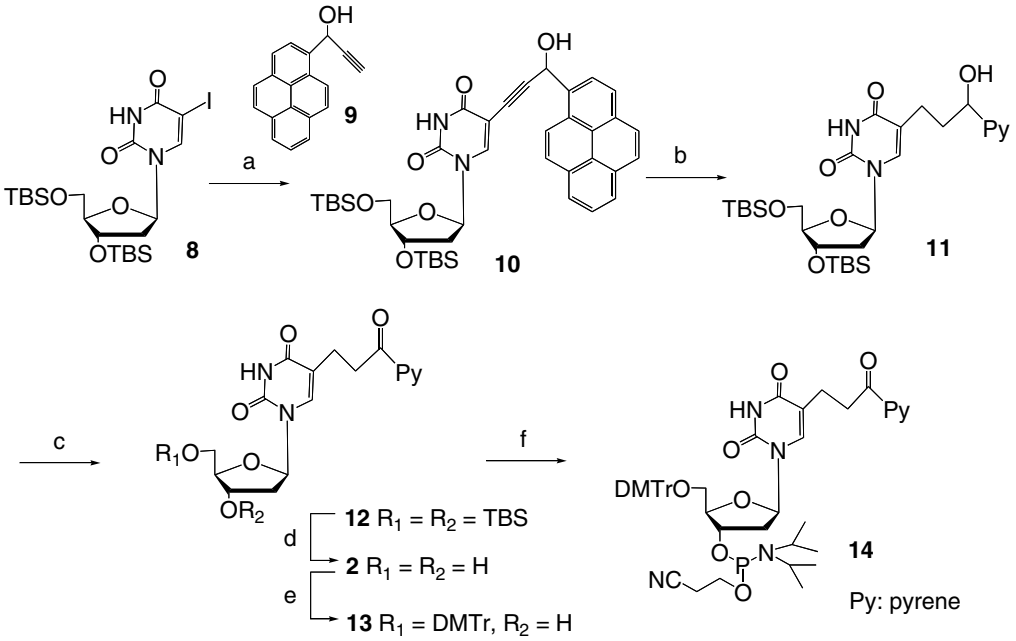


Scheme 1. Reagents and conditions: (a) $SOCl_2$, 100 °C, 2 h; (b) $AlCl_3$, pyrene, CH_2Cl_2, rt, overnight, 39% (in two steps); (c) **4**, $Pd(PPh_3)_4$, CuI, Et_3N, DMF, rt, 6 h, 88%; (d) DMTrCl, pyridine, rt, 8 h, 75%; (e) $(i\text{-}Pr_2N)_2$ $PO(CH_2)_2CN$, 1*H*-tetrazole, CH_3CN, rt, 1 h; DNA synthesis (ODN(^{AMPy}U), 16 %).

provided target nucleoside **2**.[5] By employing a reaction similar to that described for compound **7**, we obtained phosphoramidite **14** from **2**. The newly synthesized ODNs are summarized in Table 1.

There are numerous reports for fluorescence properties of pyrenecarbonyl fluorophore. It is well known that the monomer fluorescence of pyrene-1-carboxaldehyde shows a strong dependency on solvent polarity. The fluorescence in polar solvent is quite high (Φ_F in ethanol = 0.15), although the fluorescence in nonpolar solvents such as *n*-hexane is very weak ($\Phi_F < 0.001$).[2a,b] For pyrene-1-carboxaldehyde, fluorescence is emitted from the n–π^* state in nonpolar solvents. When the solvent polarity increases, the π–π^* state that lies slightly above the n–π^* state is brought below the n–π^* state by solvent relaxation during the lifetime of the excited state, and thus becomes the emitting state. Further, Armbruster and co-workers reported the fluorescence properties of a pyrene carbonyl fluorophore, 1-heptanoylpyrene, in water binary mixture with methanol.[2c] In pure methanol, the fluorescence emission was around 436 nm, but was shifted to 447 nm when the water concentration was increased to 35% of methanol. They also indicated that the fluorescence intensity increases with increasing molar fraction of water. These results suggest that BDF nucleosides containing an alkanoylpyrene fluorophore should be highly polarity-sensitive. Importantly, the wavelength of 1-heptanoylpyrene is about 40–50 nm longer than that reported for previous pyrenecarboxamide fluorophore.[4,7]

