Synthesis and Biological Activity of Novel Retinamide and Retinoate Derivatives

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Retinoic acid and its amide derivative, *N*-(4-hydroxyphenyl)retinamide (4-HPR), have been proposed as chemopreventative and chemotherapeutic agents. However, their low cytotoxic activity and water solubility limit their clinical use. In this study, we synthesized novel retinoid derivatives with improved cytotoxicity against cancer cells and increased hygroscopicity. Our syntheses were preceded by selective *O*-acylation and *N*-acylation, which led to the production of retinoate and retinamide derivatives, respectively, in one pot directly from aminophenol derivatives and retinoic acid without protection. Transcription assays in COS-1 cells indicated that the *N*-acylated derivatives (2A—5A) and 4-HPR (1A) were much weaker ligands for all three subtypes of retinoic acid receptor (RAR) than all-*trans* retinoic acid (ATRA), although they showed some selectivity for RARβ and RARγ. In contrast, the *O*-acylated retinoate derivatives (1B—5B) activated all three RAR isotypes without specificity to an extent similar to ATRA. The cytotoxicity was determined using an MTT assay with HCT116 colon cancer cells, and the IC_{50} of *N*-acylated retinamide derivative 4A and *O*-acylated retinoate derivative 5B was 1.67 μM and 0.65 μM, respectively, which are about five and 13-fold better than that of 4-HPR (8.21 μM), a prototype *N*-acylated derivative. When retinoate derivative 5B was coupled to organic acid salts, the resulting salt derivatives 5C and 5D had RAR activation and cytotoxicity similar to those of 5B. These data may delineate the relationship between the structure and function of retinoate and retinamide derivatives.

Key words retinamide; retinoate; derivative; cytotoxicity; retinoic acid receptor (RAR)

The amide analogue of retinoic acid, *N*-(4-hydroxyphenyl)retinamide (4-HPR), was developed as a therapeutic agent for various skin conditions. It is currently being assessed as a drug to prevent and treat several cancers.[1,2] Unlike all-*trans* retinoic acid (ATRA), 4-HPR selectively binds to retinoic acid receptors (RAR) β and γ and activates them less strongly than ATRA. These properties make 4-HPR less toxic and substantially less teratogenic than ATRA.[3] Therefore 4-HPR has been used as an antitumor agent in animal studies[4,5] and as a chemopreventive agent for several cancers in clinical trials.[6—8] A number of *in vitro* biological studies indicated that 4-HPR has marked cytotoxicity in various cancer cells by inducing apoptosis.[9,10]

However, 4-HPR has a major limitation in its clinical use. The plasma levels in patients receiving 200 mg of 4-HPR daily are less than 1 μM, which is far less than the effective concentration (usually 10 μM) required to induce apoptosis *in vitro*. Therefore it is necessary to use higher doses of 4-HPR or to synthesize other derivatives with better clinical efficacy. Taking the second approach, several retinamide derivatives have recently been synthesized, which have hydroxyl, carboxy, or methoxy substitutions on the terminal phenylamine ring.[11,12] Of these derivatives, 3-HPR showed the most active growth inhibition in four bladder cancer cell lines,[11] while 2-CPR, a carboxyl derivative, was the most effective in some head and neck, and lung cancer cell lines.[12]

To develop other potentially potent antitumor agents, we synthesized many retinoid derivatives. Since butyric acid has been implicated as an anticancer agent,[13] we introduced aminophenol or its butyryl derivatives into ATRA by selective *N*-acylation (carboxamide) and *O*-acylation (carboxyl ester) to produce retinamide (**1A**—**5A**) and retinoate (**1B**—**5B**) derivatives, respectively. To increase their water solubility, a selected derivative (**5B**) was coupled to organic acid salts. We determined the selectivity of these compounds in the activation of RAR subtypes and measured their cytotoxic activity against HCT116 colon cancer cells. Our data may be useful for delineating the relationship between structure and function in retinoate and retinamide derivatives.

Results and Discussion

Chemistry The synthesis of retinoid derivatives **1A**—**5A** and **1B**—**5B** is illustrated in Charts 1 and 2. As shown in Chart 1, the selective *N*- and *O*-acylation of ATRA and 4-aminophenol (4-AP) gave either the corresponding carboamide (method A) or carboester (method B). The coupling of ATRA and 4-AP in method A, which uses EDCI/DMAP as a reagent, creates 4-HPR (**1A**), which contains a carboamide bond. In contrast, the coupling of the same compounds using method B, which uses DCC/DMAP as a reagent, creates 4-APR (**1B**) with a carboester bond. As previously reported,[14] 4-HPR could also be prepared from ATRA *via* retinoyl chloride using method C. Both reactions involve acylation at the carboxy moiety of ATRA. At present, it is not clear how one acylation reaction can give rise to two different compounds. It is generally accepted that the coupling of an acid and amine in the presence of EDCI/DMAP or DCC/DMAP results in a reaction producing a carboamide bond.[15—17] In preparing 4-HPR, it is plausible that EDCI/DMAP activates the NH_2 group of 4-AP by preferentially shifting a proton to the OH group, making the NH_2 group more likely to react with the carboxy moiety of ATRA. How is 4-APR produced? ATRA can be activated by DCC/DMAP, and its activated carboxy moiety reacts with the OH group of 4-AP to form 4-APR. In the presence of DCC/DMAP, it is likely that the NH_2 group of 4-AP serves

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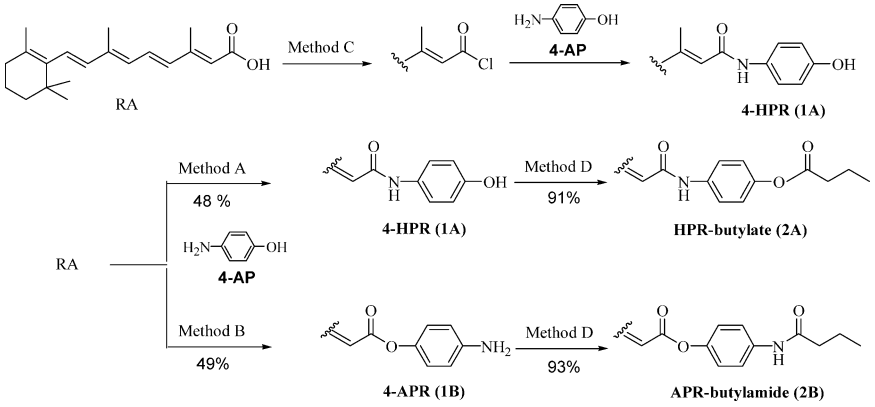
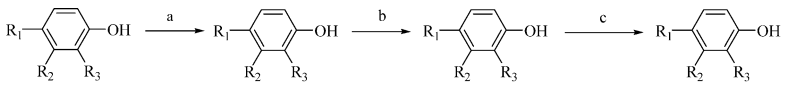


Chart 1. Synthesis of Retinamide (**1A**, **2A**) and Retinoate (**1B**, **2B**) Derivatives

Methods A, B, C, and D are described in Experimental.



