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Stereoselective synthesis of carbohydrate fused pyrano[3,2-c]pyranones as anticancer agents

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Priti Kumari[a†], Sonal Gupta[b†], Chintam Narayana[a‡], Shakeel Ahmad[b‡], Shailja Singh[b]* and Ram Sagar[a,c]*

Abstract

Pyrano[3,2-c]pyranone is an important structural motif present in many natural products exhibiting diverse biological activities. Two series of carbohydrate fusedpyrano[3,2-c]pyranone derivatives (n = 20) were efficiently synthesized starting from 2-*C*–formyl galactal and 2-*C*–formyl glucal, reacting with various 4-hydroxycoumarins in very short reaction time (10 min) under microwave assisted condition. Anticancer activity of these synthesized pyrano[3,2-c]pyranones were determined in detail through cellular assays against MCF-7 (breast), MDA-MB-231 (breast) and HepG2 (liver) cancer cell lines. The newly synthesized pyrano[3,2-c]pyranones were screened for their cell-viability and anti-proliferative activity against MCF-7, MDA-MB-231 and HepG2 cell lines. The compounds **12**, **13** and **14** exhibited high growth inhibitory potencies selectively against MCF-7 cell with half maximal inhibitory concentration (IC_{50}) values 19.9, 14.5 and 10.9 μM respectively. The compounds **12, 13**, **14**, **15** and **19** inhibited cell growth of MDA-MB-231 cell (breast) by 43, 44, 37, 31 and 45% respectively. However, no inhibitory effect was observed by these compounds in human liver cancer cell line (HepG2) and normal cell lines (HEK293, human embryonic kidney cells). Mechanistic studies showed that, these compounds alter cell morphology and cause G2/M arrest in MCF-7. Further study showed compounds **12**, **13** and **14** significantly inhibited cell migration which was accompanied by altered microtubule distribution. An enhanced accumulation of these compounds in cells was observed as compared to 4-hydroxycoumarins precursor in intracellular uptake assay. These findings confirms that, carbohydrate fused pyrano[3,2-c]pyranone are better candidates for anticancer activity.

Introduction

Cancer is one of the leading cause of death worldwide and as per World Health Organization (WHO) report there will be 26 million new cancer cases and 17 million cancer deaths estimated per year due to cancer by 2030.[1,2] Breast cancer is most common type of cancer found in women worldwide. In 2012 it was estimated that, 1,671,149 new breast cancer cases were diagnosed and 521,907 deaths occurred worldwide due to breast cancer.[3] Cancer statistics review revealed that, by end of 2017 number of new diagnosed female breast cancer cases would be around 252710, which is 15% of all new cancer cases, and estimated death would occur 40,610 in USA alone.[4] This situation becomes even more problematic with the development of resistant in breast cancer.[5] These alarming situation attracted attention of medicinal chemists and chemical biologists to, search for new molecules as chemotherapeutics with better activity and understand mechanistic aspects of resistant at molecular level through detailed studies. Therefore, efficient synthesis of drug-like small molecules has been interest of the research for medicinal chemists because, they play vital role in new drug development processes.[6] These drugs like bioactive compounds are broadly interact with enzyme or receptor and modulate their function, thus serve as important leads in medicinal chemistry.[7] The incorporation of privileged substructures into novel core skeletons is an important approach in medicinal chemistry.[8] Coumarin derivatives and fused pyrano[3,2-c]pyranones are widely present in bioactive natural products, synthetic products as well as in pharmaceutical agents (**A-C**, Fig. 1).[9] Coumarins and pyrano[3,2-c]pyranones well documented primarily for their anticancer activity, along with antidiabetic, anti-Alzheimer, anticoagulant, insecticidal, and antifungal properties.[10,11] Coumarin derivatives have been shown to exhibit growth inhibitory activity in different types of cancer like breast cancer[12] prostate cancer[13] and malignant melanoma.[14] Coumarin–monastrol fused hybrid molecules showed potent inhibitory activity against human breast cancer lines like

[a] *Department of Chemistry, Shiv Nadar University, NH-91 Dadri, GB Nagar (UP) 201314, India*
[b] *Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi-110 067,India*
[c] *Department of Chemistry, Institute of Science, Banaras Hindu University, Varanasi-221 005, India*
Corresponding Author:
Email: ram.sagar@snu.edu.in, shailja.jnu@gmail.com,

†equal contribution

‡equal contribution

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basal type MCF-7 and triple negative MDA-MB-231.[12] Most of these coumarin derivatives were shown to suppress cell proliferation by arresting cell cycle at G1 or G2/M phases thereby inducing apoptosis. Tomoda and coworkers reported that compounds bearing pyrano-pyranone skeleton inhibits the DNA synthesis by 79-91% and tumor cell growth by 93-100%. It has also been observed that extended conjugation in pyrano-pyranone molecules showed better activity and these compounds inhibit the tubulin polymerization.[15]

The above literature reports strongly support pyrano[3,2-c]pyranonesas privileged skeleton. When the privileged skeleton fused with carbohydrates and resulted hybrid molecules have many additional advantages eg. a) A better aqueous solubility, b) improved selectivity, c) improved cellular uptake, d) reduced cellular toxicityetc.[16]

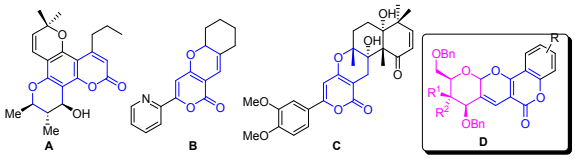

Fig. 1. Bioactive pyrano-pyranone molecules (**A-C**): (**A**) Calanolide A, anti-HIV drug; (**B**) anticancer tricyclic pyrano[3,2-c]pyranone; (**C**) Arisugacin A, a selective inhibitor of acetylcholinesterase (**D**) our anticancer pyrano[3,2-c]pyranone molecules presented in current work.

Herein, we report microwave assisted synthesis and anticancer activity of carbohydrate fused pyrano[3,2-c]pyranones (**D**) derived from the hybrid structure of 4-hydroxycoumarin and 2-*C*-formyl glycals in good to very good yield in very short reaction time (10 min). Anticancer activity of these newly synthesized fused pyrano[3,2-c]pyranones were determined by various cellular assays against MCF-7, MDA-MB-231 and HepG2 cancer cell lines. Carbohydrate fused pyrano[3,2-c]pyranones **12**, **13** and **14** exhibited high growth inhibitory potencies selectively against MCF-7 with IC_{50} in the range of 10-20 μM. The cell growth inhibition for compounds **12, 13**, **14**, **15** and **19** for MDA-MB-231 cell (breast) was found 43, 44, 37, 31 and 45% respectively. The efficient microwave assisted synthesis of these molecules and their detailed anticancer activity are presented in this paper.

Results and Discussion

Chemistry

For the efficient synthesis of privileged carbohydrate-fused pyrano[3,2-c]pyranones, we identified 4-hydroxycoumarin **1** as substrate which can be coupled with 2-*C*-formyl galactal **1a**[17] under microwave reaction condition and get transformed to the desired pyrano[2,3-c]pyranones **11** (table 1) through selective C-1,2 nucleophilic addition followed by 6π-electron electrocyclization reaction i.e. formal [3+3] cycloaddition reaction. Link and co-woekers first under thermal condition.[20] However, some of transformations reported this type of annulation reaction in 1944.[18] This reaction later explored by many research groups for synthesis of natural products and biologically active molecules.[19] The synthesis of few pyrano[3,2-c]-5(2*H*)-one was carried out earlier have faced in term of reaction time, isolated yields and substrate scope etc. Therefore, there was a need to develop an efficient and clean synthetic method for library synthesis of pyrano[3,2-c]pyranones. Herein we are reporting microwave assisted efficient synthesis of carbohydrate fused pyrano[3,2-c]pyranones and their detailed anticancer activity. The initial annulation reaction of coumarin **1** with 2-*C*-formyl galactal **1a** was performed under microwave heating condition in toluene at 80 °C it doesn't go to completion even after 20 min. whereas similar reaction in the presence of L-proline furnished carbohydrate fused pyrano[3,2-c]pyranone **11** was obtained in 90% isolated yield with highly stereoselective and regioselective manner (entry 2, Table 1). The structure of pyrano[3,2-c]pyranone was established by 1D and 2D NMR (NOE, COSY and HSQC) experiments (SI) and comparing it with earlier spectral data.[20]