Since these reported alkanoylpyrene fluorophores show highly polarity-sensitive fluorescence emission at longer wavelength, we expected that the synthesized alkanoylpyrene-labeled nucleosides ^{AMPy}U and ^{MPy}U would also exhibit polarity-sensitive fluorescence emission. Thus, we initially measured the fluorescence spectra of monomer ^{AMPy}U and ^{MPy}U in solvents of different polarities. With excitation of both ^{AMPy}U and ^{MPy}U at



Scheme 2. Reagents and conditions: (a) $Pd(PPh_3)_4$, CuI, Et_3N, DMF, rt, 6 h, 90%; (b) Pd/C, H_2, dioxane, rt, 17 h, 76%; (c) MnO_2, CH_2Cl_2, frorisil, rt, 7 h, 80%; (d) TBAF, THF, 0 °C, 1 h, 92%; (e) DMTrCl, pyridine, rt, 8 h, 75%; (f) $(i\text{-}Pr_2N)_2PO(CH_2)_2CN$, 1*H*-tetrazole, CH_3CN, rt, 1h; DNA synthesis (ODN(^{MPy}U), 13%).

Table 1. Oligonucleotides (ODNs) used in this study

	Sequences
ODN(^{AMPy}U)	5′-d(CGCAAT^{AMPy}UTAACGC)-3′
ODN(^{MPy}U)	5′-d(CGCAAT^{MPy}UTAACGC)-3′
ODN(^{nat}T)	5′-d(CGCAATTTAACGC)-3′
ODN(N)	5′-d(GCGTTANATTGCG)-3′, **N** = **T**, **C**, **G**, **A**
ODN$_{ALDH2}$(^{AMPy}U)	5′-d(TTTTCACTT^{AMPy}UAGTG-TATGCC)-3′
ODN$_{ALDH2}$(A)	5′-d(GGCATACACTAAAGTGAAAA)-3′
ODN$_{ALDH2}$(G)	5′-d(GGCATACACTGAAGTGAAAA)-3′
ODN$_{bcr/abl}$(^{AMPy}U)	5′-d(TGAAGGGCTT^{AMPy}UTGA-ACTCTG)-3′
ODN$_{bcr/abl}$(A)	5′-d(CAGAGTTCAAAAGCCCTTCA)-3′
ODN$_{abl}$(G)	5′-d(CAGAGTTCAGAAGCCCTTCA)-3′

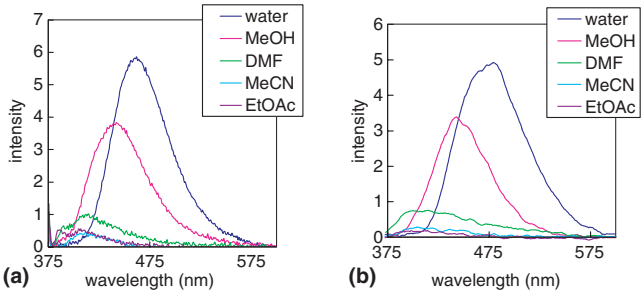


Figure 2. (a) Fluorescence spectra of ^{AMPy}U (10 μM) in water, methanol, DMF, acetonitrile, and ethyl acetate. (b) Fluorescence spectra of ^{MPy}U (10 μM) in water, methanol, DMF, acetonitrile, and ethyl acetate. Excitation wavelength was at 372 nm.

372 nm in water, strong fluorescence emission at 462 nm was observed as shown in Figure 2a and b. Upon excitation of ^{AMPy}U and ^{MPy}U in methanol, we observed medium emission at 443 nm. In contrast, the fluorescence emission of both ^{AMPy}U and ^{MPy}U in less polar solvents like DMF, acetonitrile, and ethyl acetate was very weak. As expected, these alkanoylpyrene-labeled nucleosides showed high sensitivity to the solvent polarity. It is noteworthy that the wavelength of the emitted light is about 70 nm longer than that reported for previous pyrenecarboxamide-labeled BDF nucleosides (397 nm in water).[4]