6. R_1=NH_2HCl, R_2=OH, R_3=H
7. R_1=CbzNH, R_2=OH, R_3=H
8. R_1=NO_2, R_2=H, R_3=OH
9. R_1=NO_2, R_2=H, R_3=NH_2
10. R_1=CbzNH, R_2=$OCO(CH_2)CH_3$, R_3=H
11. R_1=NO_2, R_2=H, R_3=$OCO(CH_2)_2CH_3$
12. R_1=NO_2, R_2=H, R_3=$NHCO(CH_2)_2CH_3$
13. R_1=NH_2, R_2=$OCO(CH_2)_2CH_3$, R_3=H
14. R_1=NH_2, R_2=H, R_3=$OCO(CH_2)_2CH_3$
15. R_1=NH_2, R_2=H, R_3=$NHCO(CH_2)_2CH_3$

Chart 2. Synthesis of Intermediates **6**—**15**

Conditions: (a) Cbz–Cl, TEA, DMF; (b) butyryl chloride, NMM; (c) 10% Pd/C, H_2, MeOH.

as a base to activate the OH group of 4-AP, allowing *O*-acylation and creating 4-APR as the sole product.[18,19] Therefore this method enables the efficient preparation of two different types of retinoid derivative *via* the simple, selective coupling of retinoic acid to the NH_2 or OH group without protection. The structures of 4-HPR and 4-APR were elucidated by IR spectroscopy based on the carbonyl absorption frequencies of 1634 cm^{-1} for 4-HPR and 1716 cm^{-1} for 4-APR. Method D was used to introduce a butyryl group into derivatives **1A** (4-HPR) and **1B** (4-APR), producing derivatives **2A** and **2B** in yields of 91% and 93%, respectively.

To synthesize other retinamide and retinoate derivatives, we first synthesized target compounds **13**, **14**, and **15**, as shown in Chart 2. To introduce selectively a butyryl group into the *ortho*-OH (R2) of 4-amino resorcinol **6**, a series of reactions was performed. First, the amine of **6** was protected with Cbz–Cl (1.2 eq)/TEA (1.2 eq) to obtain the *N*-Cbz **7** in 89% yield. Acylation of **7** with butyryl chloride (1.5 eq)/NMM (1.5 eq) according to method D provided the monobutyrated compound **10** in 65% yield. Nitro compounds **11** and **12** were prepared from starting materials **8** and **9** by butyrylation of the OH or NH_2 group (R3) *meta* to the nitro group, in 61% and 72% yield, respectively. Compounds **10**, **11**, and **12** were catalytically hydrogenated with 10% Pd/C in MeOH, producing the corresponding compounds **13**, **14**, and **15** in high yields. Intermediates **13**—**15** were coupled with ATRA by selective *N*- or *O*-acylation according to methods A and B to give final compounds **3A**—**5A** and **3B**—**5B**, respectively, as shown in Table 1. The structures of these compounds were verified by IR spectroscopy.

Consistent with the general notion that most synthetic retinoids are lipophilic, our retinoid derivatives (Fig. 1) were insoluble in aqueous solvents and precipitated *in vivo*. Unfortunately, this was true for derivative **5B**, which has significant biological activity (Table 2). Derivative **5B** has a terminal NH_2 group, which can form salts to increase its water solubility. Although the hydrochloride and hydrobromide salts were more hydrophilic, these compounds were very unstable

Table 1. Summary of Two Different Retinoids (Retinamide and Retinoate Derivatives) Prepared According to Methods A and B

Starting materials	Method A[a]		Method B[b]	
	Products	Yield (%)	Products	Yield (%)
4-AP	**1A**	48	**2A**	49
1A	**2A**[c]	91	**2B**[c]	93
1B				
13	**3A**	47	**3B**	52
14	**4A**	50	**4B**	45
15	**5A**	51	**5B**	43

a) Method A: EDCI (2.0 eq)/DMAP/CH_2Cl_2, room temperature, 4 h. *b*) Method B: DCC (2.0 eq)/DMAP/CH_2Cl_2, room temperature, 4 h. *c*) **2A** and **2B** were prepared from **1A** and **1B**, respectively, using Method D: butyryl chloride (1.2 eq)/NMM (1.2 eq)/0 °C, 1 h.

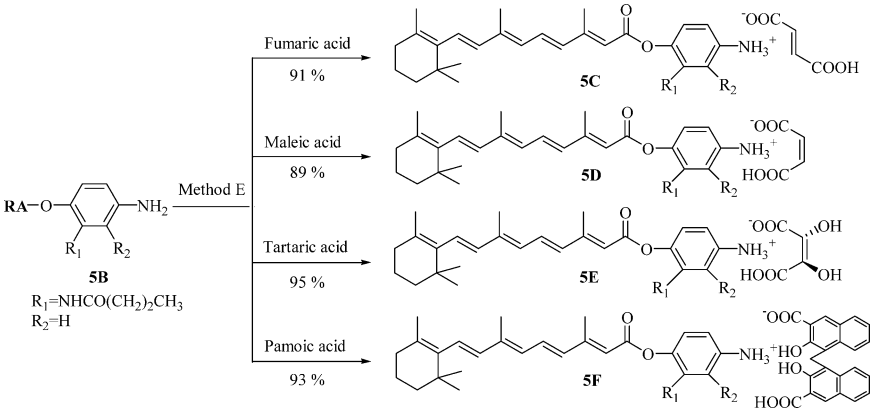


Chart 3. Synthesis of Organic Acid Salts of Retinoate Derivative **5B**

These compounds were prepared according to method E: THF, 45 °C→hexane, 10 °C.

under strongly acidic conditions. Therefore we prepared organic salts **5C**—**F** by reacting the free base form of **5B** with a stoichiometric amount of fumaric, maleic, tartaric, or pamoic acid in THF according to Method E,[20] as shown in Chart 3.

