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Promise of Retinoic Acid-Triazolyl Derivatives in Promoting Differentiation of Neuroblastoma Cells

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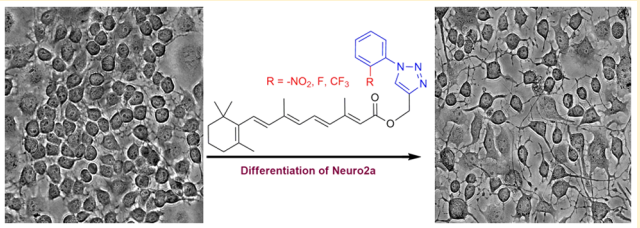
Supporting Information

ABSTRACT: Retinoic acid induces differentiation in various types of cells including skeletal myoblasts and neuroblasts and maintains differentiation of epithelial cells. The present study demonstrates synthesis and screening of a library of retinoic acid-triazolyl derivatives for their differentiation potential on neuroblastoma cells. Click chemistry approach using copper-(I)-catalyzed azide−alkyne cycloaddition was adopted for the preparation of these derivatives. The neurite outgrowth promoting potential of retinoic acid-triazolyl derivatives was studied on neuroblastoma cells. Morphological examination revealed that compounds **8a**, **8e**, **8f**, and **8k**, among the various derivatives screened, exhibited promising neurite-outgrowth inducing activity at a concentration of 10 μM compared to undifferentiated and retinoic acid treated cells. Further on, to confirm this differentiation potential of these compounds, neuroblastoma cells were probed for expression of neuronal markers such as NF-H and NeuN. The results revealed a marked increase in the NF-H and NeuN protein expression when treated with **8a**, **8e**, **8f**, and **8k** compared to undifferentiated and retinoic acid treated cells. Thus, these compounds could act as potential leads in inducing neuronal differentiation for future studies.



KEYWORDS: *Retinoic acid, azide−alkyne cycloaddition, neurite outgrowth, neuroblastoma cells, neuronal markers*

Retinoic acid (RA), a small lipophilic molecule derived from vitamin A, plays an important role in embryonic development, neuronal differentiation, neuronal patterning, and axonal growth. Studies on RA in neuronal development have led to a greater understanding of the mechanisms of neurodegenerative diseases.[1−5] It has been also observed that RA is involved in the maintenance of the differentiated state of adult neurons and neural stem cells and its altered signaling leads to the symptoms of neurodegenerative diseases.[1,6] RA-mediated differentiation in various types of cells including skeletal myoblasts and neuroblasts have been thoroughly studied.[7−10] Furthermore, it has been also studied that the clonal human neuroblastoma cell line, SH-SY5Y, differentiates and expresses neuronal markers upon exposure to RA.[11,12] Owing to the prominent role of RA in the development and regeneration of the nervous system, this molecule and its analogues have ample potential to act as therapeutic leads against various neurodegenerative disorders, pending enhancement of bioavailability in vivo.[13]

On the other hand, 1,2,3-triazole containing heterocycles have shown promising results in the treatment of various diseases and are considered as the cornerstone of medicinal chemistry.[14] These triazole-containing molecules exhibit hydrogen bonding, dipole−dipole and $\pi-\pi$ stacking interactions which enhance their solubility and binding capacity with biological targets.[15,16] It is with these reasons that 1,2,3-triazole motif is found in a number of biologically active compounds and pharmaceutical agents.[17] Among these, an anticancer compound, carboxyamidotriazole (CAI, **1**), a reverse transcriptase inhibitor, *tert*-butyldimethylsilylspiroaminooxathioledioxide (known as TSAO, **2**), a β-lactum antibiotic tazobactum (**3**), a broad spectrum antibiotic, cefatrizine (**4**), and a potent anti-HIV agent (**5**) are the representative examples (Figure 1).[18−20] Previously in our group, we have also reported the synthesis of 1,2,3-triazole-analogues of various natural products as potential anticancer agents.[21−23] Keeping in consideration the promise of 1,2,3-triazoles in drug discovery and the limitations associated with RA in neuronal differentiation, we proposed to synthesize a library of RA-triazolyl analogues and

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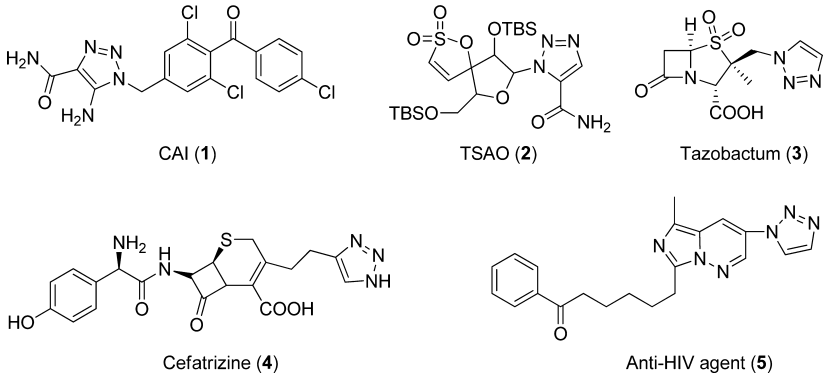
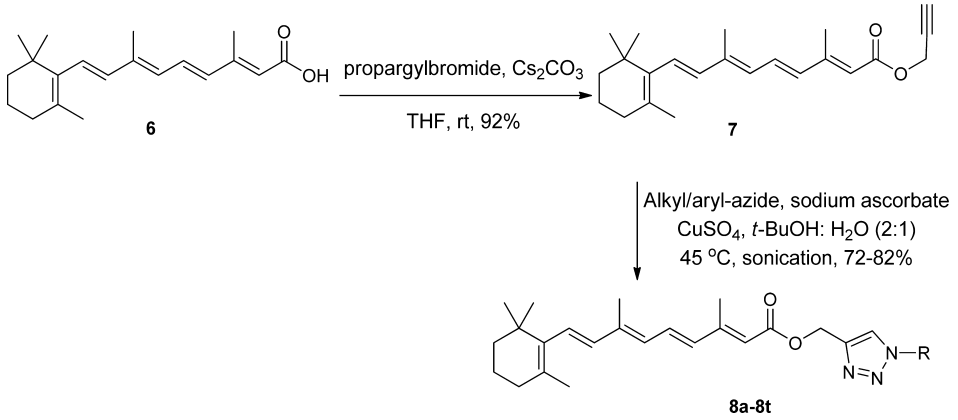


Figure 1. 1,2,3-Triazole containing drugs and bioactive entities.

