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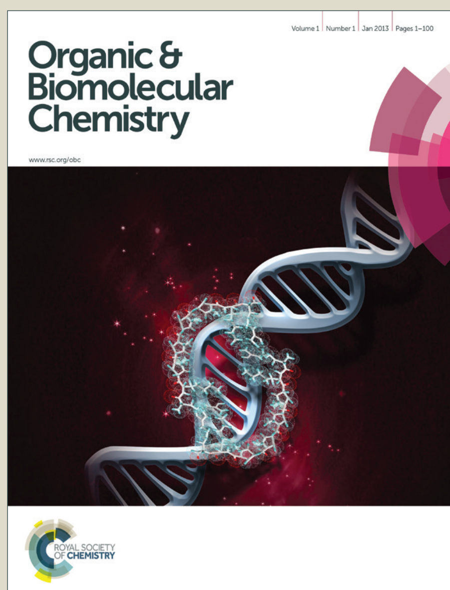

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Paper

Synthesis and P-glycoprotein induction activity of colupulone analogs†‡

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Brain amyloid-beta (Aβ) plaques are one of the primary hallmarks associated with Alzheimer's disease (AD) pathology. Efflux pump proteins located at the blood-brain barrier (BBB) have been reported to play important role in the clearance of brain Aβ, among which, the P-glycoprotein (P-gp) efflux transporter pump has been shown to play a crucial role. Thus, P-gp has been considered as a potential therapeutic target for treatment of AD. Colupulone, a prenylated phloroglucinol isolated from *Humulus lupulus* is known to activate pregnane-X-receptor (PXR), which is a nuclear receptor controlling P-gp expression. In the present work, we aimed to synthesize and identify analogs of colupulone that are potent P-gp inducer(s) with an ability to enhance Aβ transport across the BBB. A series of colupulone analogs were synthesized by modifications at both prenyl as well as acyl domains. All compounds were screened for P-gp induction activity using rhodamine 123 based efflux assay in the P-gp overexpressing human adenocarcinoma LS-180 cells, wherein all compounds showed significant P-gp induction activity at 5 µM. In the western-blot studies in LS-180 cells, compounds 3k and 5f were able to induce P-gp as well as LRP1 at 1 µM. The effect of compounds on the Aβ uptake and transport was then evaluated. Among all tested compounds, diprenylated acyl phloroglucinol 5f displayed a significant increase (29%) in Aβ transport across bEnd3 cells grown on inserts as a BBB model. The results presented here suggest the potential of this scaffold to enhance clearance of brain Aβ across the BBB and thus its promise for development as a potential anti-Alzheimer agent.

Introduction[1]

Prenylated phloroglucinols widely occur among plants of Myrtaceae and Guttiferae (Clusiaceae) families, particularly in the genera *Eucalyptus* and *Leptospermum*[1-4] and are reported to possess antimicrobial properties.[5] This class of compounds have been extensively explored synthetically due to their fascinating chemical structures and potent bioactivities.[2] The flowers of *Humulus lupulus* (hops) were historically used as a preservative and flavoring agent in beer. Hops extracts are currently marketed as a source of phytoestrogens to alleviate menopausal symptoms and as an alternative to hormone replacement therapy.[6-7] The major constituents of hops are bitter acids (12-15% of all components).[8] These bitter acids are classified as α-acids (e.g. humulone, cohumulone) and β-acids (e.g. lupulone, colupulone) (Structures are shown in Figure 1). The β-acid colupulone[8] has been reported to possess antibacterial and anticancer activities. In addition, it has been reported to stimulate expression of hepatic CYP3A enzymes[9] and pregnane-X-receptor (PXR).[10]

PXR, a member of the nuclear receptor superfamily of proteins, modulates expression of genes involved in metabolism and clearance of wide range of structurally diverse endogenous and exogenous molecules. PXR is known to regulate the expression of P-glycoprotein (P-gp), a major efflux transport protein widely distributed in various tissues such as intestinal epithelium, hepatic canalicular membrane, proximal tubular of kidneys and endothelial cells of the blood-brain barrier (BBB). A recent two independent clinical studies have reported that Alzheimer's disease (AD) patients have decreased clearance of CNS amyloid-β (Aβ) compared to healthy volunteers.[11-12] Several studies have reported the important role of P-gp in the clearance of Aβ,[13-15] thus drugs that are able to induce P-gp have the potential to emerge as novel AD therapeutics.

The bicyclic polyprenylated phloroglucinol natural product hyperforin is reported to activate PXR,[16] and induce P-gp expression,[17] and has also been reported to show memory enhancement in animal models.[18-19] Colupulone is a simplified triprenylated phloroglucinol natural product possessing potent PXR activation ability similar to hyperforin.[10] Literature precedence on colupulone[10] and related compounds[17] clearly indicates potential of this scaffold to increase Aβ clearance. So far, no medicinal chemistry efforts have been reported on this unique scaffold for P-gp induction activity. Therefore, based on the known PXR activation property of colupulone and related compounds, and as a part of our efforts in this area,[20-21] we

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initiated a medicinal chemistry program to explore its SAR for P-gp induction and evaluate its utility in Aβ clearance across the BBB. The modifications were planned at both the prenyl (site A) and acyl (site B) domains as depicted in Figure 1. A series of tetra- and di-alkylated analogs of colupulone were synthesized and evaluated for their P-gp induction activity using in vitro rhodamine 123 efflux assays in LS-180 cells. Further, the effect of these compounds on Aβ transport across BBB was also studied.

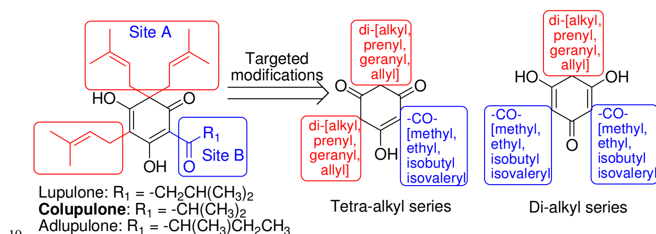
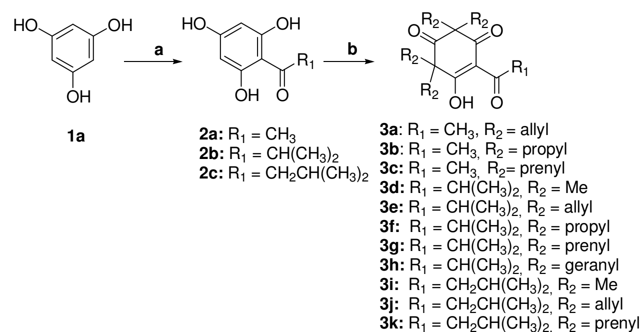


Figure 1. Chemical structures of *β*-acids from hops; and general structures of designed series for synthesis.