Effect of Retinoid Derivatives on RARs To determine the activity and specificity of our retinoid derivatives on RAR subtypes, a series of conventional transcriptional assays was performed using a retinoid responsive reporter gene in the presence of each receptor. COS-1 cells were cotransfected with a receptor expression vector (RARα, β, and γ in pSG5) and reporter gene [$(RARE)_3$-tk-CAT], and treated with increasing concentrations of ATRA, 4-HPR, or other retinoids. CAT ELISA was used to measure the amount of CAT protein induced in response to the retinoid derivatives. For each retinoid and receptor, a dose–response curve was plotted. The concentration required for 50% activation (EC_{50} value) was determined by interpolation from the curves (Table 2). ATRA, a physiological RAR ligand, strongly activated all three RARs with an EC_{50} of 1.8—5.1×10^{-9} M. As previously reported, 4-HPR (**1A**) was selectively active for RARβ and γ.[21,22] Retinamide derivatives **2A**—**5A**, like 4-HPR, were all more active for RARβ and γ with a lower V_{max} than ATRA, whereas retinoate derivatives **1B**—**5B**, except for **3B**, were as active as ATRA for all three RARs with a similar V_{max}. At present, it is not clear why **3B** is not as active as the other derivatives. The side functional group of **3B** may interfere with the coactivator binding required for RAR activation, but this remains to be determined using a coactivator as in CBP and SRC-1.[23] Organic salts **5C** and **5D** were as active as their parental compound **5B** for all RARs.

Cytotoxic Activity of Retinoid Derivatives The cytotoxic potential of the retinoid derivatives was investigated by determining their concentrations required for 50% growth inhibition (IC_{50} value) for HCT116 colon cancer cells. Following treatment of the cells with increasing concentrations of retinoid derivatives (0, 0.5, 1, 2.5, 5, 10 μM) for 48 h, proliferation was assayed using MTT, and dose–response curves were plotted. The IC_{50} value was determined by interpolation from the curves. As summarized in Table 2, the IC_{50} value of derivative **5B** was 0.65 μM for HCT116 cells, which was 13-fold better than that of 4-HPR (8.21 μM). The IC_{50} values of the other derivatives was slightly better than (**2B**, **3A**, **B**, **4A**), similar to (**1B**, **2A**), or much worse (**4B**, **5A**) than that of 4-HPR. Of the retinamide derivatives, **3A** and **4A** were superior to 4-HPR, the parental retinamide. Retinoate derivatives **2B**, **3B**, and **5B** were more cytotoxic than 4-HPR. Retinoate derivative **4B** and retinamide derivative **5A** were much less cytotoxic than 4-HPR. Interestingly, **4B** was a strong ligand for all RARs, whereas **5A** was nearly inactive for all RARs, suggesting that RAR activation is not correlated with cytotoxicity in HCT116 cells. Organic salts **5C** and **5D** were as cytotoxic as parental compound **5B** in these cells. For a better clinical outcome, we searched for retinoid derivatives with a submicromolar IC_{50} value and low side effects. Although the IC_{50} value of 4-HPR is relatively high, its relatively few side effects made it useful in clinical trials. In this regard, our derivative **5B** and its organic salt forms, the most effective of the derivatives tested, are promising cancer prodrugs, although their side effects and hygroscopicities remain to be determined in animal studies.

Table 2. EC_{50} and IC_{50} Values of Retinoid Derivatives for RAR Activation and Cytotoxicity

Retinoids	EC_{50} (M)[a] for RAR			IC_{50} (μM)[b,c] for HCT116 cells
	α	β	γ	
ATRA	5.1×10^{-9}	3.7×10^{-9}	1.8×10^{-9}	>20
1A (4-HPR)	5.3×10^{-7}	7.6×10^{-9}	9.9×10^{-9}	8.21
1B	8.6×10^{-9}	3.9×10^{-9}	2.8×10^{-9}	9.73
2A	4.6×10^{-7}	1.3×10^{-7}	1.5×10^{-7}	14.71
2B	4.8×10^{-9}	5.1×10^{-9}	4.9×10^{-9}	3.01
3A	6.4×10^{-7}	8.3×10^{-8}	8.1×10^{-8}	3.15
3B	3.2×10^{-8}	5.1×10^{-8}	5.3×10^{-8}	4.08
4A	8.3×10^{-8}	3.2×10^{-8}	2.4×10^{-8}	1.67
4B	7.5×10^{-9}	8.1×10^{-9}	7.4×10^{-9}	>20
5A	$\gg10^{-6}$	$>10^{-6}$	$>10^{-6}$	>20
5B	4.9×10^{-9}	4.4×10^{-9}	8.7×10^{-9}	0.65
5C	4.1×10^{-9}	5.2×10^{-9}	4.4×10^{-9}	0.78
5D	5.2×10^{-9}	6.1×10^{-9}	6.3×10^{-9}	0.73

a) The dose–response curves were plotted using data obtained from transcription assays with individual RAR subtypes in the presence of increasing concentrations of test retinoid. EC_{50} values, the concentrations required for 50% activation, were determined by interpolation of the curves. *b*) The dose–response curves were plotted by MTT assay as described in Experimental. IC_{50} values, the concentrations required for 50% growth inhibition, were determined by interpolation of the curves. Each experiment was performed in triplicate and repeated a minimum of three times. *c*) $p<0.05$ for all IC_{50} values.

Using data not presented here, we found that the position of the butyryl group is critical for activity, especially in the case of **5B**. A derivative with the butyryl group in the *meta*

position of the amine group inhibited HCT116 cell growth 50 times more potently than did a derivative with the butyryl in the *ortho* position. When the butyryl groups of **3A** and **5B** were removed, the cytotoxic potential was completely abrogated. Furthermore, the number of carbons in the side functional group was also important in both **3A** and **5B**. A derivative with four carbons at that position, *i.e.*, a butyryl group, was the most potent under our assay conditions.[24] These overall results suggest that increased cytotoxicity is closely associated with side chain length. Based on the structure–function relationship, we propose that a cellular target protein(s) responds to the four-carbon fatty acid chain by direct association.