Scheme 1. Synthesis of RA-Triazolyl Analogues

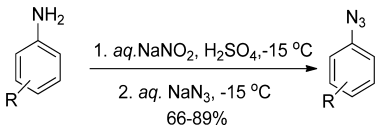


evaluate them on neuroblastoma cells for their differentiation potential.

RESULTS AND DISCUSSION

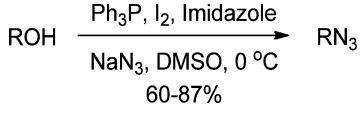
Chemistry. In light of the preceding discussion, a library of RA-triazolyl derivatives was synthesized employing click chemistry protocol and screened on neuroblastoma cells. For this purpose, carboxylic acid group of RA (**6**) was subjected to propargylation using Cs_2CO_3 under anhydrous conditions to yield the alkyne derivative **7** (Scheme 1). On the other hand, aromatic azides were prepared from their corresponding anilines via diazotization with sodium nitrite in acidic conditions followed by azidation with sodium azide (Scheme 2). Aliphatic azides were prepared by treatment of a solution of

Scheme 2. Synthesis of Aromatic Azides from Anilines



aliphatic alcohol with sodium azide in the presence of Ph_3P, I_2, and imidazole (Scheme 3). Regioselective, 1,3-dipolar cycloaddition reaction of **7** with aromatic and aliphatic azides in the presence of $CuSO_4 \cdot 5H_2O$ and sodium ascorbate in *t*-BuOH/H_2O (2:1; v/v) led to the formation of 1,4-substituted-triazolyl derivatives (**8a**–**8t**) in decent yields (Scheme 1). All the reactions were carried out under sonication at 45 °C within 2–4 h. The products were confirmed by ^{1}H NMR, ^{13}C NMR, and MS analysis. In ^{1}H NMR, cyclization of azides to form triazoles were confirmed by the resonance of H-5 of triazole ring in aromatic region, in addition to, chemical shift of other protons in aliphatic or aromatic region. The structure was further supported by ^{13}C NMR and DEPT, which showed all the expected carbon signals corresponding to RA-triazolyl derivatives.

Scheme 3. Synthesis of Aliphatic Azides from Alcohols



By employing the above reaction conditions, a series RA-triazolyl derivatives that vary at substitutions on the R-group were synthesized from a range of aromatic and aliphatic azides. It was observed that all the reactions worked smoothly under ultrasonication conditions in decent yields, Table 1.

Neurite Outgrowth Formation of Neuro2a cells Treated with RA-Derivatives. In order to determine the differentiation potential of these RA-triazolyl derivatives, Neuro2a (neuroblastoma) cells were used for the study. Cells were treated with various concentrations (1, 5, 10, and 20 μM) of **8a**–**8t** for 5 days, and neurite outgrowth property was assessed by observing the cells morphologically. It was found that below 10 μM concentration there was no significant neurite outgrowth, while above 10 μM concentration treatment was able to induce differentiation, but at the same time significant cell death was observed. Neurite outgrowth formations were markedly discernible with compounds **8a**, **8e**, **8f**, and **8k** at the concentration of 10 μM compared to

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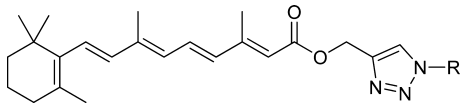
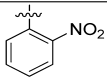
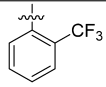
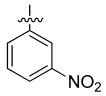
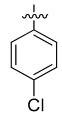
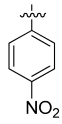
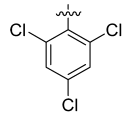
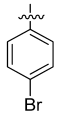
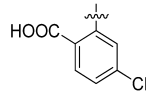
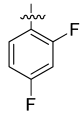
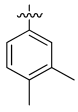
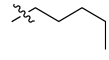
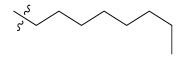
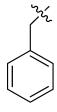
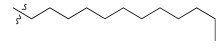
Table 1. Retinoic Acid-Triazolyl Derivatives with Varied Substitutions



Entry	R	Time (h)	(%) Yield[a]	Entry	R	Time (h)	(%) Yield[a]
8a		2	79	**8k**		2	80
8b		2	72	**8l**		2	82
8c		2	78	**8m**		2	82
8d		2.5	77	**8n**		2.5	78
8e		2	79	**8o**		2.5	73
8f		2	79	**8p**		3	76
8g		3	72	**8q**		2.5	74
8h		3	71	**8r**		4	67
8i		3	73	**8s**		4	67
8j		3	75	**8t**		4	69

[a]Yields reported are isolated yields.

undifferentiated and RA-treated cells (Figure 2). In order to confirm these results, we performed immunohistochemistry of neuronal markers such as NF-H and NeuN with compounds **8a**, **8e**, **8f**, and **8k**. From the results, it was observed that all these four compounds induced neurite outgrowth; however, compound **8a** demonstrated significant differentiation when compared to the parent RA. Additionally, Western blot analysis revealed a significant increase in the expression levels of NeuN in compounds **8a** and **8e** treated Neuro2a cells, compared to undifferentiating cells.

Since these compounds contain an ester-linkage, we were concerned about their stability within the cells owing to the presence of endogenous esterases. Therefore, we determined the stability of these compounds (**8a**, **8e**, **8f**, and **8k**) within the cells using mass spectrometry. Analysis was carried out on the cytosolic fraction of the cells after 5 days of treatment with compounds **8a**, **8e**, **8f**, and **8k**, following the procedure of Kroesen et al.[24] From the mass spectral data, it was clear that none of these compounds were hydrolyzed to RA; instead all of these RA-triazolyl derivatives remained intact under experimental conditions (see Supporting Information LC-MS data).