Such fluorescent nucleosides would be valuable for monitoring the microenvironment of DNA duplex, such as for sensing the difference in polarities between the inside and outside of DNA duplexes. Previously, we reported pyrenecarboxamide-labeled BDF nucleosides which contain a acetylene linker.[4a] When the pyrenecarboxamide fluorophore is attached to uracil at the C-6 position, via a rigid acetylene linker, the fluorophore is extruded to the outside of the groove due to base pairing with adenine into a highly polar aqueous phase and, therefore, strong fluorescence should be observed. In contrast, when the pyrene fluorophore is inside of the duplex due to the lack of base-pairing (mismatched), the BDF base exhibits no emission due to the location of the pyrene fluorophore at a highly hydrophobic site in the groove.[4a] We assumed that these solvent polarity-dependent fluorescence changes of ^{AMPy}U and ^{MPy}U would be useful for the detection of the change in the DNA microenvironment, such as SNP typing. Therefore, the measurement of the fluorescence spectra of 2.5 μM of ^{AMPy}U-, ^{MPy}U-containing duplex in sodium phosphate buffer (pH 7.0) was performed. The fluorescence spectra of ^{AMPy}U-, ^{MPy}U-containing ODNs and the duplexes containing different bases opposite BDF nucleosides were measured with excitation at

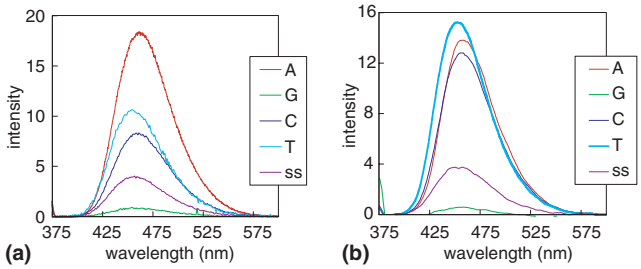


Figure 3. (a) Fluorescence spectra of **ODN**(^{AMPy}**U**) hybridized with 2.5 μM **ODN(A)**, **ODN(G)**, **ODN(C)**, or **ODN(T)** and single-stranded **ODN**(^{AMPy}**U**). (b) Fluorescence spectra of **ODN**(^{MPy}**U**) hybridized with 2.5 μM **ODN(A)**, **ODN(G)**, **ODN(C)**, or **ODN(T)** and single-stranded **ODN**(^{MPy}**U**) (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature). Excitation wavelength was at 374 nm.

374 nm. In the case of ^{AMPy}**U**-containing ODN, the fluorescence spectrum of the perfectly matched duplex [**ODN**(^{AMPy}**U**)/**ODN(A)**] showed strong fluorescence emission at 462 nm (quantum yield $\Phi_F = 0.351$).[7] In contrast, the fluorescence of the mismatched duplexes (**ODN**(^{AMPy}**U**)/ODN(**N**), **N** = C, G, T) and single-stranded ODN[**ODN**(^{AMPy}**U**)] showed weak emission ($\Phi_F = 0.177$, 0.003, 0.235, and 0.063, respectively, Fig. 3a). Although ^{AMPy}**U**-containing ODN indicated A-selective fluorescence emission, no selectivity was observed in ^{MPy}**U**-containing ODN which lack rigid acetylene linker. The fluorescence quantum yields of duplexes (**ODN**(^{MPy}**U**)/**ODN**(**N**), **N** = C, G, T, A) and single-stranded ODN[**ODN**(^{MPy}**U**)] were 0.130, 0.057, 0.144, 0.145, and 0.038, respectively (Fig. 3b). These results indicate that a long and rigid acetylene linker is indispensable to induce clear change of the microenvironment around pyrenoyl fluorophore.

In melting temperature measurements of the duplex, a high duplex stability was observed for ^{AMPy}**U**- and ^{MPy}**U**-containing duplexes. As shown in Table 2, the melting temperatures of **ODN**(^{AMPy}**U**)/**ODN(A)** and **ODN**(^{MPy}**U**)/**ODN(A)** were considerably higher than that observed for other mismatched duplexes in sodium phosphate buffer (pH 7.0), suggesting that both ^{AMPy}**U** and ^{MPy}**U** form a stable base pair with A.

The clear change in the fluorescence of ^{AMPy}**U**-containing ODN by the type of the base on the complementary strand would be very useful for SNP typing and gene sequence detection. First, we tested the SNP detection

Table 2. T_m measurement of duplex containing ^{AMPy}**U** or ^{MPy}**U** [a,b]

5'-CGC AAT **X** TAA CGC-3'
3'-GCG TTA **N** ATT GCG-5' X = ^{AMPy}**U** or ^{MPy}**U**

Entry	N	^{AMPy}U, T_m (°C)	^{MPy}U, T_m (°C)
1	A	54.4	59.9
2	G	48.5	53.8
3	C	47.9	50.7
4	T	52.3	48.1

[a] 2.5 μm DNA in 50 mM Na phosphate, 100 mM NaCl, pH 7.0.
[b] T_m for ODN(^{nat}T)/ODN(A) = 56.1 °C.