Conclusion

This paper describes a new synthetic method for retinoid derivatives that involves selectively controlling *N*-acylation (carboxamide) and *O*-acylation (carboxyl ester). Our method led to the facile synthesis of various retinoid derivatives in one pot directly from retinoic acid and aminophenol derivatives without protection. In terms of RAR activation, retinamide derivatives **2A**—**5A** functioned like 4-HPR, *i.e.*, they were specific for RAR β and γ, whereas, like ATRA, retinoate derivatives **1B**—**5B** were active for all three RARs without specificity. In cytotoxicity assays using HCT116 cells, we found that retinamide derivatives **3A** and **4A** and retinoate derivatives **2B**, **3B**, and **5B** were superior to 4-HPR, the parental retinamide. Data derived from derivatives **4B** and **5A** indicated that RAR activation is not correlated with cytotoxicity. Organic salts **5C** and **5D** were similar to parent compound **5B** in terms of RAR activation and cytotoxicity in HCT116 cells. Although the data were not shown, the increased cytotoxicity of retinoid derivatives appears to be closely associated with the side chain length of the functional group. From these overall results, we conclude that our derivative **5B** and its organic salt forms, the most effective of the derivatives tested, are promising cancer prodrugs.

Experimental

ATRA was purchased from Sigma Chemical Co. (Sigma-Aldrich Korea, Seoul). The dry DMF was stored over 4-Å sieves and degassed before use by bubbling argon through it for at least 1 h. Dry CH_2Cl_2 was obtained by distillation from CaH_2. The other commercially available reagents and solvents were used without further purification. All reactions were conducted under an Ar atmosphere, except for those reactions utilizing water as a solvent. They were monitored by TLC (Merck Kieselgel 60, F_{254}). All the products prepared were purified by flash column chromatography on silica gel 60 (Merck, 230—400 mesh). ^{1}H- and ^{13}C-NMR spectra were recorded on a Brüker AC-200F and JEOL JNM EX-400 using $CDCl_3$ and DMSO-d_6 as a solvents. All chemical shifts (δ) are quoted in ppm downfield from TMS and coupling constants (J) are given in Hz. Mass spectra were measured on an Agilent 1100 LC/MSD (API-ES) mass spectrometer, Micro MS-autospec/OA Tof (HR-EI-MS). IR spectra were recorded on Perkin Elmer 16FPC FT-IR spectrophotometer and frequencies are given in reciprocal centimeters.

Method A. (2*E*,4*E*,6*E*,8*E*)-[3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-nona-2,4,6,8-tetraenoylamino]-(4-hydroxy)phenylamide (1A, 4-HPR) To a solution of EDCI (0.13 g, 0.66 mmol) in dry CH_2Cl_2 (5 ml) was added retinoic acid (0.10 g, 0.33 mmol) in dry CH_2Cl_2 (5 ml). The solution was stirred at room temperature for 0.5 h. To this mixture were added 4-amino phenol (0.053 g, 0.33 mmol) in dry DMF (2 ml) and DMAP (cat.), and the mixture was stirred for 4—5 h. The reaction was quenched with NH_4Cl (aq.) and extracted with EtOAc (30 ml). The extracts were washed with H_2O and brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (EtOAc/hexane=1/4) to give **1A** (0.062 g, 48%) as a yellow solid. mp 170—171 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.34 (d, J=8.80, 2H), 7.13 (s, 1H), 6.98 (dd, J=14.90, 11.40, 1H), 6.77 (d, J=8.80, 2H), 6.24 (m, 4H), 5.78 (s, 1H), 5.51 (s, 1H), 2.41 (s, 3H), 2.01 (br s, 5H), 1.72 (s, 3H), 1.60 (m, 2H), 1.46 (m, 2H), 1.02 (s, 6H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 163.93, 152.96, 147.19, 142.92, 137.72, 137.07, 136.82, 135.94, 131.04, 129.95, 129.17, 129.09, 127.16, 122.88, 120.53, 114.87, 39.20, 33.85, 32.61, 28.84, 21.53, 18.79, 13.24, 12.61. IR (KBr, cm^{-1}) 2924, 2858, 1634 (NH–C=O–), 1582. MS: m/z (%)=392 (M^+, 100), 283 (13), 224 (7), 102 (18). HR-EI-MS m/z 391.2512 (Calcd for $C_{26}H_{33}NO_2$, 391.2511).

4-{(2*E*,4*E*,6*E*,8*E*)-[3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-nona-2,4,6,8-tetraenoylamino]}-phenyl Butanoate (2A) Using method D, yield: 91% as a yellow solid, mp 146—148 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.56 (d, J=7.84, 2H), 7.19 (s, 1H), 7.04 (d, J=8.80, 2H), 7.00 (dd, J=15.00, 11.40, 1H), 6.26 (m, 4H), 5.78 (s, 1H), 2.53 (t, J=7.34, 2H), 2.41 (s, 3H), 2.01 (br s, 5H), 1.78 (m, 2H), 1.72 (s, 3H), 1.60 (m, 2H), 1.46 (m, 2H), 1.04 (t, J=7.34, 3H), 1.02 (s, 6H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 172.21, 165.01, 150.48, 146.53, 139.21, 137.60, 137.14, 135.85, 135.16, 130.30, 129.77, 129.39, 128.39, 121.77, 121.07, 120.61, 115.42, 39.69, 36.26, 34.33, 33.18, 29.03, 21.83, 19.33, 18.54, 13.85, 13.74, 12.96. MS: m/z (%)=462 (M^+, 33), 461 (100), 339 (7), 282 (18). HR-EI-MS m/z 461.2931 (Calcd for $C_{30}H_{39}NO_3$, 461.2930).

2-{(2*E*,4*E*,6*E*,8*E*)-[3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-nona-2,4,6,8-tetraenoylamino]}-5-hydroxyphenyl Butanoate (3A) Yield: 47% as a yellow solid, mp 143—145 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.63 (s, 1H), 7.48 (s, 1H), 7.05 (dd, J=14.90, 11.40, 1H), 6.86 (d, J=8.60, 1H), 6.73 (d, J=2.50, 1H), 6.57 (dd, J=8.60, 2.50, 1H), 6.22 (m, 4H), 5.86 (s, 1H), 2.54 (t, J=7.30, 2H), 2.41 (s, 3H), 2.01 (br s, 5H), 1.80 (m, 2H), 1.72 (s, 3H), 1.60 (m, 2H), 1.46 (m, 2H), 1.00 (m, 3H), 1.02 (s, 6H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 172.41, 166.90, 152.86, 150.13, 149.19, 140.07, 137.67, 137.17, 134.85, 131.47, 130.11, 129.39, 128.96, 123.96, 122.46, 119.03, 113.29, 112.94, 39.57, 36.18, 34.24, 33.10, 28.94, 21.74, 19.18, 18.43, 13.83, 13.60, 12.94. IR (KBr, cm^{-1}) 3318, 2960, 2924, 1746 (O–C=O–), 1635 (NH–C=O–), 1577. MS: m/z (%)=478 (M^+, 100), 283 (40), 161 (35). HR-EI-MS m/z 477.2877 (Calcd for $C_{30}H_{39}NO_4$, 477.2879).