Expression of NF-H in Neuro2a Cells Treated with RA-Derivatives (8a, 8e, 8f, and 8k) by Immunofluorescence. To confirm the differentiation induced by **8a**, **8e**, **8f**, and **8k**, Neuro2a cells were treated with these compounds, incubated with anti-NF-H and observed for its expression. The results revealed a marked increase in the protein expression as compared to the undifferentiated cells (Figure 3). RA was taken

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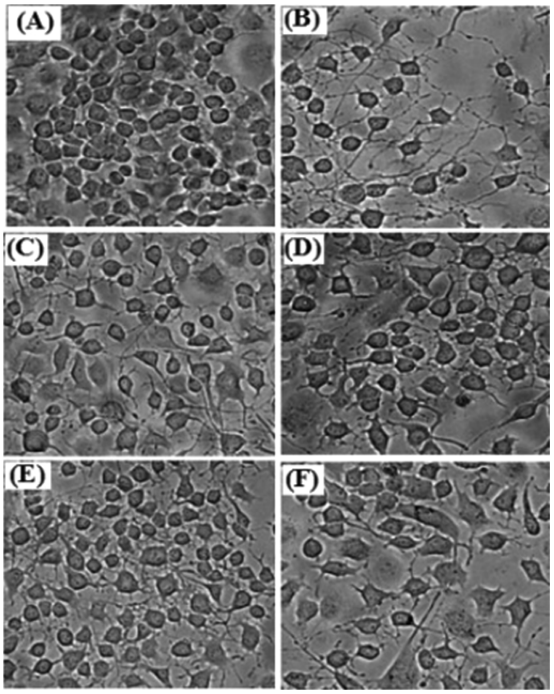

Figure 2. Formation of neurite outgrowth in Neuro2a cells after treatment with RA and RA-derivatives. (A) Undifferentiated cells, (B) cells treated with 10 μM RA, (C) cells treated with 10 μM **8a**, (D) cells treated with 10 μM **8e**, (E) cells treated with 10 μM **8f**, and (F) cells treated with 10 μM **8k**.

as positive control for the expression of NF-H. It was observed that among compounds **8a**, **8e**, **8f**, and **8k** tested for this study, compound **8a** showed significant results for differentiation compared to the parent RA.

Expression of NeuN in Neuro2a Cells Treated with RA-Derivatives (8a, 8e, 8f, and 8k) by Immunofluorescence. In addition to NF-H protein expression, we further attempted to investigate these compounds for neuronal marker NeuN expression. For this purpose Neuro2a cells were treated with **8a**, **8e**, **8f**, and **8k**, incubated with anti-NeuN and observed for its expression. The results observed were in agreement with those observed in NF-H expression. The expression of NeuN was significantly higher in the cells treated with **8a**, **8e**, **8f**, and **8k** in comparison with the undifferentiated cells (Figure 4). RA was taken as positive control throughout the experiment.

Expression of NeuN by Western Blotting. To validate this neuronal differentiation potential of these RA-derivatives (**8a**, **8e**, **8f**, and **8k**) further, change in protein expression level (NeuN) was studied by Western blotting (Figure 5). The results indicate significant increase in the expression levels of NeuN in compounds **8a** and **8e** treated Neuro2a cells, compared to undifferentiating cells (Figure 5A). β-Actin was taken as internal loading control. Densitometric analysis of Western blot (Figure 5B) was performed using ImageJ software. The results revealed that compounds **8a** and **8e** treatment resulted statistically significant increase in NeuN protein levels as compared to undifferentiated cells.

In conclusion, a series of RA-triazolyl derivatives were conceptualized and synthesized to validate their role in differentiation of neuroblastoma cells. Morphological examination revealed that compounds **8a**, **8e**, **8f**, and **8k**, among the various RA-derivatives screened, exhibited promising neurite-outgrowth inducing activity at a concentration of 10 μM compared to undifferentiated and RA-treated cells. To confirm it further, cells were studied for neuronal markers such as NF-H and NeuN after treatment with **8a**, **8e**, **8f**, and **8k**. The results revealed a marked increase in the NF-H and NeuN protein expression compared to the undifferentiated and RA-treated cells. Thus, these four compounds could act as potential leads in inducing neuronal differentiation for future studies.

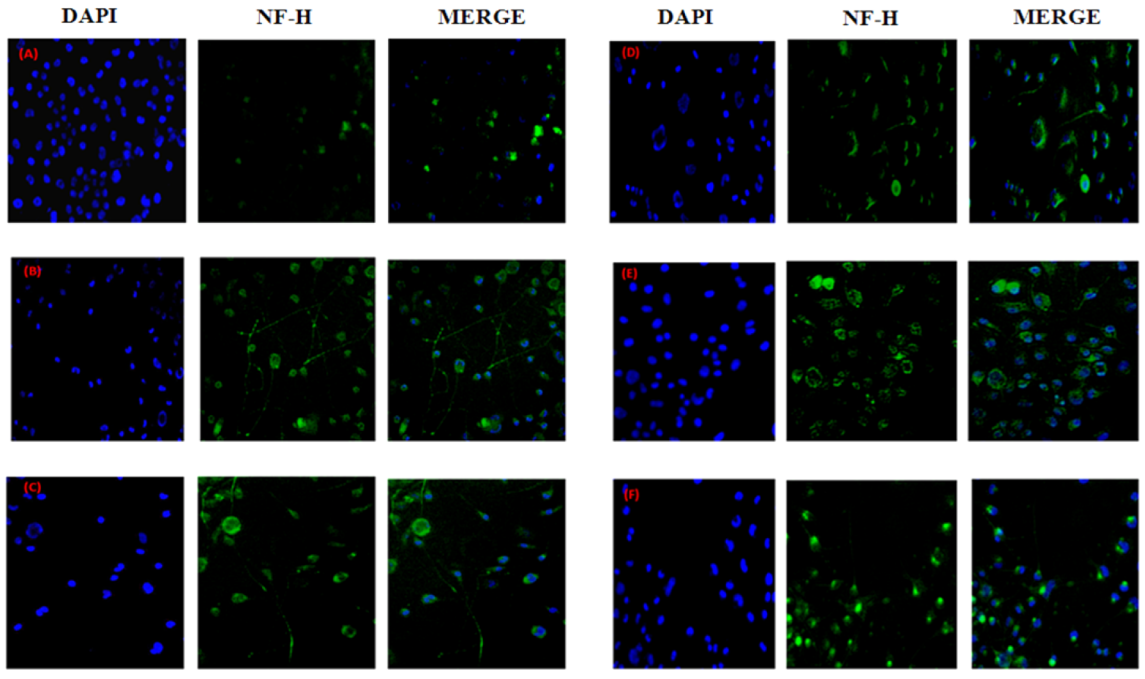

Figure 3. Expression of NF-H in Neuro2a cells after treatment with RA and RA-derivatives. (A) Undifferentiated cells, (B) cells treated with 10 μM RA, (C) cells treated with 10 μM **8a**, (D) cells treated with 10 μM **8e**, (E) cells treated with 10 μM **8f**, and (F) cells treated with 10 μM **8k**.

