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# Photocleavable Molecule for Laser Desorption Ionization Mass Spectrometry†

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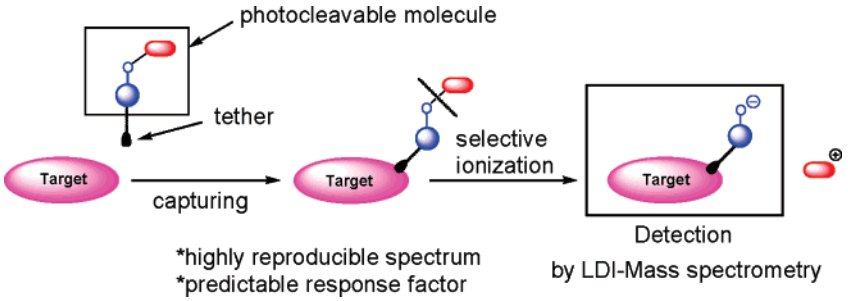


A new photocleavable molecule for laser desorption ionization mass spectrometry (LDI-MS) was designed and synthesized. The molecule exhibited high sensitivity for negative mode MS detection with good chemical stability. The molecule was successfully applied to molecular tag for (LDI-MS). Kinetic measurement of the amidation reaction and monitoring of aminolysis of acetylated sugars were demonstrated with the molecular tag.

## Introduction

Mass spectrometry is a well recognized highly sensitive characterization method for organic molecules. In particular, matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) has been successfully applied for high throughput screening for bioactive compounds and reaction conditions with minimum consumption of substrates.[1] These successes unambiguously demonstrate further potential of the MALDI-MS analysis at many stages of research. To expand its utility as a high throughput chemical and biological screening method, key technologies to be improved would be (a) variation of the response factor, (b) selectivity for the target molecule, and (c) measurement in the low mass region. As the MALDI process is known to pass through a complicated ionization mechanism in which energy and charge of irradiated matrix transfer to the target molecule,[2] the balance of signal intensity is quite sensitive to sampling conditions. Although some sophisticated techniques were presented to solve the problem with well-controlled surfaces,[3] more convenient devices would be helpful. One of the promising tools to improve sensitivity and selectivity in MALDI-MS is a molecular tag for the target molecule to assist ionization under standard MALDI conditions.[4] However, so far reported methods still require the assistance of matrix for desorption processes.

† Dedicated to the memory of Professor Dr. Yoshihiro Matsumura. Deceased on April 14, 2007.

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§ Graduate School of Biomedical Science.

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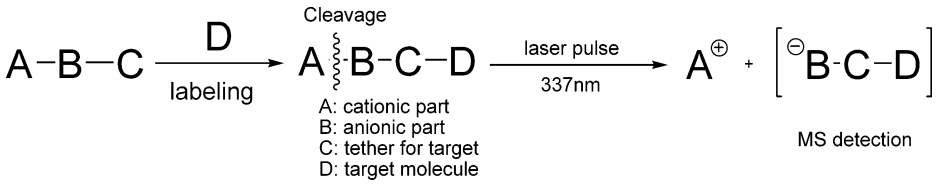
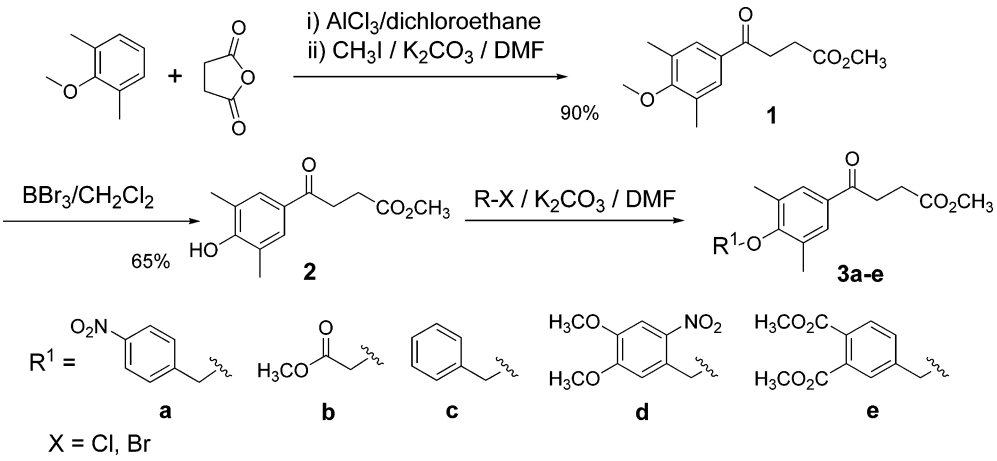
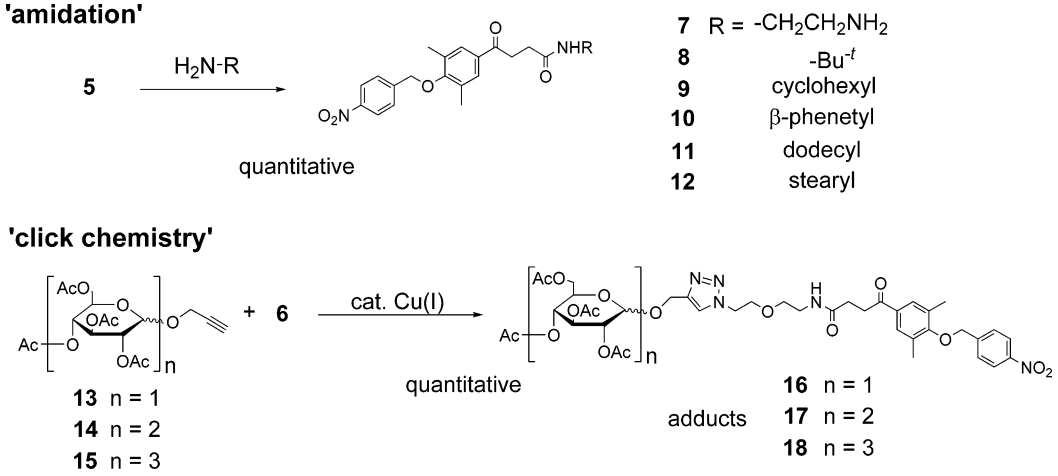
**SCHEME 1. Basic Concept of Photocleavable Molecular Tag for LDI-MS**




**SCHEME 2. Concise Synthesis of Various Photocleavable Molecules**




**SCHEME 3. Quantitative Tagging via "Amidation" and "Click Chemistry" with Derivatives 5 and 6**




We envisaged that a photocleavable molecule, which affords an MS detectable ion upon irradiation without matrix assistance, would greatly simplify the ionization mechanism and be a reliable and selective labeling device for laser desorption ionization mass spectrometry (LDI-MS). Herein we report a novel photocleavable molecular tag for LDI-MS to achieve a predictable response factor with high selectivity in the low mass region.