5-{(2*E*,4*E*,6*E*,8*E*)-[3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-nona-2,4,6,8-tetraenoylamino]}-2-hydroxyphenyl Butanoate (4A) Yield: 50% as a yellow solid, mp 165—167 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.58 (br s, 1H), 7.48 (br s, 1H), 6.92 (m, 3H), 6.60 (d, J=8.80, 1H), 6.24 (m, 4H), 5.81 (s, 1H), 2.57 (t, J=7.35, 2H), 2.41 (s, 3H), 2.01 (br s, 5H), 1.80 (m, 2H), 1.72 (s, 3H), 1.60 (m, 2H), 1.46 (m, 2H), 1.05 (m, 3H), 1.02 (s, 6H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 171.78, 164.98, 150.68, 148.41, 140.28, 138.39, 139.34, 137.17, 136.95, 135.52, 135.46, 129.44, 129.33, 127.86, 122.29, 122.17, 110.36, 108.33, 39.52, 35.54, 33.89, 32.72, 28.61, 21.42, 18.85, 18.11, 13.34, 13.24, 12.54. MS: m/z (%)=478 (M^+, 100), 391 (28), 283 (33). HR-EI-MS m/z 477.2878 (Calcd for $C_{30}H_{39}NO_4$, 477.2879).

{(2*E*,4*E*,6*E*,8*E*)-[3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-nona-2,4,6,8-tetraenoylamino]}-(3-butylamino-4-hydroxy)phenylamide (5A) Yield: 51% as a yellow solid, mp 186—190 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.31 (s, 1H), 8.97 (s, 1H), 8.70 (s, 1H), 7.92 (br s, 1H), 7.25 (dd, J=8.70, 2.31, 1H), 6.94 (dd, J=14.90, 11.40, 1H), 6.85 (d, J=8.70, 1H), 6.24 (m, 4H), 5.91 (s, 1H), 2.39 (m, 5H), 2.01 (br s, 5H), 1.72 (s, 3H), 1.67 (m, 4H), 1.46 (m, 2H), 1.02 (s, 6H), 0.98 (t, J=7.38, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 173.19, 165.28, 149.37, 144.45, 138.82, 137.70, 137.31, 135.70, 131.27, 129.83, 129.80, 129.64, 128.26, 126.25, 122.09, 118.11, 117.75, 113.75, 39.59, 38.81, 34.24, 33.08, 28.95, 21.74, 19.23, 19.21, 13.69, 13.59, 12.87. IR (KBr, cm^{-1}) 3293, 2960, 1649 (NH–C=O–), 1506. MS: m/z (%)=478 (30), 477 (M^+, 100), 212 (15), 175 (8). HR-EI-MS m/z 476.3038 (Calcd for $C_{30}H_{40}N_2O_3$, 476.3039).

Method B. 4-Amino Phenyl (2*E*,4*E*,6*E*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetraenoate (1B, 4-APR) Yield: 49% as a yellow form. To a solution of DCC (0.14 g, 0.66 mmol) in dry CH_2Cl_2 (5 ml) was added retinoic acid (0.10 g, 0.33 mmol) in dry CH_2Cl_2 (5 ml). The solution was stirred at room temperature for 0.5 h. To this mixture were added 4-amino phenol (0.034 g, 0.33 mmol) in dry DMF (2 ml) and DMAP (cat.), and the mixture was stirred for 4—5 h. The reaction was quenched with NH_4Cl (aq.) and extracted with EtOAc (30 ml). The extracts were washed with H_2O and brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (EtOAc/hexane=1/4) to give **1B** (0.064 g, 49%) as a yellow form, mp 147—150 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.10 (dd, J=14.90, 11.40) , 6.88 (d, J=8.74, 2H), 6.65 (d, J=8.75, 2H), 6.24 (m, 4H), 5.96 (s, 1H), 2.39 (s, 3H), 2.01 (br s, 5H), 1.72 (s, 3H), 1.61 (m, 2H), 1.46 (m, 2H), 1.02 (s, 6H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 166.07, 154.92, 143.88, 142.92, 140.13, 137.64, 137.19, 134.88, 131.66, 130.13, 129.41, 128.95, 122.34, 117.49, 116.14, 115.60, 39.57, 34.24, 33.09, 28.94, 21.74, 19.18, 13.99, 12.92. IR (KBr, cm^{-1}) 3462, 3375,

2919, 1716 (O–C=O–), 1582. MS: *m/z* (%)=393 (27), 392 (M^+, 100), 283 (20), 175 (8). HR-EI-MS *m/z* 391.2513 (Calcd for $C_{26}H_{33}NO_2$, 391.2511).

4-(Butyrylamino)phenyl (2*E*,4*E*,6*E*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetraenoate (2B) Using method D, yield: 93% as a yellow solid, mp 148—152 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.50 (d, *J*=8.75, 2H), 7.21 (s, 1H), 7.05 (m, 3H), 6.24 (m, 4H), 5.97 (s, 1H), 2.40 (s, 3H), 2.32 (t, *J*=7.32, 2H), 2.01 (br s, 5H), 1.72 (m, 2H), 1.70 (s, 3H), 1.61 (m, 2H), 1.46 (m, 2H), 1.02 (s, 6H), 1.00 (t, *J*=7.32, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 171.23, 165.17, 155.63, 146.76, 140.45, 137.62, 137.14, 135.40, 134.66, 132.04, 130.20, 129.34, 129.13, 122.07, 120.73, 117.02, 39.56, 39.49, 34.23, 33.09, 28.93, 21.73, 19.17, 19.02, 14.06, 13.72, 12.94. MS: *m/z* (%)=462 (M^+, 33), 461 (100), 339 (19), 283 (30). HR-EI-MS *m/z* 461.2931 (Calcd for $C_{30}H_{39}NO_3$, 461.2930).