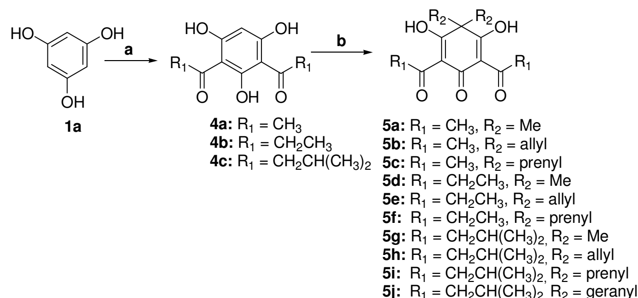
Results and discussion

Chemistry. Colupulone is a triprenylated acylphloroglucinol compound. For synthesis, we aimed to synthesize tetra- and diprenylated acylphloroglucinols. The variation in prenyl group by replacing it with geranyl or simple alkyls was also planned. These two series (*tetra-* and *di-*substituted) of acylphloroglucinols were synthesized starting from commercially available phloroglucinol (**1a**). The first step involved in their synthesis is Friedel-Craft's acylation of phloroglucinol either using $AlCl_3$ or BF_3-etherate. The use of $AlCl_3$ and nitrobenzene as a solvent primarily produced mono-acyl phloroglucinols **2a-c**, which further on treatment with alkyl halides in alkali at room temperature produced tetra-alkylated acylphloroglucinol products **3a-k** in 50-78% yields (Scheme 1).



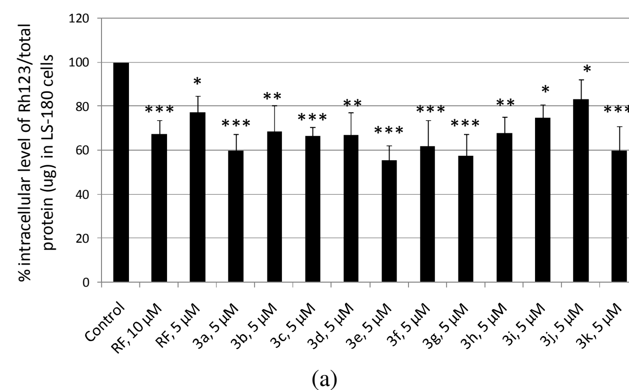
Scheme 1. Synthesis of tetraalkylated acylphloroglucinols **3a-k.** Reagents and conditions: (a) RCOCl, $AlCl_3$, CS_2, $PhNO_2$, N_2 atm, 50 °C, 1h, 50-70%; (b) R_1X, 10% KOH, rt, 30 min, 55-78%.

The treatment of phloroglucinol (**1a**) with carboxylic acids in presence of BF_3-etherate produced diacylphloroglucinols **4a-c**, which on treatment with alkyl halides in presence of NaOMe produced dialkylated acylphloroglucinols **5a-j** (Scheme 2). All products were characterized by NMR, IR, MS, HR-ESIMS analysis.

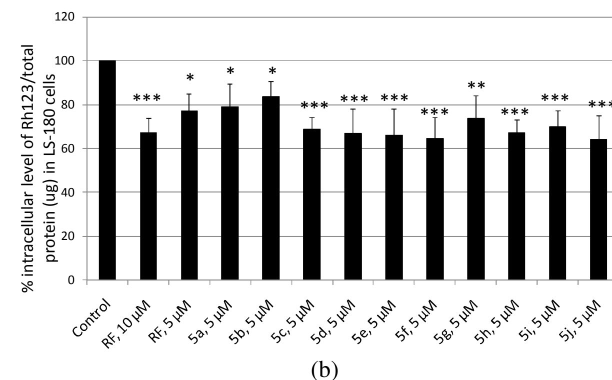


Scheme 2. Synthesis of dialkylated acylphloroglucinols **5a-j.** Reagents and conditions: (a) RCOOH, BF_3-etherate, 100 °C, 2h, 55-75%; (b) R_1X, NaOMe, 60 °C, N_2 atm, 3 h, 50-78%.

Biology. All synthesized compounds were screened for P-gp induction activity using rhodamine 123 based efflux assay in P-gp overexpressing human colorectal adenocarcinoma LS-180 cells. In MTT assay, compounds treatments up to 48 h was not toxic to LS-180 cells with IC_{50} > 10 μM; thus, for P-gp induction assay, we used 5 μM as a test concentration. LS-180 cells treated with 5 μM of each compound for 48 h displayed a significant induction in P-gp activity, as displayed by the increased efflux of rhodamine-123 (Figure 2a-b). The exact structure-activity relationship could not be established as most of the compounds displayed almost similar level of P-gp induction activity (Figure 2). However, the combination of allyl or prenyl (R_2 substituent) with isobutanoyl or propanoyl substituent ($-COR_1$ substituent) was somewhat better than others. Compounds of this combination demonstrated increased activity of P-gp as confirmed by the decreased percent of rhodamine-123 intracellular accumulation by ≥35% when compared to untreated control cells due to P-gp inducers.



(a)



(b)

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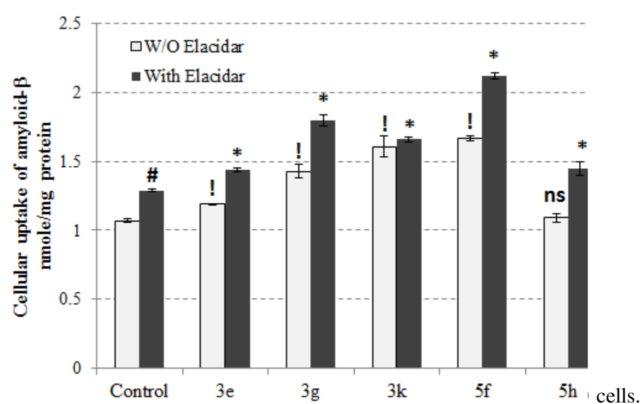
Figure 2. P-gp induction activity of tetra-alkylated (a) and dialkylated (b) acylphloroglucinols in LS-180 cells. The P-gp induction activity of compounds was tested at 5 µM and was measured in terms of the % intracellular accumulation of rhodamine 123 (Rh123)/total protein (µg) inside LS-180 cells. The decrease in % intracellular accumulation (compared to control) of Rh123 indicates induction of P-gp. Rifampicin was used as a reference P-gp inducer. On the X-asis legends, RF indicates the rifampicin. The statistical comparisons were made between control vs compounds. The *p* value <0.5 was considered to be significant. *P* value *< 0.5, **<0.01, ***<0.001. All values are shown as average of three experiments ± SD.

Available experimental data strongly suggest that impaired clearance of Aβ across the BBB might largely contribute to the formation of Aβ brain deposits and AD progression. Also, it has been demonstrated that the efflux transporter P-gp plays a substantial role in the elimination of $A\beta_{40}$ and $A\beta_{42}$ from the brain across the BBB.[13, 22-24] Accordingly, in this study we report the impact of P-gp up-regulation on the intracellular accumulation of $A\beta_{42}$ *in vitro*.