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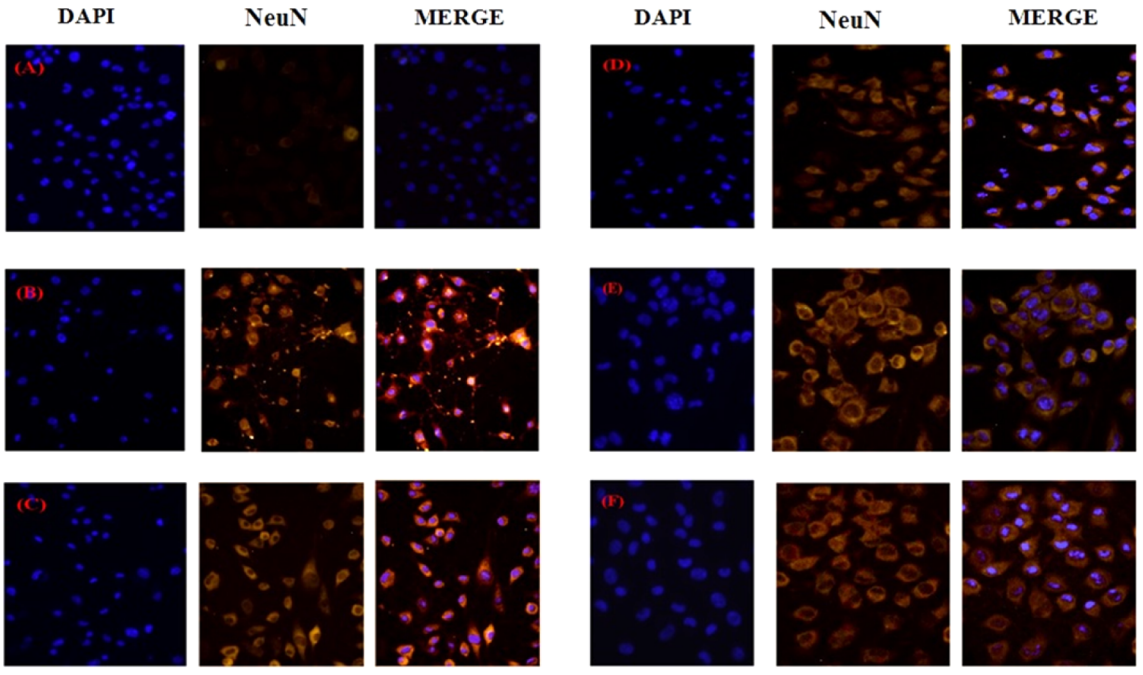

Figure 4. Expression of NeuN in Neuro2a cells after treatment with RA and RA-derivatives. (A) Undifferentiated cells, (B) cells treated with 10 μM RA, (C) cells treated with 10 μM **8a**, (D) cells treated with 10 μM **8e**, (E) cells treated with 10 μM **8f**, and (F) cells treated with 10 μM **8k**.

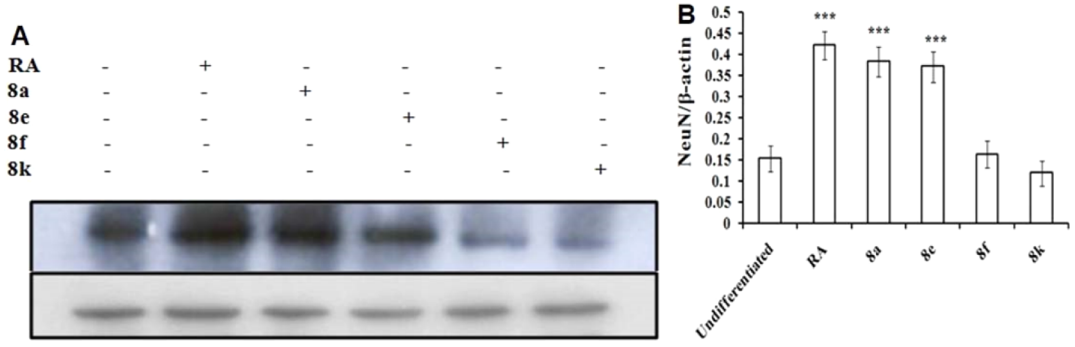

Figure 5. Expression of neuron-specific marker NeuN in Neuro2a cells after treatment with RA and RA-derivatives. Data expressed are the mean ± SD of three independent experiments. *Significant with respect to undifferentiated, ***$P < 0.001$.

METHODS

Chemistry. ^{1}H NMR and ^{13}C NMR spectra were recorded on 400 and 101 MHz Bruker Daltonics spectrometers. The chemical shifts (δ) are reported in ppm relative to tetramethylsilane (TMS) as internal standard, and coupling constants were measured in Hertz. Mass spectra were recorded on a Bruker Daltonics electrospray ionization apparatus. Column chromatography was carried out on silica gel (Qualigens, 60−120 mesh), and precoated silica gel thin layer chromatographic (TLC) plates were used for reaction monitoring. Retinoic acid was purchased from Sigma-Aldrich (Sigma, St. Louis, MO).

*Synthesis of (2E,4E,6E,8E)-Prop-2-yn-1-yl 3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**7**).* To a solution of **6** (300 mg, 0.8 mmol) in THF, cesium carbonate (600 mg, 1.6 mmol) and propargyl bromide (458 μL, 4.0 mmol) were added, and the reaction mixture was stirred at room temperature for 1 h. Progress of the reaction was monitored by TLC. After the reaction was over, the crude product was subjected to column chromatography (eluent: ethyl acetate/hexane; 5:95) to result pure compound 7 (312 mg, 92% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.03 (t, 1H, J = 12 Hz), 6.29 (d, 2H, J = 16 Hz), 6.15 (d, 2H, J = 12 Hz), 5.80 (s, 1H), 4.72 (s, 2H), 2.46 (s, 1H), 2.37 (s, 3H), 2.01 (s, 6H), 1.62 (m, 2H), 1.71 (m, 2H), 1.48 (m, 2H), 1.03 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.3, 154.6, 140.3, 137.9, 137.4, 135.0, 131.8, 130.3, 129.6, 129.1, 117.2, 78.4, 74.6, 51.4, 39.8, 34.4, 33.3, 29.1, 21.9, 19.4, 14.2, 13.1. HRMS $[M]^+$ 338.2243

Representative Procedure for the Synthesis of Azides. *Aromatic Azides.* To a solution of 2,5-dimethyl aniline (500 mg, 4.13 mmol) in 1,4-dioxane at −15 °C, 2 M sulfuric acid (10.3 mL, 20.6 mmol) was added in small installments while stirring. After 5 min, 3 M aqueous sodium nitrite solution (2.75 mL, 8.26 mmol) was added dropwise followed by careful addition of 3 M sodium azide solution (4.13 mL, 12.39 mmol). After the addition was over, the reaction mixture was allowed to attain room temperature and extracted with diethyl ether (3 × 50 mL). The combined organic layer was washed with saturated sodium bicarbonate solution, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to yield aromatic azide that was used in the next reaction without purification.