4-Amino-3-(butyryloxy)phenyl (2*E*,4*E*,6*E*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetraenoate (3B) Yield: 52% as a yellow form, mp 119—123 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.11 (dd, *J*=15.00, 11.40, 1H), 6.87 (s, 1H), 6.77 (m, 1H), 6.25 (m, 5H), 6.00 (s, 1H), 3.63 (br s, 2H), 2.48 (t, *J*=7.34, 2H), 2.42 (s, 3H), 2.01 (br s, 5H), 1.77 (m, 2H), 1.72 (s, 3H), 1.60 (m, 2H), 1.46 (m, 2H), 1.03 (s, 6H), 1.02 (m, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 172.44, 164.55, 156.47, 152.59, 142.30, 140.72, 137.79, 137.63, 137.20, 134.99, 132.40, 130.08, 129.29, 124.01, 119.21, 116.25, 113.23, 112.88, 39.57, 36.14, 34.25, 33.11, 29.68, 28.95, 21.75, 19.18, 18.43, 14.14, 13.64, 12.94. MS: *m/z* (%)=478 (M^+, 100), 382 (15), 283 (65). HR-EI-MS *m/z* 477.2878 (Calcd for $C_{30}H_{39}NO_4$, 477.2879).

4-Amino-2-(butyryloxy)phenyl (2*E*,4*E*,6*E*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetraenoate (4B) Yield: 45% as a yellow form, mp 125—127 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.11 (dd, *J*=15.00, 11.40, 1H), 6.90 (d, *J*=8.75, 1H), 6.51 (m, 2H), 6.25 (m, 4H), 5.93 (s, 1H), 3.65 (br s, 2H), 2.48 (t, *J*=7.35, 2H), 2.39 (s, 3H), 2.01 (br s, 5H), 1.72 (s, 3H), 1.65 (m, 4H), 1.46 (m, 2H), 1.03 (s, 6H), 0.97 (t, *J*=7.34, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 171.80, 164.38, 156.02, 144.92, 142.68, 140.58, 137.63, 137.13, 134.57, 134.30, 132.18, 130.26, 129.31, 129.21, 123.63, 116.39, 112.68, 110.05, 39.56, 35.89, 34.24, 33.11, 29.35, 28.95, 21.75, 19.18, 18.50, 14.06, 13.66, 12.96. MS: *m/z* (%)=478 (M^+, 100), 356 (16), 283 (40). HR-EI-MS *m/z* 477.2878 (Calcd for $C_{30}H_{39}NO_4$, 477.2879).

4-Amino-2-(butyrylamino)phenyl (2*E*,4*E*,6*E*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetraenoate (5B) Yield: 43% as a yellow form, mp 127—129 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.69 (br s, 1H), 7.27 (br s, 1H), 7.12 (dd, *J*=14.90, 11.40, 1H), 6.90 (d, *J*=8.70, 1H), 6.28 (m, 5H), 6.00 (s, 1H), 3.72 (br s, 2H), 2.41 (s, 3H), 2.32 (t, *J*=7.44, 2H), 2.01 (br s, 5H), 1.72 (s, 3H), 1.70, (m, 2H), 1.64 (m, 2H), 1.46 (m, 2H), 1.02 (s, 6H), 0.98 (t, *J*=7.38, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.96, 165.17, 156.45, 144.60, 140.86, 137.65, 137.04, 134.40, 132.51, 130.59, 130.29, 129.43, 129.19, 122.48, 116.06, 110.40, 108.32, 39.77, 39.63, 34.25, 33.11, 28.93, 21.68, 19.18, 18.92, 14.16, 13.61, 12.93. IR (KBr, cm^{-1}) 3441, 3344, 2929, 1710 (O–C=O–), 1685 (NH–C=O–), 1506. MS: *m/z* (%)=478 (20), 477 (M^+, 65), 284 (20), 283 (100), 175 (22). HR-EI-MS *m/z* 476.3040 (Calcd for $C_{30}H_{40}N_2O_3$, 476.3039).

Method C. (2*E*,4*E*,6*E*,8*E*)-[3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-nona-2,4,6,8-tetraenoylamino]-(4-hydroxy)phenylamide (1A, HPR) A mixture of dry DMF (0.077 ml, 0.99 mmol) and $SOCl_2$ (0.072 ml, 0.99 mmol) was stirred under an argon atmosphere for 1 h. ATRA was added to the solution (0.10 g, 0.33 mmol) in dry DMF (2 ml). After stirring at 0 °C for 45 min, in subdued light, the clear deep red retinoyl chloride solution was added dropwise to a cooled solution of distilled triethylamine (0.14 ml, 0.99 mmol) and 4-aminophenol (0.072 g, 0.66 mmol) in dry, degassed DMF (2 ml). The temperature was maintained at 10—15 °C during the addition. The dark-colored reaction solution was stirred at room temperature until TLC analysis indicated none remaining (about 2 h). The reaction was quenched with NH_4Cl (aq.) and extracted with EtOAc. The extracts were washed with H_2O and brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography using hexane/EtOAc (3/1) as the eluent to give **1A** (0.10 g, 78%) as a yellow solid.

(2,4-Dihydroxyphenyl)-carbamic Acid Benzylester (7) TEA (trimethylamine, 0.72 ml, 7.41 mmol) was added to a solution of 4-amino resorcinol (1.0 g, 6.18 mmol) dissolved in dry DMF (5 ml). To the resulting mixture Cbz-Cl (1.06 ml, 7.41 mmol) was added dropwise at 0 °C and stirred for 2 h. The reaction mixture was extracted with EtOAc (50 ml) and washed with H_2O (2×30 ml). The organic layer was dried over $MgSO_4$ and evaporated *in vacuo*. The crude product was crystallized from (EtOAc/hexane=1/3) to give **7** (1.42 g, 89%), as a white solid, mp 121—123 °C. ^{1}H-NMR (200 MHz, $CDCl_3$+DMSO-d_6): δ 8.96 (br s, 1H), 8.46 (br s, 1H), 7.47 (d, 1H, NH), 7.37 (m, 5H), 6.45 (d, *J*=2.50, 1H), 6.33 (dd, *J*=8.64, 2.50, 1H), 5.17 (s, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$+DMSO-d_6): δ 154.32, 145.93, 136.91, 128.60, 128.40, 128.32, 128.23, 127.69, 125.89, 122.42, 117.00, 105.62, 102.73, 65.47. MS: *m/z* (%)=260 (M^+, 100), 126 (23), 110 (21).