The most potent five compounds **3e**, **3g**, **3k**, **5f** and **5h** were further investigated for their effect on Aβ uptake. To determine the impact of P-gp up-regulation on the uptake of Aβ, the cellular uptake of $A\beta_{42}$ was evaluated following compounds treatment in the presence or absence of elacridar (2.5 µM), a P-gp inhibitor. Figure 3 shows the effect of compounds treatment on the intracellular accumulation of $A\beta_{42}$ with and without elacridar in LS-180 cells, which were compared to vehicle treated cells (control). Inhibition studies with elacridar were performed to specifically elucidate the compounds effect on P-gp. As anticipated, the co-treatment with elacridar significantly increased the intracellular levels of $A\beta_{42}$ as a result of P-gp inhibition with all compounds except **3k** (p <0.05); however, in the absence of elacridar, the increased levels of P-gp was not able to decrease $A\beta_{42}$ cellular uptake to levels lower than those with control treated cells (Figure 3). These results suggest a modulatory effect of the tested compounds, not only on P-gp function, but on other Aβ transport proteins that oppose the efflux function of P-gp. In the absence of elacridar, the compounds **3e**, **3g**, **3k** and **5f** increased the intracellular accumulation of $A\beta_{42}$ indicating a possible induction of an uptake transporter(s) of Aβ such as the low-density lipoprotein receptor-related protein 1 (LRP1). To confirm the contribution of LRP1 to the enhanced uptake of $A\beta_{42}$, Western-blot analysis was performed to evaluate compounds effect on LRP1 protein expression in addition to P-gp following cells treatment with 1 µM concentrations. The results are shown in Figure 4. All compounds were able to induce P-gp at 1 µM concentration by 1.6- to 3.6-fold compared to untreated cells. On the other hand, only compounds **3k** and **5f** were able to significantly induce LRP1 by 10 and 16%, respectively, but not compounds **3e**, **3g**, and **5h**. While the increase in LRP1 by compounds **3k** and **5f** could explain the increase in intracellular levels of $A\beta_{42}$ compared to the control (in the absence of elacridar). The increase in $A\beta_{42}$ uptake by **3g** and to a lesser extent **3e** could be related to the up-regulation of another $A\beta_{42}$ transport protein(s) other than LRP1, that could be expressed either at the luminal or abluminal side of the BBB. To test this, we conducted transport studies in a better representative model for the BBB using mouse brain endothelial cells.



cells.

The data are expressed as mean ± SD of n = 3 experiments. (**#**) Indicates significantly different from control cells in the absence of the P-gp inhibitor elacridar, (*) indicates significant difference from control cells in the presence of elacridar, and (**!**) indicates significantly different from control cells in the absence of elacridar; *P*<0.05. **ns**, not significant from control.

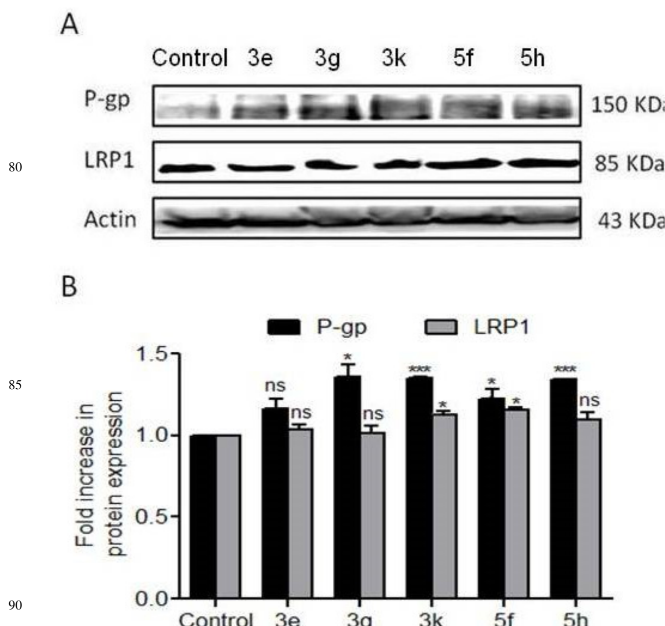


Figure 4. **(A)** Representative Western blots for P-gp and LRP1 in LS180 cells treated with the compounds at 1 µM concentration. (B) Quantitative fold increase in P-gp and LRP1 expressions. The data are expressed as mean ± SD of n = 3 independent experiments (* *P*<0.05, *** *P*<0.001)).

Compounds **3k** and **5f** were selected to evaluate their effect on Aβ transport across the mouse brain endothelial bEnd3 cells as a BBB model. These two compounds were chosen as representative compounds from the tetraalkylated acylphloroglucinols (**3k**) and dialkylatedacyl phloroglucinols (**5f**) series. Results from MTT assay indicated treatment of bEnd3 cells with the compounds **3k** and **5f** at 1 µM concentration for 48 h was not toxic to the cells and were comparable to those observed with the control treated cells. Among the two tested compounds, only compound **5f** (at 1 µM for 48 h) resulted in a significant increase (by 29%) in ^{125}I-$A\beta_{40}$ transport across the endothelial monolayer (p <0.05), while compound **3k** showed comparable ^{125}I-$A\beta_{40}$ transport to that of control cells (no treatment), which is inconsistent with the uptake study results in LS180 cells. This could be due to the effect of **3k**

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on other efflux or influx Aβ transport proteins expressed either at the luminal or abluminal side. In addition, these collective findings demonstrate that utilization of transport studies (A→B) to evaluate compounds effect on Aβ clearance is better model to identify hit compounds that are able to enhance Aβ clearance from the brain. In conclusion, these findings provide an *in-vitro* proof of concept of the potential of compound **5f** to enhance Aβ clearance from AD brains (Figure 5).

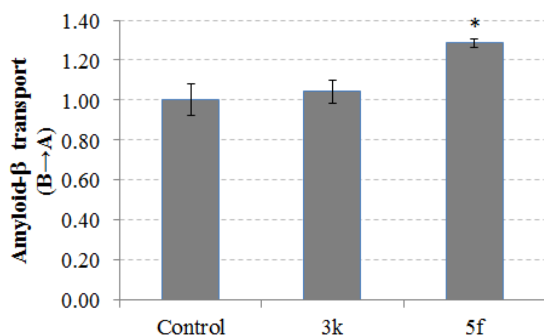

Figure 5.Effect of compounds **3k** and **5f** on ^{125}I-Aβ transport, relative to control, across bEnd3 cell-based BBB model. Asterisk (*) indicates significant difference from control (p <0.05).

Conclusion

In conclusion, the colupulone scaffold has shown promising P-gp induction activity in LS-180 cells. The diprenylated triketone compound **5f** showed increase in the Aβ efflux in LS-180 and increase in its transport across bEnd3 cells, indicating its promise to enhance Aβ clearance from AD brains by targeting P-gp. Thus, results presented in this work, indicates promise of this scaffold for development as an anti-Alzheimer agent. Further studies are in progress to evaluate the compound **5f** *in vivo* and verify effect on P-gp activity at the BBB and Aβ clearance.