## Results and Discussion

**Concept.** The concept of a photocleavable molecular tag is depicted in Scheme 1. The device molecule is composed of A, B, and C parts. Bond A−B is photocleavable and undergoes heterolysis affording cationic $A^+$ and anionic $B^-$ fragments upon irradiation. The B part bears a C part, which is a tether to capture a target molecule. Thus, anionic fragment $[B-C]^-$ generated by irradiation of the molecular tag is detectable with negative mode mass spectrometry. When the tether C captures a target molecule via an addition reaction, one can observe *m*/*z* as the sum of molecular masses of target D and B−C (Scheme 1).

Negative mode MS detection would be preferable owing to the less fragmentation tendency of anionic species to achieve quantitative analysis. Importantly, a commercial photocleavable molecule was applied for direct mass spectrometric monitoring of solid phase organic syntheses by Waldmann and Gerdes.[5] However, traditional photocleavable molecules were not suitable for our purposes because of chemical lability of the ester linkage. A new photocleavable molecule which is suited for LDI-MS detection, chemically stable, and readily accessible via standard organic synthesis was needed. Under the idea, we planned a synthetic strategy to scrutinize a new photocleavable molecule (Scheme 2).

**Design and Synthesis of a New Photocleavable Molecule.** We chose the phenacyl substructure as a photoreactive B part

(5) Gerdes, J. M.; Waldmann, H. *J. Comb. Chem.* **2003**, *5*, 814−820.

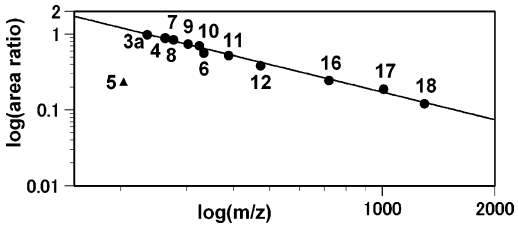


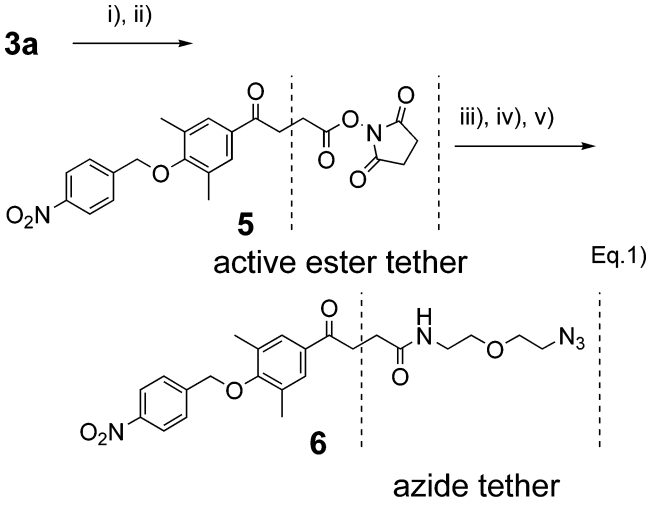

**FIGURE 1.** Dependence of relative response factors on molecular masses. Sensitivity of each compound was expressed as a ratio of signal area to **3a**. The values are average of ten spectra. An empirical equation was obtained by least-square method as $y = 725.04x^{-1.208}$), $R^2 = 0.991$.

(Scheme 1) because of its structural simplicity and synthetic accessibility. Key intermediate **2** was obtained in three steps from 2,6-dimethylanisol in high yield. The intermediate **2** was readily converted to ethereal compounds **3a**−**e** through nucleophilic substitution to afford photocleavable molecules possessing various A parts.

**Sensitivity of Photocleavable Molecule for LDI-MS.** Sensitivity of compounds **1**, **2**, and **3a**−**e** for LDI-MS were examined with a commercial MALDI-TOF spectrometer equipped with a nitrogen laser (337 nm). The results are summarized in Table 1.

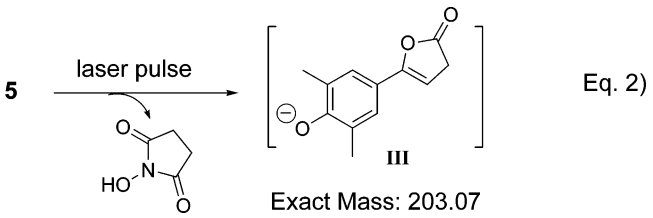
Satisfactory results were obtained with **3a** which afforded a signal at $m/z = 235.10$, corresponding to negatively charged phenolate ion **I** (run 3). Interestingly, variation of the A part was found to strongly impact on the sensitivity for LDI-MS. Relative sensitivities of **1**, **2**, and **3b**−**e** were expressed as a ratio to **3a**. Phenol derivative **2** ($R^1$ = H) showed only 6% signal intensity of **3a** in spite of relatively low O−H bond energy of **1** (run 2). Methyl group as $R^1$ also afforded a poor result (run 1). The electron withdrawing methoxycarbonyl group improved the sensitivity (run 4). It turned out that the benzyl group bearing electron withdrawing substituents such as nitro- and methoxycarbonyl groups effectively enhanced their relative intensities of signals (runs 5 to 7). Regioisomers (*m*- and *o*-nitroisomers) of **3a** afforded comparable results with **3a** in their sensitivity. Less than 6% of standard deviation was observed for **3a**, **3d**, and **3e**. The detection limit was around 20 fmol/spot for **3a**. The characteristic photocleavable ethereal C−O bond, which connects the nitrobenzyl A part and acylphenolate B part, showed good chemical stability under moderate acidic and basic conditions as well as lighting in a laboratory.