Aliphatic Azides. A mixture of octanol (4.6 mL, 3.8 mmol), triphenylphosphine (1.2 g, 4.6 mmol), iodine (1.16 g, 4.6 mmol), and imidazole (0.26 g, 3.84 mmol) was thoroughly mixed in a round-bottom flask. To this mixture was added sodium azide (0.99 g, 15.3 mmol) in DMSO and stirred at room temperature for 1 h. The reaction mixture was extracted with diethyl ether (3 × 30 mL), washed with brine, and concentrated under reduced pressure to yield crude aliphatic azide that was used in the next reaction without further purification.

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*General Procedure for the Synthesis of Compounds **8a–8t**.* To a solution of compound **7** (15 mg, 0.03 mmol) in *t*-BuOH/H_2O mixture (2:1, 2 mL), sodium ascorbate (1.2 mg, 0.006 mmol) and $CuSO_4$ (1.2 mg, 0.0045 mmol) were added at room temperature. To this mixture, aryl or aliphatic azide (0.06 mmol) was added and the reaction mixture was sonicated at 45 °C until its completion monitored by TLC. The crude mixture was extracted with ethyl acetate (3 × 50 mL), and the combined organic layer was dried over sodium sulfate. Solvent was removed under reduced pressure, and the product was purified through column chromatography (eluent: ethyl acetate/hexane; 15:85) to result in pure **8a–8t** in 72–82% yields.

*(2E,4E,6E,8E)-(1-(2-Nitrophenyl)-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8a**).* ^{1}H NMR (100 MHz, $CDCl_3$) δ 8.61 (m, 1H), 8.32 (m, 1H), 8.19 (m, 2H), 7.76 (m, 2H), 7.60 (s, 1H), 6.89 (t, 1H, *J* = 16 Hz), 6.23 (m, 2H), 5.81 (s, 1H), 5.17 (s, 2H), 2.36 (s, 3H), 2.06 (s, 3H), 2.04–2.19 (m, 4H), 1.72 (s, 3H), 1.43 (m, 2H), 1.10 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.9, 154.5, 143.5, 137.8, 135.1, 131.4, 131.1, 130.5, 126.1, 125.8, 125.0, 123.4, 122.1, 122.0, 118.5, 117.6, 117.4, 56.9, 34.5, 30.8, 29.8, 25.9, 20.4, 13.9, 13.2. HRMS $[M]^+$ 502.2578.

*(2E,4E,6E,8E)-(1-(3-Nitrophenyl)-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8b**).* ^{1}H NMR (100 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.92 (s, 1H), 7.77–7.61 (m, 3H), 6.85 (t, 1H, *J* = 16 Hz), 6.23 (m, 2H), 5.79 (m, 2H), 5.34 (s, 2H), 5.14 (s 1H), 2.32 (s, 3H), 2.02–1.93 (m, 4H), 1.75 (s, 3H), 1.62 (m, 2H), 1.42 (s, 3H), 1.23 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.8, 154.2, 143.3, 134.0, 131.3, 130.9, 130.3, 128.2, 126.8, 125.8, 125.4, 118.5, 118.0, 56.8, 41.2, 34.9, 31.8, 29.9, 26.2, 22.9, 14.2, 12.9. HRMS $[M]^+$ 502.2579.

*(2E,4E,6E,8E)-(1-(4-Nitrophenyl)-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8c**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (s, 1H), 8.43 (s, 1H), 7.97–7.97 (m, 2H), 7.81 (m, 1H), 7.43 (dd, *J* = 16, 12 Hz), 6.80 (d, 2H, J = 16 Hz), 6.58 (d, 2H, J = 16 Hz), 6.85 (s, 1H), 5.89 (s, 2H), 2.60 (s, 3H), 2.52 (s, 3H), 1.98–1.80 (m, 6H), 1.82 (s, 3H), 1.24 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.3, 157.5, 148.8, 139.7, 139.1, 136.90, 132.5, 131.9, 132.5, 129.8, 129.7, 129.0, 123.4, 131.3, 118.7, 116.8, 57.9, 39.4, 34.7, 33.5, 29.3, 21.5, 18.9, 14.3, 13.4. HRMS $[M]^+$ 502.2578.

*(2E,4E,6E,8E)-(1-(4-Bromophenyl)-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8d**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.60–7.51 (m, 4H), 6.74 (m, 1H), 6.59–6.50 (m, 3H), 6.39 (m, 1H), 5.94 (s, 1H), 5.33 (s, 2H), 2.15 (m, 2H), 2.07 (s, 3H), 1.81 (s, 3H), 1.80 (s, 3H), 1.56 (m, 2H), 1.59 (m, 2H), 0.99 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.4, 152.4, 148.1, 137. 3, 136.9, 135.4, 132.1, 132.1, 131.5, 130.8, 129.2, 121.8, 121.8, 120.5, 119.6, 118.8, 51.7, 38.5, 34.8, 33.8, 28.8, 28.8, 21.0, 20.3, 15.9, 15.9. HRMS $[M]^+$ 535.1830.

*(2E,4E,6E,8E)-(1-(2-Fluorophenyl)-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)deca-2,4,6,8-tetraenoate (**8e**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.87 (m, 1H), 7.36–7.19 (m, 3H), 6.93 (dd, 1H, *J* = 16, 12 Hz), 6.20 (d, 2H, *J* = 16 Hz), 6.05 (d, 2H, *J* = 16 Hz), 5.73 (s, 1H), 5.29 (s, 2H), 2.29 (s, 3H), 1.92 (s, 3H), 1.63–1.39 (m, 6H), 1.18 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.0, 154.2, 140.1, 137.8, 137.4, 135.0, 131.6, 130.5, 130.4, 130.3, 129.6, 129.1, 125.4, 125.4, 125.1, 117.7, 117.3, 117.1, 56.9, 39.8, 34.3, 33.2, 32.0, 29.7, 29.0, 22.9, 21.7, 19.4, 14.2, 13.0. HRMS $[M]^+$ 475.2631.