To optimize reaction condition under microwave condition and see the effect of organocatalyst, we planned and tested a series of reaction conditions using various organocatalysts and solvent systems (table 1) under microwave irradiation. To enhance the reactivity of 2-*C*-formyl galactal **1a** various secondary amine (L-proline **I**, pyrrolidine **II**, piperidine **III** and morpholine **IV**) based organocatalysts were tested. After screening a set of reaction conditions by using different organocatalysts under microwave condition, it was found that the annulation of coumarin **1** with **1a** in the presence of pyrrolidine **II** (0.5 equiv) in toluene at 80 °C affords **11** in 93% isolated yield with excellent diastereoselectivity (table 1, entry 5). When we carried out the same transformation in the presence of 0.1 equiv of pyrrolidine **II** under similar condition compound **11** was obtained in 91% isolated yield (table 1, entry 14), we obtained the identical product **11** without the loss of stereoselectivity, when different organocatalysts (**I**-**IV**) were used for this annulation reaction as observed earlier under thermal condition.[20] The isolated yield was varied with different organocatalyst without any significant difference in the reaction time (Table 1, entries 2-14).

To study solvent effects for this transformation under microwave condition, we screened toluene, ethyl acetate and acetonitrile in the presence of L-proline (Table 1, entries 2-4) and 1% acetic acid. In comparison, toluene with 1% acetic acid was found to be superior over ethyl acetate and acetonitrile. These three solvent systems were screened with other organocatalysts (**II**-**IV**) and result showed that, indeed 1% acetic acid in toluene was the best solvent among others (Table 1).

The structure of pyrano[3,2-c]pyranone was unambiguously established by 1D and 2D NMR (NOE, COSY and HSQC) experiments (Supporting Information, SI). The stereochemistry of newly generated chiral center in compound **12** was confirmed by careful NOE experiments. A NOE correlation was observed between newly generated chiral methine proton H-11a (δ 6.10) and predefined chiral methine proton H-10 (δ 3.87). This suggested that the H-11a proton is in the same face as the H-10 at the sugar part in compound **12**.

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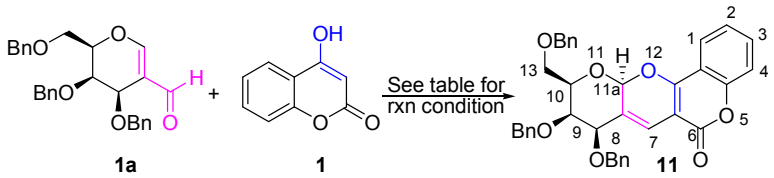
Table 1. Reaction optimization using different organocatalysts and solvents



1a **1** **11**

S. No.	Reaction conditions[a]	Yield (%)
1[b]	Toluene:AcOH, µW 80 °C, 30 min	23
2	**I**, Toluene:AcOH, µW 80 °C, 10 min	90
3	**I**, EtOAc:AcOH, µW 80 °C, 10 min	84
4	**I**, Acetonitrile:AcOH, µW 80 °C, 10 min	82
5	**II**, Toluene:AcOH, µW 80 °C, 10 min	93
6	**II**, EtOAc:AcOH, µW 80 °C, 10 min	77
7	**II**, Acetonitrile:AcOH, µW 80 °C, 10 min	55
8	**III**, Toluene:AcOH, µW 80 °C, 10 min	65
9	**III**, EtOAc:AcOH, µW 80 °C, 10 min	60
10	**III**, Acetonitrile:AcOH, µW 80 °C, 10 min	55
11	**IV**, Toluene:AcOH, µW 80 °C, 10 min	70
12	**IV**, EtOAc:AcOH, µW 80 °C, 10 min	61
13	**IV**, Acetonitrile:AcOH, µW 80 °C, 10 min	50
14[c]	**II**, Toluene:AcOH, µW 80 °C, 10 min	91

[a]0.5 equiv of organocatalyst was used in each cases, [b]Reaction was not completed, [c]0.1 equiv oforganocatalystwas used.

After having optimized reaction condition, we were interested to investigate the scope of this transformation with different substituted 4-hydroxycoumarins under microwave reaction condition. Therefore, various 4-hydroxycoumarins **1-10** were freshly prepared in laboratory starting from commercially available corresponding *ortho*-hydroxyacetophenone and diethyl carbonate (Scheme S1, SI).[21] The various 4-hydroxycoumarinswere then coupled with 2-*C* formyl galactal **1a** and 2-*C* formyl glucal **1b** under optimized reaction condition (µW, 80 °C, **II**, Toluene:AcOH, 10 min). As shown in Table 2 (entries 2-10) 2-*C* formyl galactal **1a** was successfully coupled with various substituted 4-hydroxycoumarins **2-10** to afford respective carbohydrate fused pyrano[3,2-c]pyranones **11-20** in good to very good yields with excellent stereoselectivity(dr >99:1). The substituents at the R^1, R^2, R^3 and R^4 positions on 4-hydroxycoumarins neither remarkable affected the yield nor the reaction completion time. However R^2 (= Br) substituted 4-hydroxycoumarin afforded the lesser yield (64%) as compare to other mono- and di-substituted 4-hydroxycoumarins (Table 2, entries 5, 9 and 10).

The proposed mechanism for this transformation is already discussed earlier, which involves C-1,2-addition with 4-hydroxycoumarin and 2-C-formyl galactal followed by dehydration and β-elimination. An 1-oxatriene intermediate formed after β-elimination which undergo 6-π electron electrocyclization and furnishes the corresponding pyrano[3,2-c]pyranone.[20, 22]

Further when 4-hydroxycoumarins **1** was coupled with 2-*C* formyl glucal **1b** under optimized reaction condition it afforded

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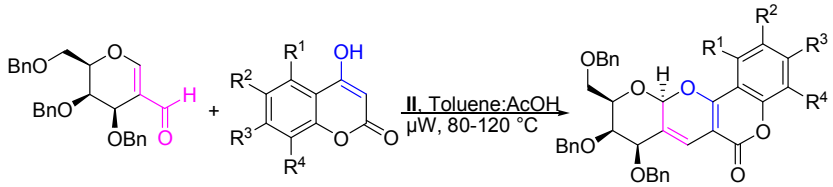
unexpectedly, epimeric mixture (1:1) of carbohydrate fused pyrano-pyranone **21a/21b** in good yield. The stereoselectivity in this case could not get restored even using different organocatalysts and conditions. As mentioned in proposed mechanism that, this type of reaction proceeds through 1-oxatriene intermediate followed by 6π-electron electrocyclization and reversibility of this cyclization was well studied and reported in the literature.[22] Based on literature we can presumed that fused pyrano[3,2-c]pyranone **21a** is equilibrating with **21b** through a common 1-oxatriene intermediate in reversible fashion and furnishing a mixture of epimers **21a/b**. The energy calculation was done to understand this equilibrium and it was found that, the energy difference between 2-C-formyl galactal derived compound pyrano[3,2-c]pyranones (**11a =11** vs. **11b**) was ΔE = 3.975 kcal/mole, whereas, that of 2-C-formyl glucal derived

Table 2. Synthesis of 2-*C* formyl galactal fused pyrano[3,2-c]pyranones **11-20**.