**Construction of the Tether Fragment.** The methoxycarbonyl group in **3a** may be used as a tether to connect various molecules of interest. As a highly reliable reaction for ligation, we examined simple amidation or "click chemistry".[6] Compound **3a** was readily converted to active ester **5** or azide **6** (eq 1). Various amide derivatives **7**−**12** were quantitatively obtained by reaction between **5** and amines. While, propargyl glycosides **13**−**15** were quantitatively coupled with **6** to afford **16**−**18** in the presence of copper(I) catalyst (Scheme 3).




**Response Factors of Tagged Molecules.** With a variety of tagged molecules in hand, LDI-MS measurement was carried out for 0.2 $\mu$L of 10 $\mu$M solution. Interestingly, relative response factors of ions generated by irradiation of these tagged molecules were found to depend only on their molecular masses in spite of their structural diversity (Figure 1).

Exceptionally, the active ester **5** decomposed upon laser pulse irradiation to afford a relatively weak signal (Figure 1, $m/z$ = 203) which corresponds to the cyclized ion **III** (eq 2).




Although accumulation of additional data is necessary for a full explanation, correlation between signal area ratio and −1.2 power of molecular mass implies that the initial velocity of ions[7] may mainly control the response factors in this simple LDI-MS. The empirical equation may allow us to estimate relative concentrations even for unknown molecules. This particular feature may be explained by comparison with MALDI-MS as depicted in Figure 2.

In the MALDI ionization process (Figure 2B), molecules of interest may receive energy and charge from the large excess of activated matrix by laser pulse irradiation. Ionization efficiency may largely depend on the structure of the target molecule and sampling state. In the tagged LDI-MS (Figure 2A), ionization may take place by a simpler mechanism. That is, only tiny amounts of tagged molecule (typically tens pmol/well) afford detectable ions quickly and directly upon irradiation if no following reaction of the ion takes place. Nice reproducibility of the spectrum and predictable response intensity of

(6) (a) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004−2021. (b) Rostovstev, V. V.; Green, L. K.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596−2599. (c) Fazio, F.; Bryan, M. C.; Blixt, O.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **2002**, *124*, 14397−14402. (d) Kolb, H. C.; Sharpless, K. B.; *Drug Discovery Today* **2003**, *8*, 1128−1137. (e) Lee, L. V.; Mitchell, M. L.; Huang, S.-J.; Fokin, V. V.; Sharpless, K. B.; Wong, C.-H. *J. Am. Chem. Soc.* **2003**, *125*, 9588−9589. (f) Hotha, S.; Kashyap, S. *J. Org. Chem.* **2006**, *71*, 364−367. (g) Gierlich, J.; Burley, G. A.; Gramlich, P. M. E.; Hammond, D. M.; Carell, T. *Org. Lett.* **2006**, *8*, 3639−3642.

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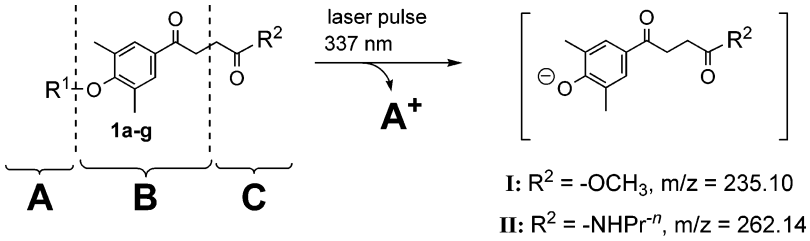
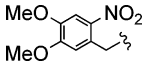
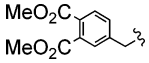
**TABLE 1. Impact of Structural Variation of A Part on Sensitivity of LDI-MS in the Presence of Internal Reference 4**




| run | $R^1$ | $R^2$ | | obsd ion | relative sensitivity[a,b] | SD (%) |
|---|---|---|---|---|---|---|
| 1 | $CH_3$- | -$OCH_3$ | **1** | **I** | 0.07 | 12.9 |
| 2 | H- | -$OCH_3$ | **2** | **I** | 0.06 | 21.7 |
| 3 | *p*-$NO_2$-$PhCH_2$- | -$OCH_3$ | **3a** | **I** | 1.00 | 5.7 |
| 4 | $CH_3O_2CCH_2$- | -$OCH_3$ | **3b** | **I** | 0.21 | 11.3 |
| 5 | $PhCH_2$- | -$OCH_3$ | **3c** | **I** | 0.16 | 12.7 |
| 6 |  | -$OCH_3$ | **3d** | **I** | 0.39 | 4.9 |
| 7 |  | -$OCH_3$ | **3e** | **I** | 1.37 | 2.9 |
| 8 | *p*-$NO_2$-$PhCH_2$- | -NHPr-*n* | **4** | **II** | 0.91 | |

[a] Internal reference of **4** was applied to compare sensitivities between **1**, **2**, and **3a**−**e**. [b] The reference **4** afforded ion **II** at $m/z = 262.14$ and **1**, **2**, and **3a**−**e** afforded ion **I** at $m/z = 235.10$, respectively. [c] Sample was prepared by premixing 10 $\mu$M solution of each substrate.

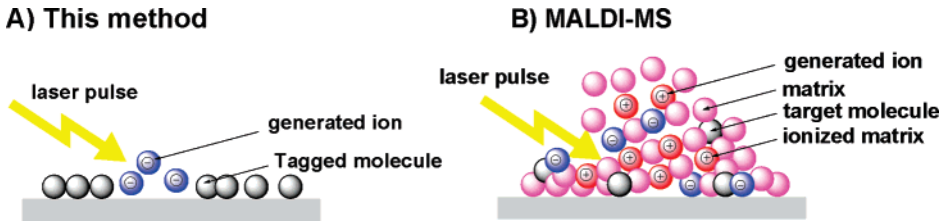



**FIGURE 2.** Schematic comparison of ionization process between tagged LDI-MS (A) and MALDI-MS (B).

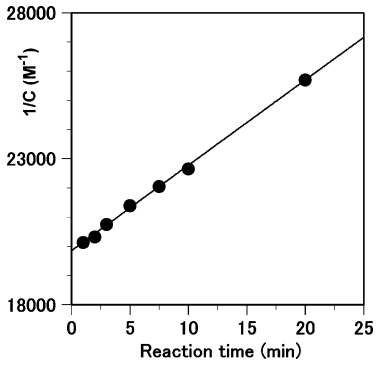



**FIGURE 3.** Analysis of reaction between active ester **5** and $\beta$-phenethylamine with second-order rate law. A linear relation between inverse of concentration and reaction time was obtained: $y = 292.1187x + 19861.52$, $R^2 = 0.998$.

signals may be considered as a result of the simplicity of the ionization mechanism.