*(2E,4E,6E,8E)-(1-(2,4-Difluorophenyl)-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8f**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 7.85 (s, 1H), 7.963–7.38 (m, 2H), 7.13 (m, 1H), 6.37 (d, 2H, *J* = 16 Hz), 6.19 (d, *J* = 12 Hz), 5.92 (s, 1H), 5.44 (s, 2H), 2.43 (s, 3H), 2.12 (s, 3H), 1.83 (s, 3H), 1.74–1.25 (m, 6H), 1.03 (s, 6H).^{13}C NMR (101 MHz, $CDCl_3$) δ 168.2, 155.3, 141.1, 139.4, 138.0, 137.8, 137.6, 135.5, 131.9, 131.0, 130.8, 129.9, 129.0, 122.8, 117.9, 117.6, 57.5, 34.0, 29.5, 22.0, 19.9, 19.5, 19.3, 14.5, 13.4. HRMS $[M + H]^+$ 493.2539.

*(2E,4E,6E,8E)-(1-(3,4-Dimethylphenyl)-1H-1,2,3-triazol-4-yl)-methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8g**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (s, 1H), 7.52 (s, 1H), 7.42–7.25 (m, 2H), 7.01 (m, 1H), 6.28 (d, 2H, *J* = 16 Hz), 6.14 (d, *J* = 12 Hz), 5.81 (s, 1H), 5.34 (s, 2H), 2.37 (s, 3H), 2.33 (s, 3H), 2.32(s, 3H), 2.00 (s, 3H), 1.71 (s, 3H), 1.71–1.26 (m, 6H), 1.03 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.0, 154.2, 140.2, 138.5, 137.9, 137.8, 137.4, 135.1, 131.6, 130.8, 130.3, 129.6, 129.1, 122.0, 118.1, 117.8, 57.1, 39.8, 34.4, 33.3, 29.2, 21.9, 20.0, 19.6, 19.4, 14.2, 13.1. HRMS $[M + H]^+$ 486.3118.

*(2E,4E,6E,8E)-(1-(Naphthalen-1-yl)-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8h**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (m, 2H), 7.96 (s, 1H), 7.63–7.58 (m, 5H), 7.02 (m, 1H), 6.29 (d, 2H, *J* = 16 Hz), 6.12 (d, 2H, *J* = 16 Hz), 5.85 (s, 1H), 5.44 (s, 2H), 2.38 (s, 3H), 2.03 (s, 3H), 1.71 (s, 3H), 1.61–1.26 (m, 6H), 1.03 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.0, 154.2, 143.6, 140.2, 137.8, 137.3, 135.0, 134.3, 133.7, 131,7, 130.6, 130.3, 129.5, 129.1, 128.6, 128.4, 128.1, 127.2, 126.4, 125.1, 123.7, 122.5, 117.8, 57.2, 39.8, 34.4, 33.3, 29.1, 21.9, 19.4, 14.2, 13.1. HRMS $[M]^+$ 507.2885.

*(2E,4E,6E,8E)-(1-(m-Tolyl)-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8i**).* ^{1}H NMR (100 MHz, $CDCl_3$) δ 8.01 (s, 1H), 7.72 (s, 1H), 7.71–7.59 (m, 3H), 6.73 (t, 1H, *J* = 16 Hz), 6.21 (m, 2H), 5.76 (m, 2H), 5.31 (s, 2H), 5.03 (s 1H), 2.21 (s, 3H), 2.01–1.89 (m, 4H), 1.73 (s, 3H), 1.60 (m, 2H), 1.41 (s, 3H), 1.19 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.7, 153.1, 142.8, 133.2, 130.8, 129.7, 129.5, 128.0, 126.6, 125.5, 124.8, 117.3, 115.4, 57.0, 40.9, 35.0, 32.0, 29.8, 26.0, 22.7, 14.1, 13.0. HRMS $[M]^+$ 471.2883.

*(2E,4E,6E,8E)-(1-Benzyl-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8j**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 (m, 6H), 6.98 (m, 1H), 6.31–6.09 (m, 4H), 5.75 (s, 1H), 5.62 (s, 2H), 4.31 (s, 2H), 2.32 (s, 3H), 2.03 (s, 3H), 1.79 (s, 3H), 1.68–1.44 (m, 6H), 1.00 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.0, 153.9, 143.4, 140.0, 137.8, 137.4, 135.0, 131.5, 130.2, 129.6, 129.0, 123.6, 117.9, 63.2, 57.2, 50.6, 39.8, 31.8, 30.4, 29.5, 29.4, 29.2, 29.1, 26.6, 25.9, 22.7, 14.2, 13.1. HRMS $[M]^+$ 471.2884.

*(2E,4E,6E,8E)-(1-(2-(Trifluoromethyl)phenyl)-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8k**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92–7.57 (m, 5H), 7.01 (m, 1H), 6.31–6.13 (m, 5H), 5.37 (s, 2H), 2.36 (s, 3H), 2.02–1.62 (m, 4H), 1.60–1.23 (m, 8H), 1.45 (s, 6H). ^{1}H NMR (101 MHz, $CDCl_3$) δ 166.7, 153.8, 143.6, 138.5, 137.4, 136.9, 135.8, 133.1, 130.4, 129.2, 127.4, 126.4, 123.8, 121.4, 118.3, 112.7, 56.9, 40.2, 34.0, 29.1, 26.1, 21.9, 19.4, 14.2, 14.1. HRMS $[M]^+$ 525.2601.

*(2E,4E,6E,8E)-(1-(4-Chlorophenyl)-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8l**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (s, 1H), 7.91 (m, 2H), 7.72 (m, 2H), 7.24 (t, 1H, *J* = 16, 12 Hz), 6.50 (m, 2H), 6.35 (m, 2H), 6.02 (s, 1H), 5.56 (s, 2H), 2.59 (s, 3H), 2.22 (s, 3H), 1.93 (s, 3H), 1.90–1.69 (m, 6H), 1.24 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.0, 154.3, 140.3, 137.8, 137.3, 135.6, 134.9, 134.8, 131.8, 130.3, 130.1, 129.5, 129.1, 121.9, 117.6, 57.0, 39.8, 34.4, 33.3, 29.1, 21.9, 19.4, 14.2, 13.1. HRMS $[M]^+$ 491.2339.