1a **1-10** **11-20**

Entry	R^1	R^2	R^3	R^4	Product [a]	Time (min)	Yield (%)
1	H	H	H	H	**11**	10	93
2	H	OCH_3	H	H	**12**	10	92
3	H	Cl	H	H	**13**	10	78
4	H	CH_3	H	H	**14**	10	95
5	H	Br	H	H	**15**	10	64
6	H	$-C_6H_4-$		H	**16**	10	77
7	H	H	F	H	**17**	10	78
8[b]	H	Cl	H	Cl	**18**	10	76
9[b]	H	H	OCH_3	OCH_3	**19**	20	80
10[b]	OCH_3	H	OCH_3	H	**20**	20	68

[a]Stereoselectively only one diastereomer was formed in each case(*drα*:β, >99:1)determined by crude ^{1}H NMR;. [b]Required temperature was 120

pyrano[3,2-c]pyranones (**21a** vs. **21b**) was ΔE = 1.240 kcal/mole. [20] This calculation results demonstrate that 2-*C*-formyl galactal derived pyrano[3,2-c]pyrone **11** is thermodynamically more favorable than its diastereomer **11b**. However, the energy difference between 2-*C*-formyl glucal derived pyrano[3,2-c]pyranones (**21a** vs. **21b**) was significantly smaller than that of galactal case therefore they keep equilibrating between **21a** and **21b** in reversible fashion.[20] We tried to isolate one stereoisomer **21a** through column but it was futile as it again appeared mixture of epimers **21a/b** in NMR. As shown in Table 3 (entries 2-10), 2-*C* formyl glucal **1b** was successfully coupled with various substituted 4-hydroxycoumarins **2-10** to afford respective pyrano[3,2-c]pyranones **22a/b-30a/b** in acceptable to good yields as epimeric mixture (dr~1:1). Similarly different substituents here at the R^1, R^2, R^3 and R^4 positions on 4-hydroxycoumarins neither remarkable affects the yield nor the reaction completion time when got coupled with 2-*C* formyl glucal **1b** (Table 3, entries 1-10). Thus library of twenty different diverse carbohydrate fused pyrano[3,2-c]pyranone compounds along with ten 4-hydroxycoumarins were prepared and anticancer activity were determined by various cellular assays and detailed biological results are discussed below.

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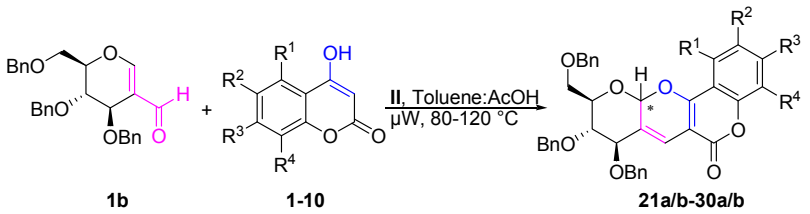
Table 3. Synthesis of 2-C formyl glucal fused pyrano[3,2-c]pyranones **21a/b-30a/b**.



1b **1-10** **21a/b-30a/b**

Entry	R^1	R^2	R^3	R^4	Product [a]	Time (min)	Yield (%)
1	H	H	H	H	**21a/21b**	15	81
2	H	OCH_3	H	H	**22a/22b**	15	76
3	H	Cl	H	H	**23a/23b**	15	84
4	H	CH_3	H	H	**24a/24b**	15	80
5	H	Br	H	H	**25a/25b**	15	73
6	H	$-C_6H_4-$		H	**26a/26b**	15	77
7	H	H	F	H	**27a/27b**	15	80
8[b]	H	Cl	H	Cl	**28a/28b**	20	54
9[b]	H	H	OCH_3	OCH_3	**29a/29b**	20	54
10[b]	OCH_3	H	OCH_3	H	**30a/30b**	20	51

[a]Diastereomeric ratio (*dr*)was ~1:1 (α:β) determined by crude ^{1}H NMR; [b]Required temperature was 120 °C

Biology

Anticancer potential of all synthesized carbohydrate fused pyrano[3,2-c]pyranones **11**-**20** and **21a/b**-**30a/b** (n= 20) along with different substituted 4-hydroxycoumarin precursors **1**-**10** (n = 10) at 50 μM was investigated by MTT assay in MCF-7 (human breast adeno carcinoma), HepG2 and MDA-MB231 cell lines. The results showed (Table 4) growth inhibition in all tested compounds in MCF-7 breast cancer cells with no effect in **25a/b**. Compounds **12, 13**, **14**, **15** and **19** were effective against MDA-MB231 cells (breast) at 20 μM. However, no significant inhibition of HepG2 (Liver heptocellular carcinoma) cells were observed with tested compounds even at higher concentration of 100 μM indicating specificity of these compounds for breast cancer cells.

Table 4. Anticancer screening results

Compounds	Growth Inhibition (%)[a] ±SD		
	MCF 7	HepG2	MDA-MB231
1	39.6 ±0.12	-29.4±0.04	4.47 ±0.00
2	33.4 ±0.65	-01.1 ±0.08	17.72 ±0.01
3	35.6 ±0.16	-15.9±0.02	2.29 ±0.02
4	23.7 ±0.09	-13.9 ±0.10	2.94 ±0.01
5	49.7 ±0.17	-45.5 ±0.11	37.37 ±0.04
6	36.5 ±0.07	-24.4 ±0.10	18.79 ±0.01
7	29.2 ±0.04	10.6±0.05	-6.78 ±0.02
8	27.8 ±0.11	01.7±0.43	10.97 ±0.03
9	27.4 ±0.06	-05.7±0.25	13.34 ±0.01
10	06.7 ±0.22	08.0±0.22	3.03±0.00
11	26.2 ±0.06	-12.9±0.26	11.58 ±0.00
12	81.4 ±0.04	-18.0±0.07	43.21 ±0.01
13	77.8 ±0.03	-8.2±0.06	44.37 ±0.01

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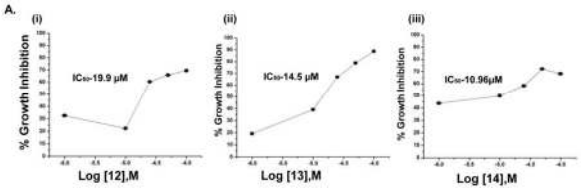
14	78.7 ±0.04	-14.9±0.08	37.66 ±0.00
15	54.2 ±0.14	01.2 ±0.48	31.55 ±0.17
16	20.7 ±0.19	04.0±0.17	6.34 ±0.0
17	24.0 ±0.12	07.6 ±0.01	15.49 ±0.00
18	15.3 ±0.27	-15.2±0.23	7.12 ±0.00
19	17.6±0.10	-04.4 ±0.22	45.84 ±0.03
20	47.0 ±0.11	04.8±0.51	1.36 ±0.01
21a/b	53.5 ±0.04	-19.1±0.05	-6.02 ±0.0
22a/b	03.1 ±0.37	19.0±0.37	4.66 ±0.00
23a/b	21.5 ±0.06	06.3 ±0.06	-1.53 ±0.00
24a/b	01.4 ±0.38	13.9 ±0.22	5.87 ±0.00
25a/b	-18.1 ±0.29	-02.8±0.37	5.85 ±0.12
26a/b	09.4 ±0.09	-03.7 ±0.14	8.12±0.00
27a/b	18.6 ±0.06	02.5 ±0.45	5.54 ±0.01
28a/b	23.7 ±0.03	06.4±0.03	8.53 ±0.00
29a/b	20.3 ±0.16	-00.4 ±0.10	0.58 ±0.00
30a/b	25.3±0.11	-08.3±0.11	1.94 ±0.01
Etoposide (5μM)	93.7 ±0.11	07.3±0.37	24.45 ±0.0

[a]50 μM, 100 μM and 20 μM concentration used for MCF-7, HepG2 and MDA-MB-231 cells respectively

From the study, it has been shown that carbohydrate fused pyrano[3,2-c]pyranone compounds **12**, **13** and **14** exhibited good inhibitory effect against MCF 7 cells among all derivatives with an IC_{50}(μM) of 19.9, 14.6 and 10.9 respectively (Fig. 2A).