Experimental section

General. All chemicals were obtained from Sigma-Aldrich Company and used as received. ^{1}H, ^{13}C and DEPT NMR spectra were recorded on Brucker-Avance DPX FT-NMR 500 and 400 MHz instruments. Chemical data for protons are reported in parts per million (ppm) downfield from tetramethylsilane and are referenced to the residual proton in the NMR solvent ($CDCl_3$, 7.26 ppm). Carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded at 125 MHz or 100 MHz: chemical data for carbons are reported in parts per million (ppm, δ scale) downfield from tetramethylsilane and are referenced to the carbon resonance of the solvent ($CDCl_3$, 77 ppm). ESI-MS and HR-ESIMS spectra were recorded on Agilent 1100 LC-Q-TOF and HRMS-6540-UHD machines. IR spectra were recorded on Perkin-Elmer IR spectrophotometer. Melting points were recorded on digital melting point apparatus.

General procedure for synthesis of monoacyled phloroglucinols 2a-c. A solution of phloroglucinol (100 mg, 1 equiv.) and anhydrous aluminium chloride (3 equiv.) in carbon disulphide (10 ml) under inert atmosphere was stirred at room temperature for 20 min. Nitrobenzene (15 ml) was added and temperature of the reaction mixture was allowed to increase to 50 °C. Acyl chloride (3 equiv.) was added and reaction mixture was stirred for a 30 min. On cooling, the reaction mixture was diluted with ethyl acetate. Water was added to the resultant mixture leading to formation of a white precipitate in the aqueous layer. The organic layer was decanted off and the remaining solid residue was washed 5-6 times with EtOAc. The combined EtOAc layer was evaporated on vacuo rotavapor and the remaining viscous oil was purified by silica gel column chromatography using n-hexane: EtOAc as an eluent to yield monoacylated phloroglucinols **2a-c** in 50-70% yield.

1-Acetyl-2,4,6-trihydroxy benzene (2a). Yield: 60%; White solid; ^{1}H NMR (CD_3OD, 400 MHz): δ 5.77 (s, 2H), 2.61 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 205.2, 172.7, 104.9, 95.7, 33.1; IR ($CHCl_3$): ν_{max} 3369, 2953, 2853, 1619, 1430, 1404, 1365, 1298, 1199, 1025 cm^{-1}; ESI-MS: *m/z* 169.00 $[M+H]^+$; HR-ESIMS: *m/z* 169.0483 calcd for $C_8H_8O_4$+H^+ (169.0495).

1-Isobutanoyl)-2,4,6-trihydroxy benzene (2b). Yield: 65%; Pale yellow sticky liquid; ^{1}H NMR (CD_3OD, 400 MHz): δ 5.72 (s, 2H), 3.89-3.86 (m, 1H), 1.04 (s, 3H), 1.02 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 212.4, 166.7, 105.3, 96.5, 40.6, 20.4; IR ($CHCl_3$): ν_{max} 3350, 2924, 2823, 1626, 1603, 1522, 1463, 1384, 1228, 1160, 1096 cm^{-1}; ESI-MS: *m/z* 197.00 $[M+H]^+$; HR-ESIMS: *m/z* 197.0805 calcd for $C_{10}H_{12}O_4$+H^+ (197.0808).

1-(3-Methyl-butanoyl)-2,4,6-trihydroxy benzene (2c). Yield: 70%; Yellow solid; ^{1}H NMR (CD_3OD, 400 MHz): δ 5.70 (s, 2H), 2.82 (d, J = 8.0 Hz, 2H), 2.15-2.05 (m, 1H), 0.86 (d, J = 8.0 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 207.1, 166.0, 165.8, 105.6, 95.8, 53.7, 44.2, 26.8, 23.2; IR ($CHCl_3$): ν_{max} 3369, 2955, 2923, 2852, 1628, 1561, 1520, 1455, 1384, 1215, 1164, 107 cm^{-1}; ESI-MS: *m/z* 211.00 $[M+H]^+$; HR-ESIMS: *m/z* 211.0962 calcd for $C_{11}H_{14}O_4$+H^+ (211.0965).

General procedure for synthesis of tetraalkylated phloroglucinols 3a-k. To the solution of monoacylated pholoroglucinols **2a-c** (1 equiv.) in 10% aq. KOH solution, was added alkyl halides (4 equiv.) and reaction mixture was allowed to stirred for 30 min at rt. It was then acidified with HCl and extracted with EtOAc. The organic layer was washed with brine solution and dried over sodium sulphate. Solvent was evaporated on vacuo rotavapor and the crude product was purified by silica gel (# 60-120) column chromatography to get tetra-alkylated products **3a-k** in 55-78% yield.

4-Acetyl-2,2,6,6-tetraallyl-5-hydroxycyclohex-4-ene-1,3-dione (3a). Yield: 60%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 18.31 (s, 1H), 5.58-5.46 (m, 4H), 5.05 (dd, J = 8.0, 20.0 Hz, 8H), 2.67-2.51 (m, 8H), 2.46 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 204.5, 200.4, 195.3, 193.2, 131.4, 130.7, 119.2, 118.4, 111.7, 63.6, 59.3, 40.0, 38.7, 26.5; IR ($CHCl_3$): ν_{max} 3421, 3080, 3011, 2981, 2926, 2854, 1715, 1668, 1640, 1555, 1433, 1363, 1028 cm^{-1}; ESI-MS: *m/z* 329.10 $[M+H]^+$; HR-ESIMS: *m/z* 329.1749 calcd for $C_{20}H_{24}O_4$+H^+ (329.1747).

4-Acetyl-5-hydroxy-2,2,6,6-tetra(prop-2-ynyl)cyclohex-4-ene-1,3-dione (3b). Yield: 76%; Brown solid; ^{1}H NMR ($CDCl_3$,

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400 MHz): δ 18.06 (s, 1H), 2.83-2.70 (m, 8H), 2.58 (s, 3H), 2.05-2.02 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 202.3, 200.9, 194.2, 191.1, 112.3, 73.3, 72.7, 62.7, 59.1, 26.7, 25.9, 24.4 ; IR ($CHCl_3$): ν_{max} 3289, 2923, 2852, 2120, 1726, 1645, 1599, 1424, 1363, 1197, 1094 cm^{-1}; ESI-MS: *m/z* 321.10 $[M+H]^+$; HR-ESIMS: *m/z* 321.1123 calcd for $C_{20}H_{16}O_4+H^+$ (321.1121).

4-Acetyl-5-hydroxy-2,2,6,6-tetrakis(3-methylbut-2-enyl)cyclohex-4-ene-1,3-dione (3c). Yield: 55%; yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 18.21 (s, 1H), 4.90–4.85 (m, 4H), 2.74-2.59 (m, 8H), 2.50 (s, 3H), 1.59-1.52 (m, 24H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 207.2, 206.4, 196.7, 193.4, 135.3, 135.5, 117.9, 117.3, 116.9, 111.5, 65.7, 64.7, 60.0, 36.1, 34.5, 32.6, 30.9, 28.7, 28.4, 24.9, 24.8, 21.7, 19.4, 18.7, 17.1, 17.0, 16.9, 16.8; IR ($CHCl_3$): ν_{max} 3391, 2982, 2928, 1720, 1666, 1550, 1453, 1362, 1259, 1028 cm^{-1}; ESI-MS: *m/z* 441.29 $[M+H]^+$; HR-ESIMS: *m/z* 441.3006 calcd for $C_{28}H_{40}O_4+H^+$ (441.2999).