Method D. 2-Benzyloxycarbonylamino-5-hydroxyphenylbutanoate (10) Compound **7** (1.01 g, 3.85 mmol) was dissolved in dry CH_2Cl_2 (15 ml) and *N*-methyl morpholine (0.62 ml, 5.77 mmol) was added. To the resulting mixture butyryl chloride (0.56 ml, 5.77 mmol) was added dropwise at 0 °C, and stirred for 30 min and the reaction was quenched with 5% $NaHCO_3$ solution and washed with 5 N HCl and H_2O. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by column chromatography (EtOAc/hexane=1/5) to give 0.83 g (65%) of **10** as a white solid, mp 112—114 °C. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.55 (d, *J*=7.24, 1H), 7.37 (m, 5H), 6.65 (d, *J*=2.74, 1H), 6.56 (s, 1H), 6.38 (s, 1H), 5.17 (s, 2H), 2.50 (t, *J*=7.35, 2H), 1.72 (m, 2H). 1.00 (t, *J*=7.42, 3H). ^{13}C-NMR (100 MHz $CDCl_3$): δ 171.65, 155.01, 145.93, 135.87, 128.59, 128.38, 128.32, 128.25, 127.95, 122.72, 121.73, 119.57, 113.62, 109.77, 67.33, 36.00, 18.39, 13.54. MS: *m/z* (%)=494 (M^+, 100), 391 (13), 283 (21).

2-Hydroxy-5-nitro Phenylbutanoate (11) Yield: 61% as a white solid, mp 108—111 °C. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 8.07 (m, 2H), 7.05 (d, *J*=8.7, 1H), 2.64 (t, *J*=7.34, 2H), 1.82 (m, 2H), 1.02 (t, *J*=7.32, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 169.01, 156.27, 141.39, 141.12, 121.82, 117.89, 117.05, 39.41, 18.83, 13.42. MS: *m/z* (%)=226 (M^+, 100), 156 (18).

***N*-(2-Hydroxy-5-nitrophenyl)butyramide (12)** Yield: 72% as a white solid, mp 122—123 °C. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 8.91 (br s, 1H), 8.80 (br s, 1H), 7.84 (m, 1H), 6.97 (dd, *J*=8.80, 0.88, 1H), 2.43 (t, *J*=7.28, 2H), 1.74 (m, 2H), 1.00 (t, *J*=7.24, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 174.56, 135.07, 125.72, 122.95, 118.06, 114.09, 111.16, 38.76, 19.08, 13.59. MS: *m/z* (%)=225 (M^+, 100), 155 (31), 110 (12), 95 (11).

2-Amino-5-hydroxyphenylbutanoate (13) To a solution of **10** (0.23 g, 0.70 mmol) in MeOH (5 ml) Pd/C (10%, 0.02 g) was added at room temperature. After stirring for 30 min under H_2, the reaction mixture was filtered on celite and evaporated *in vacuo* to give **13** (0.13 g, 96%) as a light brown oil. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.18 (d, *J*=8.60, 1H), 6.41 (d, *J*=2.60, 1H), 6.32 (dd, *J*=8.60, 2.60, 1H), 3.70 (br s, 2H), 2.36 (t, *J*=7.42, 2H), 1.73 (m, 2H), 1.00 (t, *J*=7.42, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 168.89, 147.72, 141.13, 132.24, 116.87, 113.32, 109.40, 39.59, 18.82, 13.65. MS: *m/z* (%)=196 (M^+, 100), 126 (33).

5-Amino-2-hydroxyphenylbutanoate (14) Yield: 99% as a light brown oil. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.54 (m, 1H), 6.86 (d, *J*=8.80, 1H), 6.60 (m, 1H), 2.64 (t, *J*=7.34, 2H), 1.82 (m, 2H), 1.02 (t, *J*=7.32, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 167.84, 141.53, 140.02, 139.71, 116.23, 113.30, 109.41, 39.65, 18.95, 13.59. MS: *m/z* (%)=196 (M^+, 100), 126 (26).

***N*-(5-Amino-2-hydroxyphenyl)butylamide (15)** Yield: 100% as a light brown solid. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 7.79 (br s, 1H), 6.76 (d, *J*=2.65, 1H), 6.58 (d, *J*=8.60, 1H), 6.44 (dd, *J*=8.60, 2.65, 1H), 2.36 (t, *J*=7.24, 2H), 1.69 (m, 2H), 0.98 (t, *J*=7.24, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 173.28, 140.76, 139.37, 126.40, 119.31, 113.89, 109.18, 39.35, 18.71, 13.43. MS: *m/z* (%)=195 (M^+, 100), 125 (13), 110 (21).

Method E. Fumarate Salt of 4-Amino-2-(butyrylamino)phenyl-(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetraenoate (5C) To a solution of fumaric acid (27 mg, 0.23 mmol) dissolved in dry THF (5 ml) at 40—50 °C was added to free base **5B** (0.10 mg, 0.21 mmol) in dry THF (2 ml). After stirring for 2 h at 40 °C, the solution was filtered and stirred at 10 °C until crystallization starts and then *n*-hexane (3 ml) was dropped slowly into the solution. The resulting mixture was stirred for 3 h at 0 °C and the salt formed was filtered and dried to give fumarate salt **5C** (1.10 g, 89%) as a yellow powder, mp 142—146 °C. ^{1}H-NMR (200 MHz, $CDCl_3$+DMSO-d_6): δ 7.46 (br s, 1H), 7.55 (br s, 1H), 7.10 (dd, *J*=14.90, 11.40, 1H), 6.88 (d, *J*=8.70, 1H), 6.77 (s, 2H), 6.28 (m, 5H), 6.00 (s, 1H), 2.40 (s, 3H), 2.32 (t, *J*=7.44, 2H), 2.03 (br s, 5H), 1.72 (s, 3H), 1.65 (m, 4H), 1.46 (m, 2H), 1.02 (s, 6H), 0.98 (t, *J*=7.40, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.94, 165.98, 164.99, 153.65, 146.26. 139.75, 137.23, 136.88, 135.02, 133.99, 131.95, 130.64, 129.85, 129.65, 128.23, 122.72, 117.99, 109.76, 109.20, 39.08, 38.87, 33.86, 33.33, 28.80, 21.53, 18.75, 18.70, 13.66, 13.47, 12.66. MS (API-ES, Neg, scan): *m/z* (%)=591 ([M−1], 10), 476 (11), 475 (24), 406 (29), 405 (100), 283 (58), 255 (62), 115 (9).