The structure-activity relationship (SAR) of these compounds for anticancer activity can be summarized as follows: i) in the series of galactal fused pyrano[3,2-c]pyranones, the substitution at C-2 (R^2) in coumarin ring is important for better anticancer activity; ii) 2-OMe, 2-Cl and 2-CH_3 substitution in this series shown better activity against MCF-7; iii) 2-OMe, 2-Cl, 2-CH_3 and 2-Br substituted compounds in this series shown better activity against MDA-MB-231; iv) glucal fused pyrano[3,2-c]pyranones were not very effective against used cell lines. None of these compounds shown cell growth inhibition against HepG2 cells indicating these structural frameworks has specificity towards breast cancer.



Furthermore, the effect of compounds **12**, **13** and **14** on cell viability and survival of cancer cells was determined using different approaches. The cells were treated with compounds **12**, **13** and **14** at 50 μM, which is higher concentration than their respective IC_{50} value, for 24 and 48 hours. The trypan blue staining showed > 80% reduction in number of viable cells at this concentration (Fig. 3B (i)). Additionally, we performed clonogenic assay wherein we treated cells at 50 μM were plated in fixed number and allowed to grow to form individual colonies. The survival fraction was calculated with respect to untreated cells and the results showed < 20% survival of breast cancer cells in presence of these compounds, which further confirmed cell growth inhibitory potencies of carbohydrate fused pyrano[3,2-c]pyranones compounds **12**, **13** and **14** (Fig.2B (ii))**.** Etoposide, which causes apoptosis in cancer cells, was used as positive control for these experiments.

We further investigated if this antiproliferative activity was the result of apoptotic mechanisms. MCF-7 cells treated with all three compounds **12**, **13** and **14** at 50 μM showed altered morphology, cellular shrinkage, chromatin condensation and cell death which are the characterstics of apoptosis. This phenomenon became more obvious after 48 hours of treatment as shown in Fig. 3A. Next we evaluated the effect of these compounds on cell cycle of MCF-7 cells by flow cytometry. The characterstic of cancer cells is their uncontrollable growth and proliferation capability and cell cycle arrest is an important target in oncotherapy. Treatment with compounds **12** and **14** significantly increased the population of cells in G2/M phase to 59.8% and 65.1% respectively at 50 μM as compared to 17.1% in control cells. Compound **13** showed accumulation of 42.4% cells in G2/M phase at lower concentration of 25 μM (Fig. 3B). Moreover with compound **13** treatment at 50 μM, significant increase in polyploid cells (73.6%) and apoptotic cells was observed. In addition to this, the population of G1 phase has drastically decreased in cells treated with 50 μM compounds **12**, **13** and **14** to 1.46%, 2.14% and 0.39% respectively as compared to 65.7% in untreated cells. Taken together, these results suggested that after 48 hours of treatment, compound **12**, **13** and **14** caused an obvious G2/M phase arrest which is related to reduced cell viability. MTT assay of most active compounds against MCF-7 (**12, 13** and **14**) was carried out using normal cell (HEK293, human embryonic kidney cells) and they were found non-toxic (Fig. S1, SI).

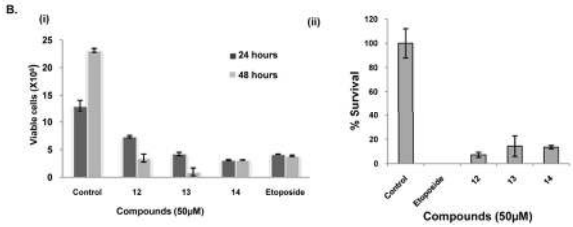


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Fig. 2. Cytotoxicity of carbohydrate fused pyrano[3,2-c]pyranones. (A) To calculate half maximal inhibitory concentration (IC_{50}) of compounds **12**, **13** or **14**, MCF 7 cells were treated with different concentrations (1-100 µM) for 48 hours. The percent growth inhibition was determined by MTT assay. IC_{50} values were determined by plotting values of percent inhibition against log concentration of each of these compounds. IC_{50} values for compounds **12**, **13** and **14** were 19.9, 14.6 and 10.98 respectively. The experiments were performed in triplicates, n=3 and ± SD value was calculated for each data point. (B) (i) Growth Inhibitory potential of **12**, **13** or **14** was evaluated towards MCF 7 cells at 50 µM. Cells were treated with compounds for 24 and 48 hours, and cell viability was determined by trypan blue exclusion assay. (ii) Survival fractions obtained by *in vitro* clonogenic assay showed significant inhibition in cells treated with these compounds.

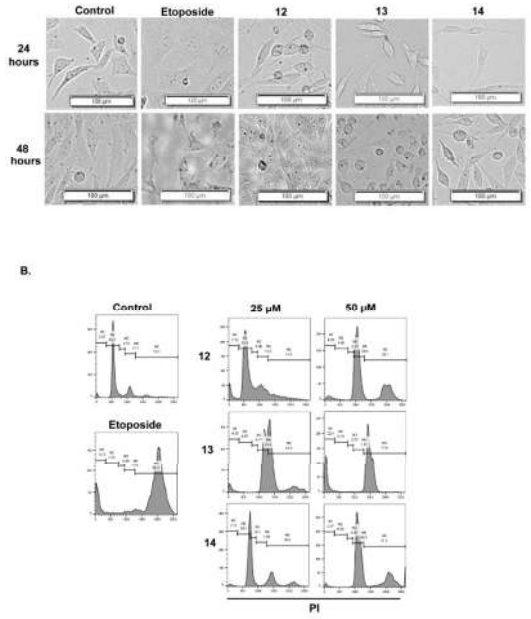

Fig. 3. Carbohydrate fused pyrano[3,2-c]pyranones induces morphological changes and modulate cell cycle. (A) MCF 7 cells treated with **12**, **13** or **14** for 24 hours and 48 hours showed significant changes in their morphology. Cellular shrinkage and dead cells were seen after treatment. (B) MCF 7 cells were treated with **12**, **13** or **14** at 25 µM and 50 µM concentrations for 48 hours and a changes in cell cycle phases were analyzed by flow cytometry. Etoposide (5 µM), was used as a positive control for the experiments. M1, M2, M3 and M4 represent apoptotic cells, G1, S and G2/M phases respectively. The percent of cells in each phase were analyzed from histogram FL2 vs cell counts. Representative images are shown from three independent experiments.

Cancerous cells migration is essential for invasion and metastasis phenotypes.[23] Herein we performed wound healing assay to determine ability of compound **12**, **13** and **14** to inhibit the migration of MCF-7 cells. The results showed that in untreated control, the cells migrated within 48 hours to fill scratched area, but treatment of these compounds significantly decreased the migration of MCF-7 cells in concentration and time dependent manner (Fig. 4).

Microtubule dynamics are critical prerequisite for mitotic spindle formation during cell division. Anticancer agents altering this microtubule dynamics drive the cancer to apoptosis.[24] Thus we had examined microtubulin distribution in cells treated with compounds **12**, **13** and **14** by immunofluorescence assay. Fluorescence microscopic images revealed tubulin aggregation in cells treated with compound **13**. Also, altered microtubule distribution was observed in elongated protrusions in cells treated with compound **12** and **14** (Fig. 5). Nocodazole, which is used as positive control for the experiment showed significant tubulin depolymerization. These findings corroborate with our cell migration results suggesting impairment of tubulin distribution affects cancer cell migration. Together our results suggest that carbohydrate fused pyrano[3,2-c]pyranones **12**, **13** and **14** affect breast cancer cell migration and induce apoptosis via microtubule distribution.

Since pyrano[3,2-c]pyranones **12**, **13** and **14** were synthesized with fused carbohydrate precursors, 2-*C* formyl galactal and 2-C formyl glucal with the aim to improve their cellular uptake by cancer cells, we monitored the time dependent intracellular uptake of these compounds in MCF-7 cells. These compounds having fluorescence properties due to conjugated double bond in pyrano[3,2-c]pyranone ring so without adding any fluorescent tag we can observe cellular uptake. Briefly, the cells were grown on coverslips and treated with of 50 µM of compounds **12**, **13** and **14** for 1 h and 4 h. The cells were then visualized for autofluorescence in UV filter under fluorescence microscopy. The results indicated higher cellular uptake of these carbohydrate fused pyrano[3,2-c]pyranones as compared to 4-hydroxycoumarin compound **5** (Fig. S2, SI).