5-Hydroxy-4-(isobutyryl)-2,2,6,6-tetramethylcyclohex-4-ene-1,3-dione (3d). Yield: 78%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 18.42 (s, 1H), 3.77-3.70 (m, 1H), 1.38 (s, 6H), 1.29 (s, 6H), 1.12 (d, *J* = 4.0 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 209.9, 208.6, 199.3, 196.8, 108.1, 56.9, 52.2, 35.2, 24.2, 23.8, 19.1; IR ($CHCl_3$): ν_{max} 3434, 2976, 2928, 1723, 1672, 1555, 1470, 1421, 1383, 1362, 1231, 1047 cm^{-1}; ESI-MS: *m/z* 253.10 $[M+H]^+$; HR-ESIMS: *m/z* 253.1601 calcd for $C_{14}H_{20}O_4+H^+$ (253.1613).

2,2,6,6-Tetraallyl-5-hydroxy-4-(isobutyryl)cyclohex-4-ene-1,3-dione (3e). Yield: 65%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 18.59 (s, 1H), 5.56-5.44 (m, 4H), 5.00 (dd, *J* = 8.0, 16.0 Hz, 8H), 3.70-3.63 (m, 1H), 2.56-2.41 (m, 8H), 1.11 (d, *J* = 4.0 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 207.5, 204.5, 196.1, 192.8, 131.4, 130.8, 119.0, 118.3, 110.5, 63.7, 59.5, 40.1, 38.6, 34.2, 18.1; IR ($CHCl_3$): ν_{max} 3400, 3079, 2979, 2927, 1715, 1669, 1639, 1555, 1433, 1293 cm^{-1}; ESI-MS: *m/z* 357.10 $[M+H]^+$; HR-ESIMS: *m/z* 357.2078 calcd for $C_{22}H_{28}O_4+H^+$ (357.2060).

5-Hydroxy-4-(isobutyryl)-2,2,6,6-tetra(prop-2-ynyl)cyclohex-4-ene-1,3-dione (3f). Yield: 55%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 18.46 (s, 1H), 3.84-3.78 (m, 1H), 2.89-2.77 (m, 8H), 2.05 (s, 4H), 1.22 (d, *J* = 4 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 208.1, 202.3, 194.8, 190.9, 111.0, 73.2, 72.5, 63.0, 59.3, 34.9, 31.9, 29.7, 29.6, 29.4, 26.1, 24.4, 22.7, 19.2, 14.2; IR ($CHCl_3$): ν_{max} 3400, 3290, 2923, 2852, 1726, 1673, 1558, 1425, 1383, 1156 cm^{-1}; ESI-MS: *m/z* 349.00 $[M+H]^+$; HR-ESIMS: *m/z* 349.1435 calcd for $C_{22}H_{20}O_4+H^+$ (349.1434).

5-Hydroxy-4-isobutyryl-2,2,6,6-tetrakis(3-methylbut-2-en-1-yl)cyclohex-4-ene-1,3-dione (3g). Yield: 68%; light yellow liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 18.61 (s, 1H), 4.92-4.86 (m, 4H), 3.66-3.59 (m, 1H), 2.65-2.59 (m, 2H), 2.54 (dd, *J* = 8.0, 12.0 Hz, 4H), 2.28 (d, *J* = 8.0 Hz, 2H), 1.60-1.50 (m, 24H), 1.16 (d, *J* = 8.0 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 208.3, 207.5, 197.8, 194.5, 136.4, 136.3, 134.6, 134.5, 119.0, 118.9, 118.4, 118.3, 112.5, 66.8, 65.8, 61.1, 37.1, 35.6, 33.7, 32.0, 29.8, 29.4, 26.0, 25.9, 22.7, 20.4, 19.2, 17.9, 17.8, 14.2; IR ($CHCl_3$): ν_{max} 3429, 2966, 2925, 2855, 1713, 1671, 1554, 1447, 1377, 1294, 1096 cm^{-1}; ESI-MS: *m/z* 469.20 $[M+H]^+$; HR-ESIMS: *m/z* 469.3329 calcd for $C_{30}H_{44}O_4+H^+$ (469.3312).

5-Hydroxy-4-(isobutyryl)-2,2,6,6-tetrakis((E)-3,7-dimethylocta-2,6-dienyl)cyclohex-4-ene-1,3-dione (3h). Yield: 72%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 18.66 (s, 1H), 5.06 (t, *J* = 8.0 Hz, 4H), 4.93 (t, *J* = 8.0 Hz, 4H), 3.73-3.66 (m, 1H), 2.67-2.62 (m, 2H), 2.57-2.50 (m, 4H), 2.38-2.32 (m, 2H), 2.01-1.89 (m, 16H), 1.68-1.56 (m, 36H), 1.18 (s, 6H) ; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 208.0, 207.2, 198.1, 194.6, 139.9, 138.5, 131.5, 131.4, 124.3, 124.1, 124.0, 123.9, 123.8, 118.8, 118.7, 118.1, 118.0, 117.7, 112.0, 109.9, 109.8, 66.8, 65.7, 61.3, 61.2, 40.1, 40.0, 36.4, 35.4, 33.8, 29.7, 29.6, 26.6, 25.7, 22.7, 19.3, 17.6, 16.4, 16.2, 16.1, 14.2; IR ($CHCl_3$): ν_{max} 3434, 2923, 2965, 1712, 1669, 1555, 1446, 1382, 1151, 1097 cm^{-1}; ESI-MS: *m/z* 741.50 $[M+H]^+$; HR-ESIMS: *m/z* 741.5829 calcd for $C_{50}H_{76}O_4+H^+$ (741.5816).

4-(3-Methylbutanoyl)-5-hydroxy-2,2,6,6-tetramethylcyclohex-4-ene-1,3-dione (3i). Yield: 60%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 18.39 (s, 1H), 2.88 (d, *J* = 4.0 Hz, 2H), 2.20-2.13 (m, 1H), 1.45 (s, 6H), 1.36 (s, 6H), 0.99 (d, *J* = 4.0 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 209.0, 202.6, 198.5, 195.9, 108.4, 55.9, 51.3, 46.2, 30.9, 28.7, 28.3, 25.0, 23.2, 22.8, 21.6; IR ($CHCl_3$): ν_{max} 3400, 2957, 2924, 2853, 1722, 1671, 1559, 1465, 1383, 1260, 1047 cm^{-1}; ESI-MS: *m/z* 267.10 $[M+H]^+$; HR-ESIMS: *m/z* 267.1588 calcd for $C_{15}H_{22}O_4+H^+$ (267.1591).