**Reaction Monitoring.** The tagged molecule allowed us to monitor the reaction profile. Figure 3 shows the LDI-MS reaction between **5** (50 $\mu$M) and $\beta$-phenethylamine (50 $\mu$M) in acetonitrile (2 mL) at 40 °C to afford amide **10**. LDI-MS measurements were carried out for a series of reaction mixtures which were collected every few minutes and quenched by excess *n*-propylamine. Only two signals corresponding to ions from **10** ($m/z = 324$) and **4** ($m/z = 262$) were observed. After correction of the area ratio of these signals by the empirical equation, which is obtained from Figure 1, a second-order rate constant ($k_2 = 292\ M^{-1}\ s^{-1}$) was obtained on the basis of the time profile of product and material concentration (Figure 3). The hydroxy succinimide generated along with the reaction did not disturb the quantitative mass analysis. Although careful examination should obviously be essential for each system, the impact of the presence of general neutral salts (organic and inorganic) on the quantitative mass analysis seems not to be so significant in this method with the simple ionization mechanism.

Aminolysis of acyl sugar **16, 17**, and **18** was demonstrated with the device. To each 100 $\mu$M of methanol solution of saccharide derivative, 100 $\mu$L, was added 400 $\mu$L of propylamine solution (100 mM) and 10 $\mu$L of dodecylamide **11** (1 mM) as an internal reference. The mixture was monitored by applying 0.2 $\mu$L of the reaction solution on a stainless steel plate every 15 min. Figure 4 shows a typical mass spectrum of partially de-acetylated products from **17**.

Total signal area was consistent to reference **11** (the signal is not shown in Figure 4) during the reaction after correction with the empirical equation obtained from Figure 1 despite significant diversity of the product functionality and polarity. Fully de-acetylated product ($m/z$=713) was observed in 92% (relative response: 0.237) after 15 h. Relative responses of in situ fully deacetylated **16′**, **17′**, and **18′** are shown in Figure 5 (red triangles).

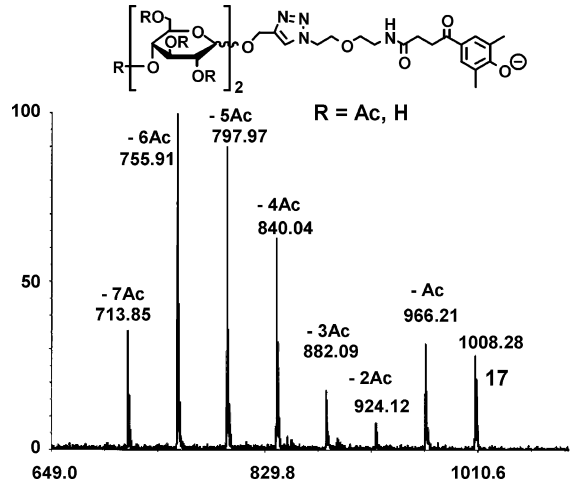



**FIGURE 4.** Mass spectrum of reaction mixture of aminolysis of **17** with excess propylamine after 5 h. The concentration of all components was estimated on the basis of each signal area ratio to internal reference **11** (signal not shown) after correction by response factor at each *m*/*z* value calculated from the empirical equation of Figure 1.

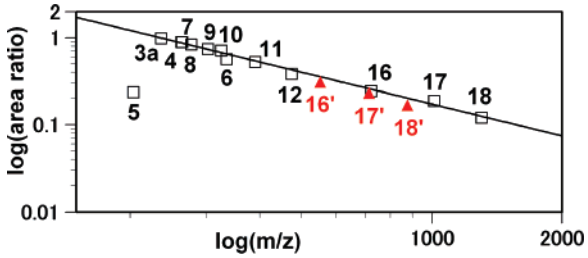



**FIGURE 5.** Consistency of relative response factors of fully deacetylated sugar derivatives **16′**, **17′**, **18′** (red triangles) to the empirical equation from Figure 1. The sensitivity of each compound was expressed as a ratio of signal area to **3a**. The values are the average of 10 spectra.

Although the experiment does not afford information of regioselectivity of the reaction, the results clearly prove the utility of the presented device to analyze multistep reactions without separation and standard substrates.

Generally, reaction monitoring with integral intensity of $^1$H NMR requires relatively high concentrations (typically more than 10 mM) and is limited to application of slower reaction rates than that of the NMR time scale. Although monitoring of low concentration reaction ($\mu$M) is possible with optical spectrometry, it is necessary to design the system so that the maximum wavelength of absorbance may shift along with the reaction progress. The tagging method with LDI-MS presented here does not require a reaction system itself, except for introduction of the photocleavable molecular tag into a molecule of interest. Thus, the presented method could provide a complementary role to existing methods.

## Conclusion

In conclusion, we have developed a new photocleavable molecule for LDI-MS. Since the molecule has high sensitivity for negative mode MS detection and good chemical stability, it was successfully applied to molecular tag for LDI-MS. With this device, one can selectively observe MS signals of only tagged molecules. Since their response factors are predictable through empirical equations, quantitative or semiquantitative treatment of signals is feasible. We believe that the device has broad potential for high throughput screening of chemical and enzymatic reactions as well as an analytical tool to obtain fruitful information with minimum consumption of resources (time and reagents). A theoretical approach to understanding the mechanism and further applications are currently under investigation.

## Experimental Section

**General Synthetic Procedure of Photocleavable Molecule 3.** To a solution of **2** (1mmol) in DMF (2 mL) was added potassium carbonate (2mmol) and *p*-nitro-benzyl bromide (1.1 mmol). The suspension was stirred for 3 h at room temperature. Then the mixture was poured onto water (30 mL) and extracted with EtOAc (1 × 20 mL). The layer was washed with 1 M aqueous HCl (3 × 15 mL) and brine successively. Then the organic portion was dried over magnesium sulfate, filtered, and evaporated in vacuo. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc = 4:1) and **3a** was obtained as a yellowish solid (95%).