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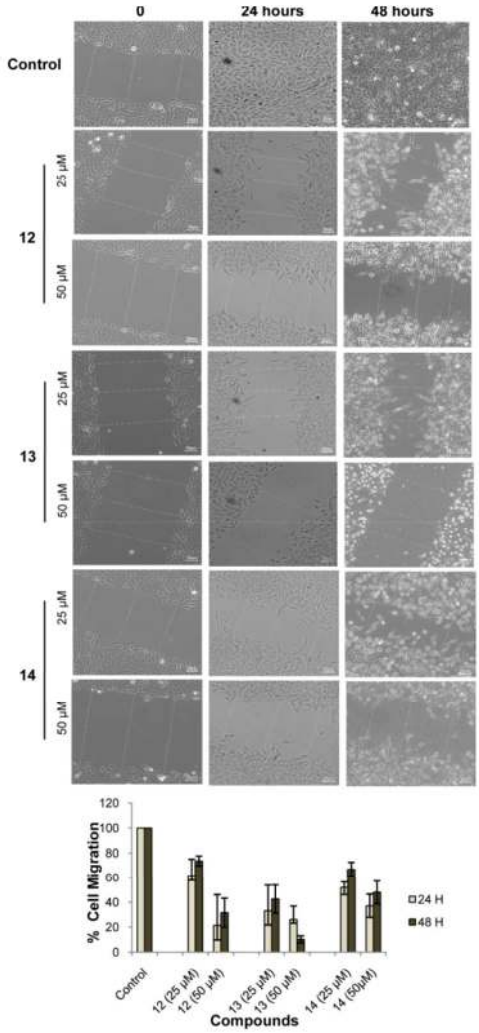

Fig. 4. Compounds 12, 13 and 14 regulate Breast Cancer Cell Migration. The cell migration was analyzed by wound healing method. Confluent monolayer of MCF 7 cells was scratched using pipette tip and the cells were incubated with indicated concentrations of these compounds for 24 and 48 hours. Untreated control closed completely after 48 hours, whereas compounds **12, 13** and **14** efficiently inhibit cell migration. The images are representative of three assays. Bar graph (bottom) represents significant cell migration inhibition by these compounds at 25 and 50 µM as compared to untreated control.

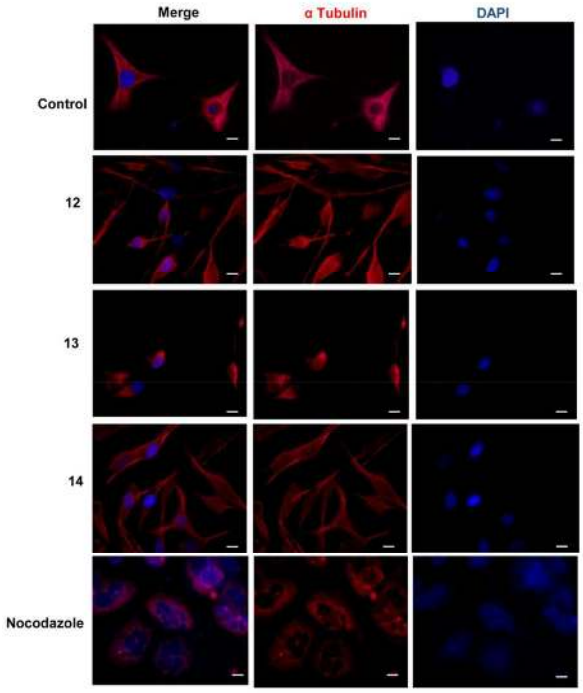

Fig. 5. Compounds 12, 13 and 14 regulate microtubules distribution. MCF 7 cells were incubated with compounds for 48 hours, permeabilized, stained with α tubulin antibody (red), and analyzed by fluorescence microscopy. Nucleuses are stained with DAPI (blue). Treated cells showed disturbed microtubule distribution. Nocodazole (1µM), positive control for the experiment causes microtubule depolymerization in MCF 7 cells. Scale bar = 10 µm.

Conclusions

In summary, we report an efficient microwave assisted synthesis of carbohydrate fused pyrano[3,2-c]pyranones. The diversely substituted on 4-hydroxycoumarins were successfully transformed to corresponding privileged carbohydrate fused pyrano[3,2-c]pyranones (n=20) within 10 min in good yield. Subsequently, we demonstrated that three of these carbohydrate fused pyrano[3,2-c]pyranone molecules possess anticancer activity at micromolar levels. Cellular uptake assay indicates that, carbohydrate moiety fusion to 4-hydroxycoumarin derivatives enhance their uptake by breast cancer cells which in turn results in their enhanced cell growth inhibitory potential than precursor 4-hydroxycoumarin compounds. We showed that, these molecules alter cell morphology and cause G2/M arrest in MCF-7. Moreover these compounds also affect cell migration and cell cycle via disturbing microtubule distribution. These findings shed light in considering these carbohyrate fused pyrano[3,2-c]pyranone molecules for developing potential anticancer chemotherapeutic agents.

Experimental Section

General Experimental Methods.

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All experiments were performed in an oven-dried apparatus and in anhydrous solvents in CEM microwave synthesiser. High resolution mass spectra obtained from a qudrapole/TOF mass spectrometer with an ESI source. Solvents were distilled by standard distillation procedures and stored in 4Å and 3Å molecular sieves. High-performance liquid chromatography (HPLC) experiments, to check purity of compounds, were carried out on a Waters Alliance System (Milford, MA) consisting of e2695 separation module and a2998 photodiode-array detector. The HPLC system was controlled with EMPOWER software (Waters Corporation, Milford, MA). ^{1}H (400 MHz), ^{13}C (100 MHz) NMR spectra was recorded with a Bruker AMX-400 MHz instrument. ^{1}H and ^{13}C chemical shifts are referenced to the solvents residual signals ($CDCl_3$: δ 7.26 for ^{1}H NMR and δ 77.16 for ^{13}C NMR) and reported in parts per million (ppm) at 25 °C. Coupling constants are expressed in hertz (Hz). Reactions were monitored by thin-layer chromatography carried out on 0.25 mm E. Merck silica gel plates (60F-254), Spots were visualized by phosphomolybdic acid and 10% H_2SO_4 in ethanol. 4-hydroxycoumarins (**1-10**), 2-*C*-formyl galactal **1a** and 2-*C*-formyl glucal **1b** were freshly prepared in laboratory. Organocatalysts (**I-IV**) used in this paper were purchased from sigma-aldrich.

General Experimental Procedure for reaction optimization: To a 10 mL of microwave vial 3,4,6-tri-*O*-benzyl-2-*C*-formyl D-galactal **1a** (100 mg, 0.225 mmol) and 4-hydroxycoumarin **1** (43.74 mg, 0.270 mmol) was added different combination of organocatalysts (**I-IV**, 0.112 mmol) in 2 mL of various solvents (viz. 1% acetic acid in toluene, EtOAc and Acetonitrile) and heated at 80°C (100W) for 10 min under microwave condition. The completion of reaction was monitored by TLC (R_f= 0.56, 3 : 7 = Ethylacetate: Hexane, v/v). After completion, reaction was quenched by adding 5 mL of $NaHCO_3$ solution and reaction mixturewas extracted with Ethylacetate (3 × 5 mL). The combined organic layer was dried over anhydrous Na_2SO_4 and combined organic layer was concentrated in vacuum to get crude product. The crude product was purified by flash column chromatography which furnished the designed product **11** in good yield (93%) as sticky solid. Different organocatalysts and solvent combinations furnished same product **11** in different yield percentage (Table 1).

General experimental procedure for synthesis of compounds 11-20 and 21a/b-30a/b.