Maleate Salt of 4-Amino-2-(butyrylamino)phenyl(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetraenoate (5D) Yield: 91% as a yellow powder, mp 122—124 °C. ^{1}H-NMR (200 MHz, $CDCl_3$+DMSO-d_6): δ 8.78 (br s, 1H), 7.62 (br s, 1H), 7.10 (dd, *J*=14.90, 11.40, 1H), 6.90 (d, *J*=8.70, 1H), 6.61 (dd, *J*=8.78, 2.63, 1H), 6.28 (m, 4H), 6.20 (s, 2H), 6.00 (s, 1H), 2.38 (s, 3H), 2.33 (t, *J*=7.44, 2H),

2.03 (br s, 5H), 1.72 (s, 3H), 1.63 (m, 4H), 1.46 (m, 2H), 1.02 (s, 6H), 0.94 (t, J=7.40, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.91, 166.01, 165.03, 153.89, 146.25, 139.73, 137.21, 136.85, 134.98, 133.93, 131.92, 130.59, 129.85, 129.62, 128.23, 122.72, 118.01, 109.76, 109.21, 39.08, 38.87, 33.86, 33.30, 28.81, 21.53, 18.75, 18.73, 13.70, 13.47, 12.68. MS (API-ES, Neg, scan): m/z (%)=591 ([M−1], 12), 476 (10), 475 (21), 406 (30), 405 (100), 255 (62), 232 (40), 115 (30).

Tartarate Salt of 4-Amino-2-(butyrylamino)phenyl(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetraenoate (5E) Yield: 95% as a yellow solid, mp 131—135 °C. ^{1}H-NMR (200 MHz, $CDCl_3$+DMSO-d_6): δ 7.89 (br s, 1H), 7.51 (br s, 1H), 7.09 (dd, J=14.90, 11.40, 1H), 6.86 (d, J=8.70, 1H), 6.24 (m, 5H), 6.02 (s, 1H), 4.52 (s, 2H), 2.39 (s, 3H), 2.32 (t, J=7.38, 2H), 2.03 (br s, 5H), 1.72 (s, 3H), 1.63 (m, 4H), 1.46 (m, 2H), 1.02 (s, 6H), 0.94 (t, J=7.40, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 173.12, 170.98, 164.01, 153.68, 146.21, 139.77, 137.26, 136.91, 135.04, 131.98, 130.66, 129.87, 129.68, 128.25, 122.75, 118.00, 109.83, 109.27, 72.16, 39.00, 38.87, 33.88, 33.36, 28.80, 21.53, 18.73, 18.70, 13.68, 13.50, 12.69. MS (API-ES, Neg, scan): m/z (%)=625 ([M−1], 8), 476 (16), 475 (42), 406 (18), 405 (100), 283 (36), 255 (41), 149 (21).

Pamoate Salt of 4-Amino-2-(butyrylamino)phenyl(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-2,4,6,8-nonatetraenoate (5F) Yield: 93% as a yellow powder, mp 110—112 °C. ^{1}H-NMR (200 MHz, $CDCl_3$+DMSO-d_6): δ 8.43 (s, 2H), 8.20 (d, J=8.70, 2H), 7.70 (d, J=8.71, 2H), 7.56 (br s, 1H), 7.34 (t, J=8.71, 2H), 7.18 (t, J=8.70, 2H), 7.10 (dd, J=14.90, 11.40, 1H), 6.83 (d, J=8.70, 1H), 6.24 (m, 5H), 6.00 (s, 1H), 4.88 (s, 2H), 2.38 (s, 3H), 2.31 (t, J=7.38, 2H), 2.03 (br s, 5H), 1.72 (s, 3H), 1.63 (m, 4H), 1.46 (m, 2H), 1.02 (s, 6H), 0.95 (t, J=7.40, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 172.55, 171.11, 164.90, 153.87, 153.66, 143.89, 139.82, 137.22, 136.89, 136.11, 134.98, 133.37, 132.04, 131.33, 130.84, 130.05, 129.84, 129.65, 128.61, 126.69, 123.32, 122.95, 120.48, 117.89, 114.72, 111.02, 110.43, 67.01, 39.08, 38.87, 33.85, 32.62, 28.78, 25.12, 21.51, 19.88, 18.77, 13.69, 13.49, 12.66. MS (API-ES, Neg, scan): m/z (%)=864 ([M−1], 6), 476 (17), 475 (32), 406 (25), 405 (100), 387 (14), 283 (16), 255 (15).

RAR Transactivation Assay Transient transfections were carried out using a liposome-based method with Lipofectamine, according to the manufacturer's instructions (Gibco BRL). Subsequent transcription assays were carried out using a CAT ELISA kit (Roche Molecular Biochemicals), as previously described.[22] Briefly, COS-1 cells (1×10^6 cells) maintained in Dulbecco's modified Eagle's essential medium supplemented with charcoal-treated 10% FBS were plated in 60-mm dishes 5 h before transfection. After overnight transfection with the reporter gene [$(RARE)_3$-tk-CAT] and receptor expression vector (RARα, β, and γ in pSG5), the cells were washed and fed with complete medium supplemented with increasing concentrations of ATRA, 4-HPR, or other retinoids as indicated in the text. All data presented represent the means of at least three independent transfections.

Cytotoxicity Assay The antiproliferative effect of retinoid derivatives was monitored using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). HCT116 colon cancer cells were seeded at an initial density of 3000 cells/well in 96-well plates and treated with medium containing various concentrations of retinoid derivatives. DMSO controls (0.01%) did not affect cell proliferation. After 48 h, 50 μl of MTT solution (2 mg/ml in PBS) was added to the culture medium and the reaction mixture was incubated at 37 °C in a 5% CO_2 atmosphere for 4 h. The MTT solution was aspirated and 150 μl of DMSO was added. The optical density was measured spectrophotometrically at 550 nm. Each experiment was performed in triplicate and repeated a minimum of three times.

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