*(2E,4E,6E,8E)-(1-(2,4,6-Trichlorophenyl)-1H-1,2,3-triazol-4-yl)-methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8m**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (s, 1H), 7.71 (s, 1H), 7.66 (s, 1H), 7.24 (m, 1H), 6.50 (d, 2H, *J* = 16 Hz), 6.35 (d, 2H, *J* = 12 Hz), 6.03 (s, 1H), 5.57 (s, 2H), 2.59 (s, 3H), 2.22 (s, 3H), 1.92, (s, 3H), 1.83–1.69 (m, 6H), 1.24 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.3, 154.5, 140.3, 137.4, 137.5, 137.6, 135.3, 132.0, 130.6, 130.2, 129.5, 129.1, 129.0, 120.8, 117.7, 57.1, 39.7, 34.3, 33.2, 29.1, 21.9, 19.4, 14.0, 13.3. HRMS $[M]^+$ 457.2726.

*(2E,4E,6E,8E)-(1-(2-Bromophenyl)-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8n**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (s, 1H), 7.72 (s, 1H), 7.57 (d, 2H, *m*), 7.63 (m, 1H), 7.22 (m, 1H), 6.52 (d, 2H, *J* = 16 Hz), 6.33 (d, 2H, *J* = 12 Hz), 6.08 (s, 1H), 5.59 (s, 2H), 2.60 (s, 3H), 2.24 (s, 3H), 1.93, (s, 3H), 1.85–1.70 (m, 6H), 1.23 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.5, 154.3, 140.4, 137.9, 137.7, 137.6, 134.8, 132.0, 131.0, 130.2, 129.8, 129.0, 129.0, 121.1, 117.9, 56.9, 38.2, 34.5, 33.4, 29.0, 22.0, 19.5, 14.4, 13.4. HRMS $[M]^+$ 535.1828.

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*4-Chloro-2-(4-((((2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoyl)oxy)methyl)-1H-1,2,3-triazol-1-yl)benzoic Acid (**8o**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.03 (s, 1H), 8.21 (s, 1H), 7.39 (m, 1H), 7.07 (m, 2H), 6.32 (m, 2H), 6.18 (m, 2H), 5.84 (s, 1H), 5.40 (s, 2H), 2.41 (s, 3H), 2.04 (s, 3H), 1.75 (s, 3H), 1.65−1.30 (m, 6H), 1.06 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.9, 154.7, 150.2, 140.4, 137.8, 137.3, 135.3, 134.8, 132.0, 130.4, 129.5, 129.3, 121.9, 121.6, 120.7, 120.5, 119.8, 117.3, 56.7, 39.8, 34.4, 33.3, 29.1, 21.9, 19.4, 14.2, 19.1. HRMS $[M]^+$ 535.2235.

*(2E,4E,6E,8E)-(1-Phenyl-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8p**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (s, 1H), 7.71 (d, 2H, J = 8 Hz), 7.66 (d, 2H, J = 8 Hz), 7.64 (m, 1H), 7.24 (m, 1H), 6.50 (d, 2H, J = 16 Hz), 6.35 (d, 2H, J = 12 Hz), 6.03 (s, 1H), 5.57 (s, 2H), 2.59 (s, 3H), 2.22 (s, 3H), 1.92, (s, 3H), 1.83−1.69 (m, 6H), 1.24 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.0, 154.2, 140.2, 137.8, 137.3, 137.1, 135.0, 131.7, 130.3, 129.9, 129.5, 129.1, 129.0, 120.8, 117.7, 57.1, 39.7, 34.3, 33.2, 29.1, 21.9, 19.4, 14.0, 13.1. HRMS $[M]^+$ 457.2726.

*(2E,4E,6E,8E)-(1-(3-Bromophenyl)-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8q**).* ^{1}H NMR (100 MHz, $CDCl_3$) δ 8.00 (s, 1H), 7.70 (s, 1H), 7.69−7.61 (m, 3H), 6.74 (t, 1H, J = 16 Hz), 6.19 (m, 2H), 5.78 (m, 2H), 5.29 (s, 2H), 5.01 (s 1H), 2.19 (s, 3H), 1.98−1.87 (m, 4H), 1.72 (s, 3H), 1.58 (m, 2H), 1.43 (s, 3H), 1.20 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.3, 152.9, 143.3, 133.4, 131.5, 129.6, 129.7, 128.4, 126.8, 125.6, 124.9, 117.3, 115.4, 57.0, 40.9, 35.0, 32.0, 29.8, 26.0, 22.9, 14.6, 13.5. HRMS $[M]^+$ 535.1829.

*(2E,4E,6E,8E)-(1-Pentyl-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8r**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (s, 1H), 6.74- 6.47 (m, 5H), 6.37 (s, 1H), 5.90 (s, 1H), 5.33 (s, 2H), 4.19 (m, 1H), 3.99 (m, 1H), 2.15−2.12 (m, 5H), 1.83 (s, 3H), 1.82 (s, 3H), 1.81 (m, 4H), 1.42 (m, 2H), 1.29−1.25 (m, 4H), 0.98 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.4, 152.4, 140.5, 137.6, 137.3, 136.9, 135.1, 131.6, 131.5, 130.8, 129.2, 124.2, 118.8, 51.7, 38.5, 34.8, 33.8, 29.8, 28.8, 28.8, 26.8, 22.9, 21.0, 20.3, 15.9, 15.9, 14.0. HRMS $[M]^+$ 451.3196.

*(2E,4E,6E,8E)-(1-Octyl-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8s**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (s, 1H), 6.68 (6.74−6.43, 5H), 5.89 (s, 1H), 5.28 (s, 2H), 4.29 (m, 1H), 3.86 (m, 1H), 2.18 (m, 2H), 2.12 (s, 3H), 1.34−1.27 (m, 16H), 1.03 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.0, 153.1, 141.2, 138.0, 137.5, 136.6, 135.4, 132.2, 132.0, 131.1, 129.4, 124.5, 119.0, 52.2, 39.2, 34.3, 32.0, 29.5, 29.2, 29.1, 28.4, 27.9, 27.3, 23.1, 21.4, 20.1, 16.2, 16.0, 14.2. HRMS $[M]^+$ 493.3664.

*(2E,4E,6E,8E)-(1-Dodecyl-1H-1,2,3-triazol-4-yl)methyl 3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoate (**8t**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (s, 1H), 6.74 (6.74−6.43, 5H), 5.93 (s, 1H), 5.32 (s, 2H), 4.26 (m, 1H), 3.88 (m, 1H), 2.15 (m, 2H), 2.09 (s, 3H), 1.82−1.65 (m, 6H), 1.53 (m, 2H), 1.31−1.25 (m, 16H), 1.00 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.4, 152.4, 140.5, 137.6, 137.3, 136.9, 135.1, 131.6, 131.5, 130.8, 129.2, 124.2, 118.8, 51.7, 38.5, 33.8, 31.6, 29.0, 28.9, 28.9, 28.9, 28.9, 28.9, 28.8, 28.8, 27.7, 26.9, 22.9, 21.0, 20.3, 15.9, 15.9, 14.0. HRMS $[M]^+$ 549.4291.