A mixture of 3,4,6-tri-*O*-benzyl-2-C-formyl D-galactal **1a** (200 mg, 0.45 mmol), various 4-hydroxycoumarins **1-10** (0.54 mmol) and pyrrolidine **II** (8 mg, 0.224 mmol) was heated in microwave vial using 2 mL of toluene: AcOH (1:0.01) at 80°C (100W) for 10 min under microwave condition. The progress of reaction was monitored by TLC (3:7 = Ethylacetate: Hexane,v/v). After completion, reaction mixture was quenched by adding 5 mL of $NaHCO_3$ solution and reaction mixture was extracted with Ethylacetate (3 × 5 mL). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuum to get crude product. The crude product was purified by flash column chromatography to obtain the pure products **11-20** in good to very good yield (Table 2). The similar reaction protocol was adopted for the synthesis of compounds **21a/b**-**30a/b** starting from 3,4,6-tri-*O*-benzyl-2-*C*-formyl D-glucal **1b** and respective 4-hydroxycoumarin **1-10** (Table 3). Compounds **21a/b**-**30a/b** were isolated as inseparable mixture (1:1) of two epimers. Therefore in ^{1} H NMR spectra of these compounds protons were integrated and reported as doubled number of protons. The required temperature for preparation of **18-20** and **28a/b-30a/b** was 120 °C.

(8R,9R,10R,11aS)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-8,9,10,11a-tetrahydro-6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (11). Sticky solid, R_f = 0.56 (3:7, Ethylacetate:Hexane),^{1}H NMR (400 MHz, $CDCl_3$): δ 7.86 (d, *J* = 7.2 Hz, 1H, ArH), 7.61 (s, 1H, ArH), 7.52 (t, *J* = 14.8 Hz,1H), 7.39-7.28 (m, 16H, ArH), 7.03 (s, 1H, H-7), 6.09 (s, 1H, H-11a), 4.94 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.82 (d, *J* = 12 Hz, 1H, CH_2Ph), 4.67 (d, *J* = 11.6 Hz,1H, CH_2Ph), 4.62 (d, *J* = 12 Hz, 1H, CH_2Ph), 4.47 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.41 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.15 (s, 1H, H-8), 3.98 (m, 1H, H-9), 3.86 (t, *J* = 11.6 Hz, 1H, H-10), 3.62 (dd, *J* = 6.0 Hz and *J* = 9.6 Hz, 1H, H-13a), 3.51 (dd, *J* = 6.4 Hz, *J* = 9.6 Hz, 1H, H-13b).^{13}C NMR (100 MHz, $CDCl_3$): δ 160.7 (C-6), 157.2, 156.2, 152.7 (ArqC), 138.1, 138.0, 137.6, 137.5, 132.2, 128.6, 128.5, 128.4, 128.2, 128.0, 127.9, 127.7, 127.5, 127.4, 126.7, 124.2, 122.8 (ArC), 116.7, 114.5, 112.6 (C-7), 99.6, 96.9 (C-11a), 79.4 (C-8), 76.3 (C-9), 75.2 (C-10), 74.4,73.6, 71.7 (3 × CH_2Ph), 68.7 (C-13). HRMS (ESI), *m/z* calcd for $C_{37}H_{32}O_7[M+H]^+$ 589.2226; Found: 589.2221.

(8R,9R,10R,11aS)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-2-methoxy-8,9,10,11a-tetrahydro 6H-pyrano[3'2':5,6]pyrano[3,2-c]benzopyran-6-one (12): Off white amorphous solid, R_f = 0.51 (7:3, Ethylacetate: Hexane), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39-7.27 (m, 15H, ArH), 7.24 (s, 1H, ArH), 7.22 (s, 1H, ArH), 7.09 (dd, *J* = 3.2 Hz and *J* = 8.8 Hz, 1H), 7.03 (d, *J* = 2.0 Hz, 1H, H-7), 6.10 (s, 1H, H-11a), 4.94 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.82 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.69 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.64 (d, *J* = 9.6 Hz, 1H, CH_2Ph), 4.48 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.41 (d, *J* = 11.6 Hz,1H, CH_2Ph), 4.15 (brs, 1H, H-8), 3.98 (d, *J* = 1.6 Hz ,1H, H-9), 3.88 (s, 1H, H-10), 3.83 (s, 3H, OCH_3), 3.63 (dd,*J* = 6.4 Hz and *J* = 9.6 Hz, 1H, H-13a), 3.49 (dd, *J* = 6.0 Hz and *J* = 9.6, 1H, H-13b). ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.8 (C-6), 156.0, 147.3, 138.1, 137.5 (ArqC), 128.6, 128.5,128.4, 128.2, 128.0, 127.9, 127.7, 127.6, 127.5, 127.4, 126.6, 120.8 (ArC), 117.9, 114.8, 112.8 (C-7), 104.0, 99.7, 96.9 (C-11a), 79.3 (C-8), 76.3 (C-9), 75.2 (C-10), 74.3, 73.6, 71.7 (3 × CH_2Ph), 68.8 (C-13), 55.9 (OCH_3). HRMS(ESI), calcd, m/z $C_{38}H_{34}O_8$, $[M+K]^+$ 657.1885;Found: 657.1948.

(8R,9R,10R,11aS)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-2-chloro-8,9,10,11a-tetrahydro 6H-pyrano[3'2':5,6]pyrano[3,2-c]benzopyran-6-one (13): Yellow amorphous solid, R_f = 0.62 (3:7, Ethylacetate : Hexane),^{1}H NMR (400 MHz, $CDCl_3$): δ 7.83 (d, *J* = 2.0 Hz, 1H, H-1), 7.45 (dd, *J* = 8.8 Hz and *J* = 2.0 Hz, 1H, H-3), 7.37-7.23 (m ,16H, ArH), 7.00 (d, *J* = 1.6 Hz, 1H, H-7), 6.08 (s, 1H, H-11a), 4.93 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.80 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.66 (d, *J* =11.8 Hz, 1H, CH_2Ph), 4.62 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.48 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.41 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.15 (brs, 1H,

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H-8), 4.01 (brs, 1H, H-9), 3.86 (t, *J* = 6.0 Hz, 1H, H-10), 3.61 (dd, *J* = 6.0 Hz and *J* = 9.2 Hz, 1H, H-13a), 3.51 (dd, *J* = 6.4 Hz and *J* = 9.6 Hz, 1H, H-13b).^{13}C NMR (100 MHz, $CDCl_3$): δ 160.1 (C-6), 156.0, 151.0, 138.0, 137.5, 137.4 (Arq C), 132.1, 129.8, 128.6, 128.4, 128.2, 128.0, 127.9, 127.7, 127.6, 127.4, 122.2 (ArC), 118.1, 115.6, 112.3 (C-7), 100.4, 96.9 (C-11a), 79.3 (C-8), 76.4 (C-9), 75.2 (C-10), 74.4, 73.6, 71.7 (3 × CH_2Ph), 68.6 (C-13). HRMS (ESI), m/z calcd. for $C_{37}H_{31}ClO_7$, $[M+Na]^+$ 645.1651; Found:645.1724.

(8R,9R,10R,11aS)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-2-methyl-8,9,10,11a-tetrahydro 6H-pyrano[3'2':5,6]pyrano[3,2-c]benzopyran-6-one (14): Yellow amorphous solid, R_f = 0.57(7:3, Ethylacetate:Hexane).^{1}H NMR (400 MHz, $CDCl_3$): δ 7.65 (s, 1H, ArH), 7.39-7.18 (m, 17H, ArH), 7.02 (s, 1H, H-7), 6.08 (s, 1H, H-11a), 4.94 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.82 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.68 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.62 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.47 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.41 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.15 (brs, 1H, H-8), 4.00 (d, *J* = 2.0 Hz, 1H, H-9), 3.86 (t, *J* = 6.4 Hz, 1H, H-10), 3.63 (dd, *J* = 6.0 Hz and *J* = 9.6 Hz, 1H, H-13a), 3.53 (dd, *J* = 6.4 Hz and *J* = 9.6 Hz, 1H, H-13b), 2.38 (s, 3H, CH_3). ^{13}C NMR (100MHz, $CDCl_3$): δ 160.8 (C-6), 156.2, 150.9, 140.8, 138.1, 137.6, 137.5 (ArqC), 133.9, 133.2, 128.6, 128.5, 128.4, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 127.4, 126.9, 126.4, 122.3 (ArC), 116.4, 114.1, 112.7 (C-7), 99.5, 96.9 (C-11a), 79.3 (C-8), 76.3 (C-9), 75.1 (C-10), 74.4, 73.6, 71.7 (3 × CH_2Ph), 68.6 (C-13), 20.8 (CH_3), HRMS(ESI), m/z calcd. For $C_{38}H_{34}O_7$, $[M+Na]^+$ 625.2197; Found : 625.2253.