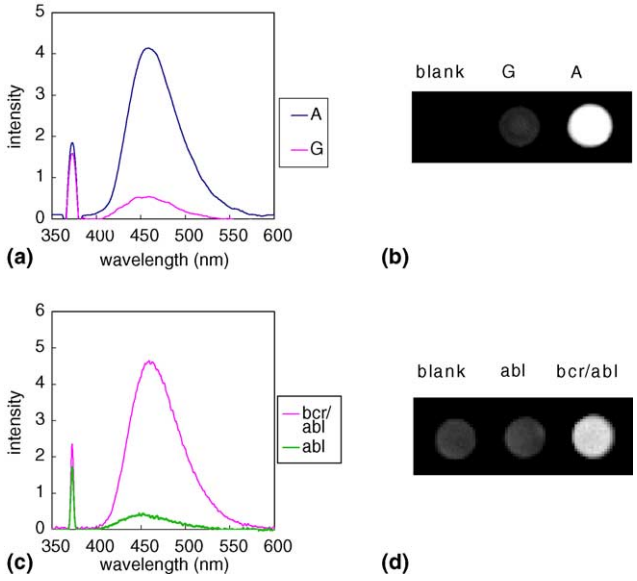


Figure 4. (a) Fluorescence spectra of 2.5 μM **ODN**$_{ALDH2}$(^{AMPy}**U**) hybridized with 2.5 μM **ODN**$_{ALDH2}$**(A)** or **ODN**$_{ALDH2}$(G) (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature). (b) Detection of fluorescence image. A volume of 2.5 μM **ODN**$_{ALDH2}$**(A)** or **ODN**$_{ALDH2}$**(G)** was hybridized with 2.5 μM **ODN**$_{ALDH2}$(^{AMPy}**U**). Fluorescence was observed with a fluorescence imager, Versa Doc Imaging System (BioRad), equipped with a 290–365 nm transilluminator. The image was taken through a 380 nm long pass emission filter. (c) Fluorescence spectra of 2.5 μM **ODN**$_{bcr/abl}$ (^{AMPy}**U**) hybridized with 2.5 μM **ODN**$_{bcr/abl}$**(A)** or **ODN**$_{abl}$**(G)**. (d) Detection of fluorescence image. A volume of 2.5 μM **ODN**$_{bcr/abl}$**(A)** or **ODN**$_{abl}$**(G)** was hybridized with 2.5 μM **ODN**$_{bcr/abl}$(^{AMPy}**U**).

of the A/G (A-allele/G-allele) SNP sequence of alcohol dehydrogenase 2 (ALDH2)[8] by using ^{AMPy}**U**-containing BDF probe. We added BDF probe **ODN**$_{ALDH2}$(^{AMPy}**U**) to a solution of the target sequence, **ODN**$_{ALDH2}$**(A)** (A-allele) and **ODN**$_{ALDH2}$**(G)** (G-allele), and incubated these solutions at room temperature for 1 min. The sample solutions were then illuminated at 360 nm, and the fluorescence images were taken through a 380 nm cutoff filter. A strong fluorescence emission was observed for the **ODN**$_{ALDH2}$(^{AMPy}**U**)/**ODN**$_{ALDH2}$**(A)** duplex, whereas the mismatched duplex [**ODN**$_{ALDH2}$ (^{AMPy}**U**)/**ODN**$_{ALDH2}$**(G)**] showed considerable fluorescence quenching (Fig. 4a and b). The fluorescence quantum yield of the matched duplex (^{AMPy}**U**/A $\Phi_F = 0.110$) was approximately 10 times stronger than that observed for the mismatched one (^{AMPy}**U**/G, $\Phi_F = 0.012$).