Reagents and Chemicals. All *trans*-retinoic acid (Cat. No. R 2625), Dulbecco's minimal essential medium (Cat. No. D1152), penicillin-G sodium salt (Cat. No. 3032), streptomycin sulfate (Cat. No. 1400000), sodium pyruvate (Cat. No. P5280), phosphate buffered saline (Cat. No. P3813), RIPA buffer (Cat. No. R0278), anti-NF-H (Cat. No. N5389), anti-mouse FITC conjugated (Cat. No. F0257), bicinchoninic acid (BCA) assay kit (B9643), BSA, DAPI, and Triton X-100 were purchased from Sigma. FBS (fetal bovine serum; Cat. No 26140-079) and anti-rabbit Alexa Fluor 546 conjugate (Cat. No. A-11010) were obtained from Invitrogen. Anti-Neu N (Cat. No. D3S3I), β-actin (Cat. No. 8H10D10), and anti-mouse HRP-linked antibody (Cat. No. 7076) were purchased from Cell Signaling Technology. Immobilon Western Chemilumenescent HRP substrate (Cat. No. WBKLSO100) and PVDF membrane were obtained from Millipore (Bedford, MA). Prestained protein ladder was obtained from Bio Rad (Cambridge, MA).

Cell Lines and Culture. The Neuro2a mouse neuroblastoma cell line was obtained from the National Centre for Cell Sciences, Pune-India. Cells were seeded into flasks, plates, or dishes in Dulbecco's minimal essential medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin in a humid atmosphere of 5% CO_2 and 95% air at 37 °C. The media was changed on alternative days.

Immunoflorescence. In order to determine the expression of different neuronal markers after treating the Neuro2a cells with different RA-derivatives, the cells were grown in 6-well plates at a density of 1 × 10^5 cells per well in complete DMEM. After 24 h, the media was changed and the cells were treated with different concentrations of RA-derivatives. The serum level of the media was reduced to 1% from 10% which becomes favorable for the differentiation. The media having final concentration of 10 μM retinoic acid, **8a**, **8e**, **8f**, and **8k**, respectively, was changed every day continuously for 5 days. On day 6, the media was aspirated, and cells were washed twice with PBS and covered up to a depth of 2−3 mm with 4% formaldehyde diluted in warm PBS and allowed to fix for 15 min at room temperature. After fixation, the cells were rinsed 5 times with 1× PBS and permeabilized with triton X-100 (0.5%) for 10 min, washed again with 1x PBS three times for 5 min each and blocked in blocking buffer (BSA 1% in PBS) for 60 min. The blocking buffer was aspirated and cells were incubated with primary antibody (1:200 ratio) overnight at 4 °C. After overnight incubation, the cells were washed 6 times with 1× PBS for 5 min each and incubated with fluorochrome-conjugated secondary antibody (1:1000) for 1 h at room temperature. Then the cells were washed with 1× PBS in the dark three times for 5 min each, and finally 1 μL/mL DAPI (1 mg/mL stock) was added and cells observed under the EVOS FLoid cell imaging station with the desired filter.

Western Blotting. To determine the effect of RA-derivatives on the expression levels of NeuN (neuronal marker), Neuro2a cells were grown in 90 mm dishes in complete DMEM for 24 h. The media was changed and the cells were treated with various concentrations of RA-derivatives. The serum level of the media was reduced to 1% from 10% which becomes favorable for the differentiation. The media having a final concentration of 10 μM of **8a**, **8e**, **8f**, and **8k**, respectively, was changed each day continuously for 5 days. On day 6, the media was aspirated, and cells were washed with PBS twice, scraped, and centrifuged at 3000 rpm for 5 min at 4 °C. The pellet was washed with PBS and subjected to lysis with RIPA (radio-immunoprecipitation assay) lysis buffer supplemented with protease inhibitor cocktail for 25−30 min on ice. The lysates were centrifuged at 14 000 rpm for 15 min at 4 °C to pellet cell debris. The supernatant containing the soluble protein was carefully transferred to precooled tubes on ice for analysis. Protein estimation was done by using a commercially available bicinchoninic acid (BCA) assay kit (Sigma, Saint Louis, MO) according to the manufacturer's instructions. For NeuN expression, which is a 54 kDa protein, an equal amount of protein (60 μg) was loaded onto 12% SDS-PAGE in different wells. Prestained protein ladder (Bio Rad) covering a broad range of molecular weights (10−210 kDa) was run in parallel and used to determine the molecular weights of the detected proteins. The proteins were transferred onto a PVDF membrane. The membrane was blocked with blocking buffer (5% BSA in TBST) for 2 h in order to minimize nonspecific binding and then incubated with anti-NeuN (1:500) overnight at 4 °C. Following primary antibody incubation, the membrane was washed three times with TBST (pH 7.7) for 5 min each and incubated with secondary HRP linked antibody for 1 h. Following incubation, the membrane was washed three times with TBST (pH 7.7) for 15 min each. Subsequently, the membrane bound antibodies were visualized with ECL detection reagent (Millipore) according to the manufacturer's instructions. The X-ray films were scanned, and the optical densities of the bands were analyzed by densitometry using ImageJ software.

Mass Spectrometric Analysis of RA, 8a, 8e, 8f, and 8k in Whole Cell Lysates. Neuro2a cells were seeded in 60 mm tissue culture dishes and incubated with a 10 μM concentration of RA, **8a**, **8e**, **8f**, and **8k** for 5 days. Following incubation, the cells were scraped

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and pelleted down by centrifugation at 3000 rpm for 5 min. The cell pellets were lysed in $CHCl_3/CH_3OH$ (1:2, v/v) according to the method of Kroesen et al.[24] and centrifuged at 14000 rpm for 15 min, and then the supernatant was transferred to the LC-MS sample vials for LC-MS analysis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00267.

^1H and ^{13}C NMR spectra of all the compounds and LC-MS data of active compounds in cytosol (PDF)

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#A.M.L. and N.J.D. contributed equally to this work. A.M.L and N.J.D carried out the experimental work and partially wrote the manuscript; A.H. designed few experiments and facilitated the work; W.A.S facilitated the work; M.A. and B.A.B. designed the experiments and wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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