**3a.** White solid; mp 125−128 °C. IR (KBr, cm $^{-1}$): 1748, 1678, 1595, 1523, 1342, 1325, 1150, 981, $^1$H NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 2.33(6H, s), 2.76(2H, t, $J$ = 6.6 Hz), 3.28(2H, t, $J$ = 6.6 Hz), 3.70(3H, s), 4.96(2H, s), 7.65(2H, d, $J$ = 8.0 Hz), 7.70(2H, s), 8.27(2H, d, $J$ = 8.0 Hz). $^{13}$C NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 16.6, 28.1, 33.3, 51.8, 72.4, 123.7, 127.6, 129.2, 131.1, 132.7, 144.3, 147.6, 159.4, 173.3, 197.2. HRMS *m*/*z* calcd for $C_{20}H_{21}NO_6$, 371.1369; found, 371.1369.

**3b.** Light yellow solid; mp 127−129 °C. IR (KBr, cm $^{-1}$): 1755, 1725, 1680, 1214, 1151, 1071, 844. $^1$H NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 2.35(6H, s), 2.74(2H, t, $J$ = 6.8 Hz), 3.27(2H, t, $J$ = 6.8 Hz), 3.70(3H,s), 3.84(3H, s), 4.56(2H, s), 7.67(2H, s). $^{13}$C NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 16.3, 28.0, 33.1, 51.6, 52.1, 68.7, 129.1, 131.0, 133.0, 159.3, 168.9, 173.3, 197.2, HRMS *m*/*z* calcd for $C_{16}H_{20}O_6$, 308.1260; found, 308.1253.

**3c.** White solid; mp 53−55 °C. IR (KBr, cm $^{-1}$): 3032, 1726, 1678, 1597, 1359, 1223, 1151, 972, 843. $^1$H NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 2.33(6H, s), 2.76(2H, t, $J$ = 6.8 Hz), 3.28(2H, t, $J$ = 6.8 Hz), 3.71(3H, s), 4.85(2H, s), 7.36−7.47(5H, m), 7.69(2H,s). $^{13}$C NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 16.5, 28.1, 33.2, 51.7, 74.1, 127.8, 128.2, 128.5, 129.1, 131.5, 137.0, 160.1, 173.4, 197.4. HRMS *m*/*z* calcd for $C_{20}H_{22}O_4$, 326.1518; found, 326.1504.

**3d.** Yellow solid; mp 127−130 °C. IR (KBr, cm $^{-1}$): 2937, 1737, 1678, 1582, 1525, 1441, 1324, 1276, 1223, 1154, 875, 796. $^1$H NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 2.33(6H, s), 2.77-(2H, t, $J$ = 6.8 Hz), 3.30(2H, t, $J$ = 6.8 Hz), 3.72(3H, s), 4.00(3H, s), 4.06(3H, s), 5.28(2H, s), 7.66(1H, s), 7.72(2H, s), 7.80(1H, s). $^{13}$C NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 16.5, 28.1, 33.3, 51.8, 56.4, 56.5, 70.3, 108, 109.0, 129.2, 129.8, 131.3, 132.8, 138.6, 147.8, 154.1, 159.6, 173.4, 197.3. HRMS *m*/*z* calcd for $C_{22}H_{25}$-$NO_8$, 431.1580; found, 431.1581.

**3e.** Light yellow solid; mp 93−95 °C. IR (KBr, cm $^{-1}$): 2953, 1726, 1597, 1439, 1293, 1151, 1071, 983. $^1$H NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 2.32(6H, s), 2.76(2H, t, $J$ = 6.8 Hz), 3.28(2H, t, $J$ = 6.8 Hz), 3.71(3H, s), 3.93(6H, s), 4.91(2H, s), 7.65-(1H, d, $J$ = 7.8 Hz), 7.70(2H, s), 7.80(2H, d, $J$ = 7.8 Hz). $^{13}$C NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 16.5, 28.0, 33.2, 51.8, 52.6, 52.7, 72.5, 127.3, 129.2, 129.3, 129.4, 131.2, 131.3, 132.5, 132.7, 140.8, 159.6, 167.6, 167.9, 173.4, 197.4. HRMS *m*/*z* calcd for $C_{24}H_{26}O_8$, 442.1628; found, 442.1629.

**4-[3,5-Dimethyl-4-(4-nitro-benzyloxy)-phenyl]-4-oxo-butyric Acid 2,5-Dioxo-pyrrolidin-1-yl Ester (5).** To a solution of **3a** (1mmol) in THF (2 mL) was added aqueous sodium hydroxide (5% 5 mL). The solution was stirred for 3 h at room temperature. Then the mixture was neutralized by 1 M HCl solution (10 mL) and extracted with EtOAc (3 × 20 mL). Then the organic portion was dried over magnesium sulfate, filtered, and evaporated in vacuo.

The corresponding carboxylic acid was obtained as a white solid. The crude carboxylic acid was dissolved in $CH_2Cl_2$ (3 mL), and *N*-hydroxysuccinimide (2mmol) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC, 1.5 mmol) was added successively. The solution was stirred for 3 h at room temperature. Then the mixture was poured onto water and extracted with $CH_2Cl_2$ (3 × 5 mL). Then the organic portion was dried over magnesium sulfate, filtered, and evaporated in vacuo. **5** was obtained as white solid (95%). **5:** White solid; mp 194−197 °C. IR (KBr, cm $^{-1}$): 3450, 1737, 1679, 1525, 1347, 1206, 1155, 1063, 983, 854. $^1$H NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 2.33(6H, s), 2.85(4H, s), 3.07(2H, t, $J$ = 6.8 Hz), 3.39(2H, t, $J$ = 6.8 Hz), 4.96(2H, s), 7.66(2H, d, $J$ = 8.4 Hz), 7.70(2H, s), 8.27(2H, d, $J$ = 8.4 Hz). $^{13}$C NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 16.5, 25.44, 25.6, 32.9, 72.4, 123.8, 127.7, 129.3, 131.4, 132.3, 144.4, 147.7, 159.8, 168.4, 169, 196. HRMS *m*/*z* calcd for $C_{23}H_{22}N_2O_8$, 454.1376; found, 454.1367.