Next, we examined the detection of *bcr/abl* cancer gene sequence.[9] As a result of the hybridization of BDF probe with **ODN**$_{bcr/abl}$**(A)** (cancer gene sequence), a strong emission was obtained for the addition of **ODN**$_{bcr/abl}$(^{AMPy}**U**), whereas the emission from the **ODN**$_{bcr/abl}$(^{AMPy}**U**)/**ODN**$_{abl}$**(G)** (normal sequence) duplex was negligible. This ^{AMPy}**U**-containing BDF probe facilitates the distinction of adenosine on a target DNA by a drastic fluorescence change at longer wavelength than that for previous pyrenecarboxamide-labeled BDF.[4] The fluorescence quantum yield of the ^{AMPy}**U**-containing BDF probe is also enhanced as compared with that of reported BDF probes, benzopyridopyrimidine

(BPP, $\Phi = 0.035$)[3a] and naphthopyridopyrimidine (NPP, $\Phi = 0.096$).[3d] These fluorescence properties of ^{AMPy}U are favorable for the application as an SNP typing probe. However, when BDF probes containing the -C^{AMPy}UC- sequence were used, the fluorescence emission was strongly quenched by the flanking C/G base pairs. This indicates that there are some limitations for the sequence to use in this method using ^{AMPy}U-containing BDF probe.

In conclusion, we have developed novel alkanoylpyrene-labeled BDF nucleosides, ^{AMPy}U and ^{MPy}U. These nucleosides show a strong fluorescence dependency on solvent polarity at long wavelength. BDF probes containing ^{AMPy}U selectively emit fluorescence only when the base opposite BDF base is adenine. The homogeneous SNP typing method using ^{AMPy}U-containing BDF probes is a powerful alternative to conventional SNP typing as well as gene detection.

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5. Spectroscopic data for selected compounds are provided. Compound **1**: ^{1}H NMR (pyridine-d_5, 400 MHz) δ 2.61–2.75 (m, 2H), 2.97 (t, 2H, $J = 7.6$ Hz), 3.49 (t, 2H, $J = 7.6$ Hz), 4.11 (dd, 1H, $J = 2.4$, 11.6 Hz), 4.21 (dd, 1H, $J = 2.8$, 11.6 Hz), 4.48 (ddd, 1H, $J = 2.4$, 2.8, 3.4 Hz), 5.02 (m, 1H), 6.93 (dd, 1H, $J = 6.0$, 6.4 Hz), 8.00–8.40 (m, 9H), 9.23 (d, 1H, $J = 9.6$ Hz); ^{13}C NMR (pyridine-d_5, 100 MHz) δ 14.7, 40.9, 41.2, 61.3, 70.6, 73.8, 85.4, 88.5, 92.1, 99.9, 124.0, 124.1, 124.6, 124.8, 125.8, 126.1, 126.3, 126.4, 126.9, 128.9, 129.2, 129.2, 130.3, 130.8, 131.8, 133.4, 143.0, 150.3, 162.6, 201.7; FABMS (Glycerol/CH_3OH), *m/z* 509 ($[M+H]^+$), HRMS calcd for $C_{30}H_{25}O_6N_2$ ($[M+H]^+$) 509.1712, found 509.1709. Compound **2**: ^{1}H NMR (pyridine-d_5, 400 MHz) δ 2.67–2.71 (m, 2H), 3.11 (t, 2H, $J = 7.6$ Hz), 3.60 (t, 2H, $J = 7.6$ Hz), 4.16 (dd, 1H, $J = 2.4$, 11.6 Hz), 4.25 (dd, 1H, $J = 2.4$, 11.6 Hz), 4.49 (ddd, 1H, $J = 2.4$, 3.2, 3.4 Hz), 5.06 (m, 1H), 7.06 (dd, 1H, $J = 6.4$, 6.8 Hz), 8.01–8.50 (m, 9H), 9.22 (d, 1H, $J = 9.2$ Hz); ^{13}C NMR (pyridine-d_5, 100 MHz) δ 23.3, 41.3, 41.6, 62.3, 71.4, 85.5, 89.0, 113.6, 124.7, 124.7, 125.3, 125.6, 126.4, 126.7, 126.9, 127.1, 127.6, 129.6, 129.7, 129.8, 130.9, 131.5, 132.9, 134.0, 137.6, 151.8, 164.8, 203.7; FABMS (DTT/CH_3OH), *m/z* 485 ($[M+H]^+$), HRMS calcd for $C_{28}H_{25}O_6N_2$ ($[M+H]^+$) 485.1717, found 485.1713.
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