(8R,9R,10R,11aS)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-2-bromo-8,9,10,11a-tetrahydro 6H-pyrano[3'2':5,6]pyrano[3,2-c]benzopyran-6-one (15): Light yellow amorphous solid, R_f = 0.71 (3:7, Ethylacetate:Hexane), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 (d, *J* = 2.0 Hz, 1H, ArH), 7.59 (dd, *J* = 8.8 Hz and *J* = 2.0 Hz, 1H, ArH), 7.38-7.28 (m,15H, ArH), 7.18 (d, *J* = 8.4 Hz, 1H, ArH), 7.00 (s, 1H, H-7), 6.09 (s, 1H, H-11a), 4.94 (d, *J* = 11.6Hz, 1H, CH_2Ph), 4.80 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.67 (d, *J* =12.4 Hz, 1H, CH_2Ph), 4.62 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.48 (d, *J* = 12.0 Hz,1H, CH_2Ph), 4.41 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.16 (brs, 1H, H-8), 4.01 (s, 1H, H-9), 3.86 (t, *J* = 12.0 Hz, 1H, H-10), 3.62 (dd, *J* = 6.4 Hz and *J* = 9.2Hz, 1H, H-13a), 3.51 (dd, *J* = 6.0 Hz and *J* = 9.6 Hz, 1H, H-13b), ^{13}C NMR (100MHz, $CDCl_3$): δ 160.0 (C-6), 154.9, 151.4, 138.0, 137.5, 137.4, 134.9 (ArqC), 128.6, 128.4, 128.2, 128.0, 127.9, 127.7, 127.6, 127.4, 125.2 (ArC), 118.4, 117.1, 116.0, 112.3 (C-7), 100.4, 96.9 (C-11a), 79.3 (C-8), 76.4 (C-9), 75.2 (C-10), 74.4, 73.6, 71.7 (3 × CH_2Ph), 68.5 (C-13). HRMS (ESI), m/z, calcd.for $C_{37}H_{31}BrO_7$, $[M+Na]^+$ 689.1145; Found: 689.1167.

(8R,9R,10R, 11aS)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-8,9,10,11a–terahydro6H-2,3-benzo[g]pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (16): Amorphous solid, R_f = 0.62 (3:7 Ethylacetate : Hexane).^{1}HNMR (400 MHz, $CDCl_3$):δ 8.53 (s,1H, ArH), 7.83 (d, *J* = 8.8 Hz, 2H, ArH), 7.67-7.63 (m, 3H, ArH), 7.40-7.25 (m, 15H, ArH), 7.08 (s, 1H, H-7), 6.13 (s, 1H, 11a), 4.96 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.84 (d, *J* = 12 Hz, 1H, CH_2Ph), 4.69 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.64 (d, *J* = 12.8 Hz, 1H, CH_2Ph), 4.48 (d, *J* = 12.0Hz, 1H, CH_2Ph), 4.41 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.17 (s, 1H, H-8), 4.01(s, 1H, H-9), 3.87 (t, *J* = 11.6 Hz, 1H, H-10), 3.64 (dd, *J* = 6.4 Hz and *J* = 8.8 Hz, 1H, H-13a), 3.52 (dd, *J* = 6.4 Hz and *J* = 9.2 Hz, 1H, H-13b).^{13}C NMR (100 MHz, $CDCl_3$): δ 160.6 (C-6), 157.2, 150.2, 138.1, 138.1, 137.6, 137.5, 135.0 (ArqC), 128.7, 128.6, 128.4, 128.2, 128.0,127.8, 127.7,127.4, 127.1, 126.2, 124.2, 122.8, 122.6 (ArC), 118.2, 112.7 (C-7), 109.8, 99.3, 97.0 (C-11a), 79.4 (C-8), 76.2 (C-9), 75.2 (C-10), 74.4, 73.6, 71.7 (3 × CH_2Ph), 68.7 (C-13) HRMS(ESI), m/z calcd. for $C_{41}H_{34}O_7$, $[M + Na]^+$ 661.2197; Found : 661.2222.

(8R,9R,10R,11aS)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-3-fluoro-8,9,10,11a-tetrahydro 6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (17): Off white solid, R_f = 0.75 (3:7 Ethyl acetate : Hexane).^{1}H NMR (400 MHz, $CDCl_3$):δ 7.77 (dd, *J* = 6.0 Hz and *J* = 6.4 Hz, 1H, H-4), 7.31-7.17 (m, 15H,ArH), 6.95-6.91 (m, 3H, ArH), 6.00 (s, 1H, H-7), 4.86 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.73 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.59 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.54 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.40 (d, *J* = 11.2 Hz, 1H, CH_2Ph), 4.33 (d, *J* = 12 Hz, 1H, CH_2Ph), 4.07(s, 1H, H-8), 3.91 (s, 1H, H-9), 3.78 (t, *J* = 6.0 Hz, 1H, H-10), 3.54 (dd, *J* = 6.4 Hz and *J* = 9.2 Hz, 1H, H-13a), 3.42 (dd, *J* = 6.4 Hz and *J* = 9.4 Hz, 1H, H-13b).^{13}C NMR (100 MHz, $CDCl_3$): δ 160.4 (C-6), 156.9, 153.9, 137.0, 137.5, 137.4 (ArqC), 128.6, 128.4, 128.0, 127.9, 127.7, 127.4, 126.9, 126.6, 124.7, 124.6 (ArC), 112.5, 112.3, 111.2 (C-7), 104.4, 104.1, 98.7, 96.8 (C-11a), 79.3 (C-8), 76.2 (C-9), 75.2 (C-10), 74.4, 73.6, 71.7 (3 × CH_2Ph), 68.7 (C-13). HRMS(ESI), m/z calcd. for $C_{37}H_{31}FO_7$, $[M+Na]^+$ for 629.1946; Found: 629.1971.

(8R,9R,10R,11aS)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-2,4-dichloro-8,9,10,11a-tetrahydro 6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (18): Yellow amorphous solid. R_f = 0.78 (3:7, Ethylacetate:Hexane), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (s, 1H, H-1), 7.55 (s, 1H, H-4), 7.37-7.25 (m, 14H ArH), 6.99 (s, 1H, H-7), 6.08 (s, 1H, H-11a), 4.93 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.80 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.68 (d, *J* = 10 Hz, 1H,CH_2Ph), 4.62 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.47 (d, *J* = 12 Hz, 1H, CH_2Ph), 4.41 (d, *J* = 12 Hz, 1H, CH_2Ph), 4.15 (s, 1H, H-8), 3.98 (d, *J* = 9.2Hz, 1H, H-9), 3.85 (brs, 1H, H-10), 3.59 (dd, *J* = 6.4 Hz and *J* = 8.8 Hz, 1H, H-13a), 3.51 (dd, *J* = 6 Hz and *J* = 8.8 Hz, 1H, H-13b), ^{13} C NMR(100 MHz, $CDCl_3$): δ 159.0 (C-6) 154.5, 137.9, 137.5, 137.3 (ArqC), 128.6, 128.5, 128.4, 128.2, 128.0, 127.9, 127.8, 127.6, 127.4, 126.9, 122.5, 120.8 (ArC), 116.6, 112.1 (C-7), 100.9, 96.9 (C-11a), 79.2 (C-8), 76.4 (C-9), 75.3 (C-10), 74.5, 73.6, 71.8 (3 × CH_2Ph), 68.5 (C-13). HRMS(ESI), calcd. m/z, for $C_{37}H_{30}Cl_2O_7$, $[M + Na]^+$,679.1261; Found: 679.1305.