4-(3-Methylbutanoyl)-2,2,6,6-tetraallyl-5-hydroxycyclohex-4-ene-1,3-dione (3j). Yield: 66%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 18.50 (s, 1H), 5.55-5.44 (m, 4H), 5.01 (dd, *J* = 8.0, 16.0 Hz, 8H), 2.79 (d, *J* = 8.0 Hz, 2H), 2.57-2.45 (m, 4H), 2.42 (d, *J* = 8.0 Hz, 4H) 2.19-2.09 (m, 1H), 0.92 (d, *J* = 8.0 Hz, 6H); ^{13}C NMR ($CDCl_3$,100 MHz): δ 205.8, 203.9, 196.9, 194.0, 132.4, 131.8, 120.1, 119.4, 112.7, 64.8, 60.2, 47.3, 41.2, 39.5, 25.5, 22.7; IR ($CHCl_3$): ν_{max} 3400, 3079, 2958, 2925, 28.52, 1715, 1669, 1639, 1555, 1435, 1384, 1039 cm^{-1}; ESI-MS: *m/z* 371.20 $[M+H]^+$; HR-ESIMS: *m/z* 371.2230 calcd for $C_{23}H_{30}O_4+H^+$ (371.2217).

4-(3-Methylbutanoyl)-5-hydroxy-2,2,6,6-tetrakis(3-methylbut-2-enyl)cyclohex-4-ene-1,3-dione (3k). Yield: 67%; Brown sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 18.48 (s, 1H), 4.88 (t, *J* = 8.0 Hz, 4H), 2.80 (d, *J* = 8.0 Hz, 2H), 2.66-2.61 (m, 2H), 2.55-2.48 (m, 4H), 2.33-2.25 (m, 2H), 2.22-2.15 (s, 1H), 1.61-1.53 (m, 24H), 1.97 (*J* = 4.0 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 207.6, 203.7, 197.4, 136.1, 134.6, 118.9, 118.3, 113.6, 65.5, 61.0, 47.7, 36.8, 33.9, 31.9, 26.0, 25.8, 25.7, 22.7, 19.7, 17.9, 17.8, 14.0; IR ($CHCl_3$): ν_{max} 3434, 2926, 2871, 1740, 1656, 1545, 1451, 1384, 1212, 1164 cm^{-1}; ESI-MS: *m/z* 483.20 $[M+H]^+$; HR-ESIMS: *m/z* 483.3464 calcd for $C_{31}H_{46}O_4+H^+$ (483.3469).

General procedure for synthesis of diacyledphloroglucinols 4a-4c. A solution of phloroglucinol (1 equiv.) and acids (4 equiv.) in BF_3-etherate were refluxed at 100 °C for 2.5 h. The reaction mixture was cooled to room temperature poured into crushed ice and extracted with ethyl acetate (100 ml × 3). The combined organic layers were evaporated on vacuo rotavapor and the crude product was purified by silica gel (# 100-200) column

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chromatography to yield diacylated phloroglucinols **4a-c** in 55-75% yields.

1,3-Diacetyl -2,4,6-trihydroxy benzene (4a). Yield: 60%; White solid; ^{1}H NMR (CD_3OD, 400 MHz): δ 5.83 (s, 1H), 2.61 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 205.2, 172.4, 104.9, 95.7, 33.1, 31.0; IR ($CHCl_3$): ν_{max} 3391, 2923, 1620, 1429, 1366, 1291, 1197, 1025 cm^{-1}; ESI-MS: *m/z* 210.90 $[M+H]^+$; HR-ESIMS: *m/z* 211.0603 calcd for $C_{10}H_{10}O_5+H^+$ (211.0601).

1,3-Dipropanoyl-2,4,6-trihydroxy benzene(4b). Yield: 55%; Pale Yellow solid; ^{1}H NMR (CD_3OD, 400 MHz): δ 5.76 (s, 1H), 3.09-3.04 (m, 4H), 1.13 (t, *J* = 8 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 208.2, 172.1, 104.6, 95.8, 38.4, 8.8; IR ($CHCl_3$): ν_{max} 3264, 2939, 1622, 1588, 1420, 1399, 1274, 1191, 1034 cm^{-1}; ESI-MS: *m/z* 239.00 $[M+H]^+$; HR-ESIMS: *m/z* 239.0916 calcd for $C_{12}H_{14}O_5+H^+$ (239.0914).

1,3-Di-(3-Methyl-butanoyl)-2,4,6-trihydroxy benzene (4c). Yield: 75%; Pale yellow solid; ^{1}H NMR (CD_3OD, 400 MHz): δ 5.80 (s, 1H), 2.94 (d, *J* = 4.0 Hz, 4H), 2.23 (s, 2H), 0.98 (d, *J* = 4.0 Hz, 12H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 208.1, 173.4, 170.3, 105.7, 96.6, 54.5, 26.8, 23.6; IR ($CHCl_3$): ν_{max} 3252, 2958, 2931, 1620, 1416, 1340, 1272, 1195 cm^{-1}; ESI-MS: *m/z* 295.10 $[M+H]^+$; HR-ESIMS: *m/z* 295.1535 calcd for $C_{16}H_{22}O_5+H^+$ (295.1540).

General procedure for synthesis of dialkylated phloroglucinols 5a-j. The sodium metal (0.15 g) was added to methanol (3 ml) to form a NaOMe solution. To this solution was added the metanolic solution of diacylated phloroglucinol (1 mmol) followed by alkyl halides (5 mmol). The solution was refluxed under N_2 atmosphere for 3 h. Then, the reaction mixture was poured into 4N HCl to make pH of reaction mixture acidic (pH 6.0). Reaction mixture was extracted with ethyl acetate and organic layer was washed with 4N HCl followed by brine solution and finally dried over sodium sulphate. Solvent was evaporated on vacuo rotavapor and the crude product was purified by silica gel (# 60-120) column chromatography to yield products **5a-j** in 50-78% yield.

2,6-Diacetyl-3,5-dihydroxy-4,4-dimethylcyclohexa-2,5-dienone (5a). Yield: 77%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 19.22 (s, 1H), 18.81 (s, 1H), 2.65 (s, 3H), 2.54 (s, 3H), 1.38 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 202.2, 201.3, 200.7, 194.9, 187.5, 106.3, 105.5, 52.1, 29.2, 27.7, 24.7, 24.3; IR ($CHCl_3$): ν_{max} 3391, 2982, 2928, 1720, 1666, 1550, 1453, 1362, 1259, 1028 cm^{-1}; ESI-MS: *m/z* 238.90 $[M+H]^+$; HR-ESIMS: *m/z* 239.0915 calcd for $C_{12}H_{14}O_5+H^+$ (239.0914).

2,6-Diacetyl-4,4-diallyl-3,5-dihydroxycyclohexa-2,5-dienone (5b). Yield: 70%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 19.29 (s, 1H), 18.95 (s, 1H), 5.50-5.42 (m, 2H), 5.02-4.92 (m, 4H), 2.77-2.68 (m, 6H), 2.61 (t, J = 8.0 Hz, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 201.2, 200.1, 198.6, 196.9, 190.9, 185.7, 130.3, 129.5, 128.8, 118.1, 117.6, 117.0, 106.5, 57.9, 53.1, 40.9, 27.5; IR ($CHCl_3$): ν_{max} 3435, 3080, 3006, 2927, 2854, 1660, 1642, 1550, 1456, 1363, 1028 cm^{-1}; ESI-MS: *m/z* 291.00 $[M+H]^+$; HR-ESIMS: *m/z* 291.1223 calcd for $C_{16}H_{18}O_5+H^+$ (291.1227).