**N-[2-(2-Azido-ethoxy)-ethyl]-4-[3,5-dimethyl-4-(4-nitro-benzyloxy)-phenyl]-4-oxo-butyramide (6).** To a solution of **5** (1mmol) in $CH_2Cl_2$ (3 mL) was added 2-(2-aminoethoxy)ethanol (2 mmol). The solution was stirred for 3 h at room temperature. Then the mixture was poured onto water and extracted with $CH_2Cl_2$ (3 × 5 mL). Then the organic portion was dried over magnesium sulfate, filtered, and evaporated in vacuo. The residue was dissolved in pyridine (5 mL) and added methanesulfonyl chloride (1.2 mmol). The mixture was stirred for 12 h at room temperature. To the mixture was added EtOAc (20 mL), and this was washed with 1 M HCl (20 mL × 3) and brine (20 mL × 1). Then the organic portion was dried over magnesium sulfate, filtered, and evaporated in vacuo. The resulting residue was dissolved in DMF (5 mL) and added sodium azide (3mmol). The suspension was stirred for 12 h at room temperature. To the mixture was added EtOAc (20 mL), and this was washed with 1 M HCl (20 mL × 3) and brine (20 mL × 1). Then the organic portion was dried over magnesium sulfate, filtered, and evaporated in vacuo. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc = 1:1 to 1:2), and **6** was obtained as a yellowish solid (70%). **6:** yellow solid; mp 112−115 °C. IR (KBr, cm $^{-1}$): 2103, 1755, 1641, 1343, 1233, 1052. $^1$H NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 2.33(6H, s), 2.63(2H, t, $J$ = 6.8 Hz), 3.32(2H, t, $J$ = 6.8 Hz), 3.40(2H, t, $J$ = 4.8 Hz), 3.50(2H, t, $J$ = 4.8 Hz), 3.58(2H, t, $J$ = 4.8 Hz), 3.69(2H, t, $J$ = 4.8 Hz), 4.96(2H, s), 7.66(2H, d, $J$ = 8.4 Hz), 7.71(2H, s), 8.28(2H, d, $J$ = 8.8 Hz). $^{13}$C NMR (500 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm); 16.6, 30.2, 33.8, 39.2, 50.6, 69.9, 70.14, 72.4, 123.8, 127.7, 129.3, 131.2, 132.9, 144.5, 147.6, 159.5, 172.2, 198.2. HRMS *m*/*z* calcd for $C_{23}H_{27}N_5O_6$, 469.1961; found, 469.1959.

**General Synthetic Procedure of Amides (4 and 7 to 12).** To a solution of **5** (1 mmol) in $CH_2Cl_2$(3 mL) was added amine (1.2 mmol). The solution was stirred for 3 h at room temperature. Then the mixture was poured onto water and extracted with $CH_2Cl_2$ (3 × 5 mL). Then the organic portion was dried over magnesium sulfate, filtered, and evaporated in vacuo. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc = 5:1 to 1:1) and **4** and **7**−**12** were obtained as white solids.

**4-[3,5-Dimethyl-4-(4-nitro-benzyloxy)-phenyl]-4-oxo-*N*-propyl-butyramide (4).** White solid; mp 170−172 °C. IR (KBr, cm $^{-1}$): 2961, 1678, 1641, 1527, 1343, 1322, 1150, 982, 854. $^1$H NMR (300 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 0.93(3H, t, $J$ = 7.2 Hz), 1.50−1.58(2H, m), 2.32(6H, s), 2.63(2H, t, $J$ = 6.6 Hz), 3.23-(2H, t, $J$ = 6.6 Hz), 3.34(2H, t, $J$ = 6.6 Hz), 4.96(2H, s), 5.99(1H, s), 7.66(2H, d, $J$ = 8.4 Hz), 7.71(2H, s), 8.28(2H, d, $J$ = 8.4 Hz). $^{13}$C NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 11.3, 16.5, 22.8, 30.4, 34.0, 41.3, 72.4, 123.8, 127.7, 129.3, 1321.2, 132.9, 144.4, 147.7, 159.6, 172.0, 198.5. HRMS *m*/*z* calcd for $C_{22}H_{26}N_2O_5$, 398.1842; found, 398.1837.

***N*-(2-Amino-ethyl)-4-[3,5-dimethyl-4-(4-nitro-benzyloxy)-phenyl]-4-oxo-butyramide (7).** White solid; mp 123−125 °C. IR (KBr, cm $^{-1}$): 3363, 2898, 1665, 1514, 1346, 1296, 1151, 1007.65, 850. $^1$H NMR (300 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 2.32(6H, s), 2.63(2H, t, $J$ = 6.6 Hz), 2.84(2H, t, $J$ = 6.3 Hz), 3.31−3.36(4H, m), 4.96(2H, s), 6.19(1H, s), 7.66(2H, d, $J$ = 9.0 Hz), 7.71(2H, s), 8.29(2H, d, $J$ = 9.0 Hz). $^{13}$C NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm); 16.5, 30.3, 33.9, 41.3, 42.0, 72.4, 123.8, 127.7, 129.3, 131.2, 132.8, 144.4, 147.6, 159.5, 172.4, 198.5. HRMS *m*/*z* calcd for $C_{21}H_{25}N_3O_5$, 399.1794; found, 399.1774.

***N*-tert-Butyl-4-[3,5-dimethyl-4-(4-nitro-benzyloxy)-phenyl]-4-oxo-butyramide (8).** White solid; mp 124−125 °C. IR (KBr, cm $^{-1}$): 2969, 1678, 1642, 1530, 1344, 1152, 983, 853; $^1$H NMR (300 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm); 1.35(9H, s), 2.32(6H, s), 2.54(2H, t, $J$ = 6.6 Hz), 3.30(2H, t, $J$ = 6.6 Hz), 4.95(2H, s), 5.61-(1H, s), 7.66(2H, d, $J$ = 9.0 Hz), 7.69(2H, s), 8.28(2H, d, $J$ = 9.0 Hz); $^{13}$C NMR(400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm); 16.6, 28.9, 31.3, 34.1, 51.4, 72.7, 124.4, 128.3, 130.0, 131.8, 133.6, 145.2, 148.4, 160.3, 172.2, 199.5; HRMS *m*/*z* calcd for $C_{23}H_{28}N_2O_5$ 412.1998 Found 412.1996.