2,6-Diacetyl-3,5-dihydroxy-4,4-bis(3-methylbut-2-enyl)cyclohexa-2,5-dienone (5c). Yield: 76%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 19.23 (s, 1H), 18.85 (s, 1H), 4.71 (t, *J* = 8.0 Hz, 2H), 2.63 (s, 6H), 2.54 (s, 4H), 1.49 (s, 12H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 206.4, 200.2, 195.9, 193.8, 135.2, 133.7, 117.9, 117.2, 112.6, 64.6, 59.8, 59.4, 36.9, 35.6, 32.8, 28.6, 26.4, 24.9, 24.8, 16.8; IR ($CHCl_3$): ν_{max} 3400, 2969, 2925, 2856, 1659, 1550, 1452, 1384, 1364, 1200, 1083 cm^{-1}; ESI-MS: *m/z* 347.10 $[M+H]^+$; HR-ESIMS: *m/z* 347.1844 calcd for $C_{20}H_{26}O_5+H^+$ (347.1853).

3,5-Dihydroxy-4,4-dimethyl-2,6-dipropionylcyclohexa-2,5-dienone (5d). Yield: 72%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 19.47 (s, 1H), 18.98 (s, 1H), 3.16-3.13 (m, 2H), 3.03-2.97 (m, 2H), 1.42 (s, 6H), 1.19-1.13 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 209.0, 204.8, 199.6, 197.3, 187.0, 107.8, 55.3, 50.7, 33.7, 32.1, 28.7, 23.4, 22.8, 8.0; IR ($CHCl_3$): ν_{max} 3331, 2981, 2939, 2877, 2856, 1721, 1670, 1563, 1471, 1381, 1339, 1225 cm^{-1}; ESI-MS: *m/z* 267.00 $[M+H]^+$; HR-ESIMS: *m/z* 267.1220 calcd for $C_{14}H_{18}O_5+H^+$ (267.1227).

4,4-Diallyl-3,5-dihydroxy-2,6-dipropionylcyclohexa-2,5-dienone (5e). Yield: 66%; Brown sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 19.55 (s, 1H), 19.05 (s, 1H), 5.50-5.46 (m, 2H), 5.04-4.94 (m, 4H), 3.18 (d, *J* = 8.0 Hz, 2H), 3.08 (d, *J* = 4.0 Hz, 2H), 2.80-2.75 (m, 2H), 2.66-2.60 (m, 2H), 1.20-1.16 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 206.2, 204.9, 198.2, 192.7, 188.0, 131.7, 131.0, 119.7, 119.1, 107.9, 59.9, 43.2, 42.5, 35.1, 33.6, 29.7, 8.9, 8.5; IR ($CHCl_3$): ν_{max} 3400, 2980, 2926, 1682, 1657, 1555, 1451, 1380, 1260, 1186, 1063 cm^{-1}; ESI-MS: *m/z* 319.10 $[M+H]^+$; HR-ESIMS: *m/z* 319.1538 calcd for $C_{18}H_{22}O_5+H^+$ (319.1540).

3,5-Dihydroxy-4,4-bis(3-methylbut-2-enyl)-2,6-dipropionylcyclohexa-2,5-dienone (5f). Yield: 50%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 19.48 (s, 1H), 18.91 (s, 1H), 4.73 (t, *J* = 8.0 Hz, 2H), 3.14-3.10 (m, 4H), 2.71-2.58 (m, 4H), 1.51 (s, 12H), 1.21-1.10 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 205.9, 205.0, 203.4, 198.4, 192.5, 191.8, 187.2, 134.6, 116.7, 111.6, 107.0, 59.3, 37.0, 36.3, 34.0, 33.8, 32.5, 28.7, 24.8, 16.8, 7.9, 7.7; IR ($CHCl_3$): ν_{max} 3400, 2973, 2925, 1656, 1551, 1379, 1337, 1259, 1212, 1085 cm^{-1}; ESI-MS: *m/z* 375.10 $[M+H]^+$; HR-ESIMS: *m/z* 375.2166 calcd for $C_{22}H_{30}O_5+H^+$ (375.2166).

1,1'-(4,6-Dihydroxy-5,5-dimethyl-2-oxocyclohexa-3,6-diene-1,3-diyl) bis(3-methylbutan-1-one) (5g). Yield: 78%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 19.44 (s, 1H), 19.02 (s, 1H), 2.98 (d, *J* = 8.0 Hz, 2H), 2.86 (d, *J* = 8.0 Hz, 2H), 2.22-2.11 (m, 2H), 1.41 (s, 6H), 0.98-0.95 (m, 12H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 203.6, 201.8, 200.4, 193.8, 186.9, 105.5, 104.5, 51.3, 48.2, 46.5, 25.2, 24.8, 24.4, 23.7, 23.2, 21.7, 21.6; IR ($CHCl_3$): ν_{max} 2959, 2930, 1667, 1544, 1469, 1448, 1385, 1367, 1217, 1165, 1096 cm^{-1}; ESI-MS: *m/z* 323.10 $[M+H]^+$; HR-ESIMS: *m/z* 323.1856 calcd for $C_{18}H_{26}O_5+H^+$ (323.1853).

2,6-Bis(3-methylbutanoyl)-4,4-diallyl-3,5-dihydroxy cyclohexa-2,5-dienone (5h). Yield: 73%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 19.58 (s, 1H), 19.09 (s, 1H), 5.52-5.46 (m, 2H), 5.04-4.94 (m, 4H), 3.01 (d, *J* = 8 Hz,

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2H), 2.95 (d, J = 4.0 Hz, 2H), 2.79-2.74 (m, 2H), 2.65-2.60 (m, 2H), 2.23-2.16 (m, 2H), 1.00-0.98 (m, 12H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 203.7, 201.3, 196.8, 190.7, 186.1, 129.6, 128.9, 117.4, 116.9, 111.0, 106.5, 57.9, 53.0, 47.5, 47.1, 46.1, 41.0, 40.3, 27.6, 23.8, 20.1; IR ($CHCl_3$): ν_{max} 2959, 2929, 2872, 1659, 1643, 1546, 1454, 1386, 1367, 1299, 1265, 1086 cm^{-1}; ESI-MS: m/z 375.10 [M+H]$^+$; HR-ESIMS: m/z 375.2175 calcd for $C_{22}H_{30}O_5+H^+$ (375.2166).

2,6-Bis(3-methylbutanoyl)-3,5-dihydroxy-4,4-bis(3-methylbut-2-enyl)cyclohexa-2,5-dienone (5i). Yield: 66%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 19.55 (s, 1H), 19.01 (s, 1H), 4.77 (t, J = 8.0 Hz, 2H), 3.04-2.94 (m, 4H), 2.71-2.65 (m, 4H), 2.23-2.11 (m, 2H), 1.55 (s, 12H), 1.00-0.97 (m, 12H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 205.5, 203.5, 199.0, 192.9, 188.3, 131.8, 119.1, 108.7, 60.2, 55.2, 49.8, 49.3, 48.3, 43.3, 42.5, 29.8, 26.0, 25.9, 25.7, 22.8, 22.7, 22.6; IR ($CHCl_3$): ν_{max} 3434, 2926, 2871, 1740, 1656, 1545, 1451, 1384, 1212, 1164 cm^{-1}; ESI-MS: m/z 431.10 [M+H]$^+$; HR-ESIMS: m/z 431.2795 calcd for $C_{26}H_{38}O_5+H^+$ (431.2792).

2,6-Bis(3-methylbutanoyl)-3,5-dihydroxy-4,4-bis((E)-3,7-dimethylocta-2,6-dienyl)cyclohexa-2,5-dienone (5j). Yield: 69%; Pale yellow sticky liquid; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 19.56 (s, 1H), 19.05 (s, 1H), 5.33 (t, J = 8.0 Hz, 2H), 5.08 (t, J = 8.0 Hz, 2H), 3.92 (d, J = 4.0 Hz, 4H), 2.99-2.92 (m, 1H), 2.78-2.62 (m, 1H), 2.10-1.82 (m, 12H), 1.74-1.51 (m, 24H), 0.98-0.94 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 204.8, 203.0, 200.1, 193.8, 188.6, 140.4, 139.4, 131.6, 123.8, 120.5, 117.4, 109.9, 68.7, 57.8, 49.9, 48.2, 39.8, 39.6, 39.1, 26.7, 26.37, 25.7, 25.6, 25.5, 22.7, 22.6, 22.4, 17.7, 16.4; IR ($CHCl_3$): ν_{max} 3368, 2962, 2926, 1656, 1545, 1451, 1383, 1329, 1087 cm^{-1}; ESI-MS: m/z 567.30 [M+H]$^+$; HR-ESIMS: m/z 567.4048 calcd for $C_{36}H_{54}O_5+H^+$ (567.4044).

Cell culture and treatments. Human colorectal adenocarcinoma LS-180 cells were purchased from ECACC, England. These cells were grown in MEM growth medium. The media for cell line was supplemented with 1% MEM non-essential amino acids along with 10% FCS, 100 U penicillin G and 100 µg/ml of streptomycin. Cells were grown in 5% CO_2 at 37 °C with 95% humidity. All the test compounds were dissolved in DMSO for treatment of LS-180 cells, while the untreated control cultures received only the vehicle (DMSO< 0.2%).

P-gp-induction assay in LS-180 cells. All synthesized compounds were screened for their ability to induce P-gp using rhodamine 123 cell exclusion method.[20] In this method, the P-gp function was evaluated in terms of intracellular rhodamine 123 accumulation.[25] Briefly, the protocol used was as follows: Colorectal LS-180 cells were seeded at a density of 2×10^4 per well of 96 well plate and were allowed to grow for next 24 h. Cells were further incubated with the test compounds, and were diluted to a final concentration of 5 µM and rifampicin (positive control) to a final concentration of 5 and 10 µM in complete media for 48 h. The final concentration of DMSO was kept at 0.1%. Drugs were removed and cells were incubated with HANKS buffer for 40 minutes before further incubation with HANKS buffer (containing 10 µM of Rh123 as a P-gp substrate) for 90 minutes. At the end of Rh123 treatment cells were washed four times with cold PBS followed by cell lysis for 1 h using 200 µl of lysis buffer (0.1% Triton X 100 and 0.2 N NaOH). A total of 100 µl of lysate was used for reading fluorescence of Rh123 at 485/529 nm. Samples were normalized by dividing fluorescence of each sample with total protein present in the lysate.

Cell viability assay. The cell proliferation assay was evaluated in human colorectal adenocarcinoma LS-180 cells. Cells (1×10^4) were seeded into each well of 96-well microplate for 24 h. Cells were treated with 10 µM of each compound for 48 h. The MTT dye was then added to each well 4 h prior to the termination of experiment. Formazan crystals were dissolved in DMSO before taking absorbance at 570 nm. Cell viability of the untreated control sample was considered to be 100%, while viability of test samples was calculated using the following formula:

$$\%\ \text{cell viability} = \frac{\text{OD (test)}}{\text{OD (control)}} \times 100$$

In addition, the MTT assay was performed in the mouse brain endothelial bEnd3 cells following compounds treatment with 1 µM of compounds **3k** and **5f** for 48 h.

Aβ_{42} uptake studies in LS-180 cells. Selected compounds were tested for their ability to induce P-gp using Aβ_{42} as a substrate.[26] In this method, P-gp function was evaluated in terms of Aβ accumulation. Briefly, the protocol used is as follows: Colorectal LS-180 cells were seeded at 50% confluency in a 48 well plate. Cells were further incubated with the test compounds, at a final concentration of 1 µM in complete media for 48 h. Test compounds were removed, and cells were washed twice with fresh media. Cells were incubated with media containing 100 nM Aβ_{42} with and without 2.5 µM elacridar (a P-gp inhibitor) for 30 min. At the end of Aβ treatment cells were washed three times with cold PBS followed by cell lysis for 30 min using 100 µl of RIPA buffer. A total of 70 µl of lysate was used for intracellular levels of Aβ_{42} using ELISA method as described by us previously.[27] Total protein concentration in lysate was used for sample normalization.

^{125}I-Aβ_{40} transport studies across bEnd3 cells as a BBB model. Transport studies were performed as reported previously.[27-28] In brief, transwell polyester membrane inserts, 6.5 mm diameter with 0.4 µm pores, were coated with collagen. The mouse brain endothelial cell line bEnd3 cells, used as a model for BBB, were plated onto coated inserts at a seeding density of 50,000 cells/cm^2, medium was changed every other day. On day 4 from seeding, cells were treated with the compounds at 1 µM for 48 h. Then transport studies of ^{125}I-Aβ_{40} on day 6 were performed. Basolateral to apical (B→A) transport studies were initiated by addition of 0.1 nM ^{125}I-Aβ_{40} and 0.05 mM^{14}C-inulin to the basolateral compartment. At the end of incubation period (30 min), media in both compartments and cells were separately collected for ^{125}I-Aβ_{40} analysis and inulin measurement using a Wallac 1470 Wizard Gamma Counter (PerkinElmer Inc., Waltham, MA), and Wallac 1414 WinSpectral Counter (PerkinElmer Inc.), respectively. The effect of compound treatment on the clearance of Aβ_{40} was compared to that of control (no treatment).

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Statistical analysis. Data is expressed as mean ± SD of three independent experiments unless otherwise indicated. The comparisons were made between control and treated groups or the entire intra group using Bonferroni test through Instat-2 software. p -values <0.5 were considered significant.

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Abbreviations

Aβ, amyloid-beta; AD, Alzheimer's disease; BBB, blood-brain barrier; bEnd3, endothelialpolyoma middle T antigen transformed cells of cerebral cortex of the brain; LS-180, intestinal human colon adenocarcinoma cell line; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); P-gp, P-glycoprotein; PXR, pregnane-X-receptor; Rh123, rhodamine 123; SAR, structure-activity relationship.

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