***N*-Cyclohexyl-4-[3,5-dimethyl-4-(4-nitro-benzyloxy)-phenyl]-4-oxo-butyramide (9).** White solid; mp 207−210 °C. IR (KBr, cm $^{-1}$): 2933, 2854, 1640, 1527, 1343, 1302, 1152, 987, 855. $^1$H NMR (300 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 1.11−1.23(3H, m), 1.29−1.38(3H, m), 1.68−1.73(2H, m), 1.88−1.92(2H, m), 2.32-(6H, s), 2.58(2H, t, $J$ = 6.6 Hz), 3.31(2H, t, $J$ = 6.6 Hz), 3.74−3.76(1H, m), 4.95(2H, s), 5.61(1H, s), 7.65(2H, d, $J$ = 8.4 Hz), 7.74(2H, s), 8.28(2H, d, $J$ = 8.4 Hz). $^{13}$C NMR(500 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 16.5, 24.8, 25.5, 30.7, 33.1, 34.1, 48.3, 72.4, 123.8, 127.7, 129.3, 131.2, 133.0, 144.4, 147.7, 159.6, 171.1, 198.5. HRMS *m*/*z* calcd for $C_{25}H_{30}N_2O_5$, 438.2155; found, 438.2148.

**4-[3,5-Dimethyl-4-(4-nitro-benzyloxy)-phenyl]-4-oxo-*N*-phenethyl-butyramide (10).** White solid; mp 176−178 °C. IR (KBr, cm $^{-1}$): 2942, 1678, 1632, 1527, 1342, 1233, 1211, 1149, 981, 855. $^1$H NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 2.33-(6H, s), 2.57(2H, t, $J$ = 6.8 Hz), 2.82(2H, t, $J$ = 6.8 Hz), 3.30(2H, t, $J$ = 6.8 Hz), 3.53(2H, q, $J$ = 6.4 Hz), 4.96(2H, s), 5.74(1H, s), 7.20−7.32(5H, m), 7.66(2H, d, $J$ = 8.8 Hz), 7.70(2H, s), 8.28(2H, d, $J$ = 8.8 Hz). $^{13}$C NMR(400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 16.5, 30.3, 33.9, 35.7, 40.68, 72.4, 123.8, 126.4, 127.6, 128.7, 129.3, 131.2, 132.8, 138.9, 144.4, 147.6, 159.5, 172.0, 198.3. HRMS *m*/*z* calcd for $C_{27}H_{28}N_2O_5$, 460.1998; found, 460.1996.

**4-[3,5-Dimethyl-4-(4-nitro-benzyloxy)-phenyl]-*N*-dodecyl-4-oxo-butyramide (11).** White solid; mp 147−149 °C. IR (KBr, cm $^{-1}$): 3088, 2918, 2850, 1642, 1528, 1343, 1234, 1150, 983, 855. $^1$H NMR (300 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 0.88(3H, t, $J$ = 6.6 Hz), 1.25(15H, s), 1.50(3H, t, $J$ = 7.2 Hz), 2.48(6H, s), 2.60-(2H, t, $J$ = 6.6 Hz), 3.24(2H, q, $J$ = 6.6 Hz), 3.33(2H, t, $J$ = 6.6 Hz), 4.95(2H, s), 5.77(1H, t, $J$ = 9.0 Hz), 7.65(2H, d, $J$ = 8.7 Hz), 7.74(2H, s), 8.82(2H, d, $J$ = 8.7 Hz). $^{13}$C NMR(500 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 16.5, 24.8, 25.5, 30.7, 33.1, 34.1, 48.3, 72.4, 123.8, 127.7, 129.3, 131.2, 133.0, 144.4, 147.7, 159.6, 171.1, 198.5. HRMS *m*/*z* calcd for $C_{31}H_{44}N_2O_5$, 524.3250; found, 524.3234.

**4-[3,5-Dimethyl-4-(4-nitro-benzyloxy)-phenyl]-*N*-octadecyl-4-oxo-butyramide (12).** White solid; mp 147−149 °C. IR (KBr, cm $^{-1}$): 3088, 2918, 2850, 1642, 1528, 1343, 1212, 1150, 983, 855. $^1$H NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 0.88(6H, t, $J$ = 6.6 Hz), 1.25(22H, s), 1.44−1.49(4H, m), 2.32(6H, s), 2.60(2H, t, $J$ = 6.6 Hz), 3.24(2H, q, $J$ = 6.6 Hz), 3.32(2H, t, $J$ = 6.6 Hz), 4.95(2H, s), 5.74(1H, s), 7.65(2H, d, $J$ = 9.0 Hz), 7.71(2H, s), 8.28(2H, d, $J$ = 9.0 Hz). $^{13}$C NMR(500 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 14.1, 16.5, 22.7, 26.9, 29.3, 29.6, 29.7, 30.5, 31.9, 34.1, 39.7, 72.4, 123.8, 127.7, 129.4, 131.2, 132.9, 144.4, 147.7, 159.6, 172.0, 198.5. HRMS *m*/*z* calcd for $C_{37}H_{56}N_2O_5$, 608.4189; found, 608.4177.

**General Synthetic Procedure of Azide Coupling ("Click Chemistry") (16 to 18).** To a solution of **13**, **14**, or **15** (1mmol) and **6** (1mmol) was added copper sulfate solution and sodium ascorbate (2.8 mL). The mixture was stirred for 12 h at room temperature. Then the mixture was poured onto water and extracted EtOAc (10 mL × 3). Then the organic portion was dried over magnesium sulfate, filtered, and evaporated in vacuo. The residue

was purified by silica gel column chromatography ($CHCl_3/CH_3OH$ = 50:1 to 10:1) and **16** to **17** were obtained as white solids, respectively.

**16 (96%).** Light yellow solid; mp 45−48 °C. IR (KBr, cm $^{-1}$): 2956, 1755, 1676, 1347, 1228, 1153, 1040. $^1$H NMR(400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 1.98−2.20(14H, m), 2.32(6H, s), 2.63-(2H, t, $J$ = 6.8 Hz), 3.32(2H, t, $J$ = 6.8 Hz), 3.75−3.85(3H, m), 3.74−3.80(1H, m), 3.80−3.92(2H, m), 4.08−4.18(1H, m), 4.22−4.32(1H, m), 4.46(2H, s), 4.65−4.90(2H, m), 4.96(2H, s), 4.99−5.04(1H, m), 5.12(1H, t, $J$ = 9.2 Hz), 5.22(1H, t, $J$ = 9.2 Hz), 6.37(1H, t, $J$ = 5.6 Hz), 7.66(2H, d, $J$ = 8.8 Hz), 7.71(2H, s), 7.75(1H, s), 8.28(2H, d, 8.8 Hz). $^{13}$C NMR(400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 16.4, 20.5, 20. 6, 20.6, 30.0, 33.7, 39.1, 50.1, 61.8, 63.1, 68.3, 68.9, 69.8, 71.3, 71.8, 72.3, 72.6, 100.0, 123.7, 123.8, 127.6, 129.2, 131.1, 132.8, 144.1, 144.4, 147.6, 159.5, 169.3, 169.4, 170.1, 170.6, 172.3, 198.2. FAB HRMS *m*/*z* calcd for $C_{40}H_{49}N_5O_{16}$, 855.3174; found, 855.3164.

**17 (95%).** White solid; mp 94−96 °C. IR (KBr, cm $^{-1}$): 2958, 1755, 1675, 1529, 1369, 1348, 1234.90, 1153, 1043. $^1$H NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 1.98−2.04(17H, m), 2.10(3H, s), 2.15(3H, s), 2.32(6H, s), 2.62(2H, t, $J$ = 6.8 Hz), 3.32(2H, t, $J$ = 6.8 Hz), 3.43(1H, q, $J$ = 5.6 Hz), 3.53−3.54(2H, m), 3.69−3.75(1H, m), 3.86(2H, t, $J$ = 4,8 Hz), 3.95−4.07(3H, m), 4.18−4.32(2H, m), 4.49−4.63(3H, m), 4.72(1H, d, $J$ = 8.2 Hz), 4.81−4.93(3H, m), 4.96(2H, s), 5.06(1H, t, $J$ = 10.2 Hz), 5.26(1H, t, $J$ = 8.8 Hz), 5.33−5.42(2H, m), 6.31(1H, t, $J$ = 5.6 Hz), 7.66(2H, d, $J$ = 8.2 Hz), 7.71(2H, s), 7.73(1H, s), 8.28(2H, d, $J$ = 8.8 Hz). $^{13}$C NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 16.5, 20.5, 20.5, 20.6, 20.8, 30.1, 33.7, 39.1, 50.1, 61.5, 62.6, 63.1, 68.0, 68.4, 68.9, 69.3, 69.8, 70.0, 72.1, 72.3, 72.4, 72.7, 75.2, 95.5, 99.6, 123.8, 123.9, 127.64, 129.3, 131.2, 132.9, 144.1, 144.4, 147.6, 159.5, 169.3, 169.7, 169.8, 170.1, 170.4, 170.5, 172.3, 198.2. HRMS *m*/*z* calcd for $C_{52}H_{65}N_5O_{24}$, 1143.4020; found, 1143.4116.

**18 (95%).** White solid; mp 98−100 °C. IR (KBr, cm $^{-1}$): 2961, 1755, 1675, 1530, 1370, 1348, 1235, 1152, 1037. $^1$H NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 1.96−2.10(27H, m), 2.15(3H, s), 2.18(3H, s), 2.32(6H, s), 2.62(2H, d, $J$ = 6.8 Hz), 3.32(2H, t, $J$ = 6.8 Hz), 3.43(1H, q, $J$ = 5.4 Hz), 3.53−3.54(2H, m), 3.74−3.77(1H, m), 3.86(2H, t, $J$ = 4.8 Hz), 3.92−4.07(4H, m), 4.17−4.33(3H, m), 4.46−4.57(4H, m), 4.70−4.75(2H, m), 4.81−4.92(3H, m), 4.96(2H, s), 5.07(1H, t, $J$ = 10.2 Hz), 5.24−5.29(2H, m), 5.34−5.42(3H, m), 6.28(1H, t, $J$ = 5.6 Hz), 7.66(2H, d, 8.8 Hz), 7.70-(2H, s), 7.73(1H, s), 8.28(2H, d, 8.8 Hz). $^{13}$C NMR (400 MHz, $CDCl_3$, TMS, rt) $\delta$ (ppm): 16.4, 20.4, 20.5, 20.6, 20.7, 20.7, 20.8, 30.0, 33.6, 39.1, 50.0, 61.3, 62.2, 62.7, 63.1, 67.8, 68.4, 68.8, 68.9, 69.2, 69.7, 70.0., 70.3, 71.6, 72.0, 72.2, 72.3, 72.4, 73.7, 75.0, 95.5, 95.6, 99.5, 123.7, 123.9, 127.6, 129.2, 131.1, 132.8, 144.0, 144.4, 147.5, 159.4, 169.3, 169.5, 169.7, 169.7, 169.9, 170.2, 170.4, 170.4, 170.4, 170.5, 172.2, 198.2. HRMS *m*/*z* calcd for $C_{64}H_{81}N_5O_{32}$, 1431.4865; found, 1431.4855.

**LDI-MS Measurement:** Spectra were acquired in negative mode, linear operational mode with delayed extraction type, and optimized laser rate type. Other settings are as follows: accelerating voltage, 20,000V; grid voltage, 95%; grid wire voltage 0, 0.05%; delay time, 130 ns; stainless steel plate (PLATE1; 45 well) was used for all measurement. Typically 10 M solution of tagged molecule was prepared. And 0.2 $\mu$L of sample was applied on each circle and dried under ambient condition. Acetonitrile or dichloromethane was used as the solvent. A premixed solution (1:1 mixture) was used for the comparison of signal response factors. The reported value was obtained as an average of each 10 spectra.

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**Supporting Information Available:** Synthetic procedure of **2** and **13**−**15** and NMR data and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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