(8R,9R,10R, 11aS)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-3,4-dimethoxy-8,9,10,11a-tetrahydro 6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one(19): Yellow amorphous solid, R_f = 0.5 (3:7 Ethyl acetate :Hexane), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56 (d, *J* = 8.8 Hz, 1H, H-2), 7.39-7.27 (m, 15H, ArH), 7.00(d, *J* = 1.6 Hz,1H), 6.87 (d, *J* = 8.8 Hz, 1H,H-1), 6.04 (s, 1H, H-11a), 4.94 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.82 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.66 (d, *J* = 12.0 Hz ,1H, CH_2Ph), 4.61 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.47 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.41 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.13 (s, 1H, H-8), 4.00 (d, *J* = 2.0 Hz, 1H, H-9), 3.96 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 3.84 (t, *J* = 12.0 Hz, 1H, H-10), 3.62 (dd, *J* = 6.0 Hz and *J* = 9.6 Hz,1H, H-13a), 3.51 (dd, *J* = 6.4 Hz and *J* = 9.6 Hz, 1H, H-13b). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.4, 155.5, 154.8, 145.8, 137.1, 136.5, 135.0 (ArqC), 127.5, 127.4, 127.2, 126.9, 126.8, 126.3, 125.94, 124.51, 116.9, 111.7 (C-7),

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107.9, 107.4, 96.5, 95.8 (C-11a), 78.3 (C-8), 75.2 (C-9), 74.1 (C-10), 73.3, 72.5, 70.7 (3 × CH_2Ph), 67.7 (C-13), 60.5 (OCH_3), 55.3 (OCH_3). HRMS(ESI), calcd. m/z, $C_{39}H_{36}O_9$, $[M+H]^+$ 649.2439; Found : 649.2424.

(8R,9R,10R,11aS)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-1,3-dimethoxy-8,9,10,11a-tetrahydro 6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (20): Light yellow amorphous solid, R_f = 0.43 (3:7, Ethylacetate:Hexane), ^{1}H NMR (400 MHz,$CDCl_3$): δ 7.64 (s, 1H, ArH), 7.40-7.32 (m, 13H, ArH), 7.13-7.00 (m, 2H, ArH), 6.48 (brs, 1H, ArH), 6.28 (d,*J* = 13.6 Hz, 1H), 6.04 (d, *J* = 9.2 Hz, 1H, H-2), 5.01 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.83 (d, *J* = 10.8 Hz, 1H, CH_2Ph), 4.75 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.69 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.63 (d,*J* = 13.2 Hz, 1H, CH_2Ph), 4.45 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.30 (s, 1H, H-8), 4.17 (m, 1H, H-9), 4.00 (s, 1H, H-10), 3.88 (s, 3H, OCH_3),3.86 (s, 3H, OCH_3), 3.61(m, 1H, H-13a), 3.20 (m,1H, 13b).^{13}C NMR(100 MHz, $CDCl_3$): δ 167.7, 166.0, 163.4, 158.1, 157.5, 157.2, 138.7, 138.4, 138.0, 137.7, 136.1 (ArqC), 128.5, 128.4, 128.1, 128.0, 128.3, 128.1, 128.0, 128.0, 127.90, 127.8, 127.6, 127.5, 127.3, 127.1, 126.8 (ArC), 112.9 (C-7), 106.07, 100.5, 95.1, 95.0 (C-11a), 93.3, 93.1, 76.2 (C-8), 75.0 (C-9), 74.0 (C-10), 72.9, 72.1, 71.6 (3×CH_2Ph), 68.2 (C-13), 56.2 (OCH_3), 55.7 (OCH_3). HRMS(ESI), calcd., m/z $C_{39}H_{36}O_9$, $[M+H]^+$ 649.2439; Found: 649.2476.

(8R,9S,10R)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-8,9,10,11a-tetrahydro-6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (21a/b): Off white solid, R_f = 0.56 (3:7,Ethylacetate:Hexane),^{1}HNMR (400 MHz,$CDCl_3$): δ 7.87-7.85 (m,4H, ArH), 7.52-7.12 (m, 34H, ArH), 6.89 (s, 1H, H-7 of 21a), 6.80 (s, 1H, H-7 of 21b), 6.56 (s, 1H, H-11a of 21b), 5.95 (s, 1H, H-11a of 21a), 4.88 (d, *J* = 10.8 Hz, 2H, CH_2Ph,), 4.66 (d, *J* = 10.8 Hz, 2H, CH_2Ph), 4.60 (d, *J* = 12.0 Hz, 2H, CH_2Ph), 4.56 (d, *J* = 3.2 Hz, 1H, CH_2Ph), 4.50 (d, *J* = 11.2Hz, 2H, CH_2Ph), 4.44 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.36 (d, *J* = 12.0Hz, 1H, CH_2Ph), 4.30 (d, *J* = 11.6 Hz,1H, CH_2Ph) 4.16 (d, *J* = 8.0 Hz, 2H), 3.92-3.57 (m, 8 H), ^{13}C NMR (100 MHz,$CDCl_3$): δ 160.5, 159.5, 156.1, 153.4, 152.8, 138.0, 137.7, 137.5, 137.4, 137.2,135.4, 132.9, 132.3, 131.7, 128.6, 128.4, 128.1, 127.9, 127.7, 127.6, 127.4, 127.1, 127.0, 124.3, 124.2, 123.4, 122.9, 121.4, 116.9, 116.7, 114.4, 111.2, 109.0, 100.3, 99.7, 99.2, 97.2, 96.2, 82.0, 80.9, 78.7, 78.5, 76.6, 75.8, 75.1, 73.5, 73.3, 73.1, 71.5, 70.2, 68.8, 68.6. HRMS(ESI) calcd, for m/z, $C_{37}H_{32}O_7$ $[M + H]^+$ 589.2226; Found:589.2221.

(8R,9S,10R)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-2-methoxy-8,9,10,11a-tetrahydro-6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (22a/b): Yellow amorphous solid, R_f = 0.71 (3:7, Ethylacetate:Hexane),^{1}H NMR (400 MHz, $CDCl_3$):δ 7.41-7.15 (m, 36H, ArH), 6.94 (s,1H), 6.85 (s,1H), 6.61 (s, 1H), 6.01 (s, 1H), 4.92 (m, 2H, CH_2Ph), 4.70 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.65 (d,*J* = 12.0 Hz, 1H, CH_2Ph), 4.59 (brs, 2H, CH_2Ph), 4.53 (d, *J* = 11.6 Hz,3H, CH_2Ph), 4.47 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.39 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.33 (d, *J* = 11.2 Hz,1H, CH_2Ph), 4.21 (d, *J* = 8.8 Hz, 1H), 3.98 (m,2H), 3.85 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.80-3.58 (m, 7H).^{13}C NMR (100 MHz, $CDCl_3$): δ 160.7, 160.6, 159.3, 156.1, 156.0, 155.9, 148.0, 147.3 , 137.9, 137.7, 137.6, 137.4, 137.3,137.1, 128.5, 128.4, 128.3, 128.1, 128.0, 127.9, 127.8, 127.7, 126.9, 123.0, 121.8, 121.3, 121.0, 118.1, 117.9, 111.3, 104.3, 104.1, 100.3, 99.8, 99.2, 96.2, 81.9, 80.9, 78.7, 78.5, 76.5, 75.1, 73.5, 73.4, 73.3, 73.0, 71.5, 70.2, 68.8, 68.6, 56.0, 55.9 (2 × OCH_3). HRMS(ESI), calcd. m/z, $C_{38}H_{34}O_8$, $[M + H]^+$, 619.2326; Found: 619.2363.

(8R,9S,10R)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-2-chloro-8,9,10,11a-tetrahydro-6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (23a/b): Yellow amorphous solid, Rf = 0.71 (3:7, Ethylacetate:Hexane), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (s, 2H), 7.50-7.18 (m, 34H, ArH), 6.93 (s, 1H, ArH), 6.83 (s, 1H), 6.63 (s, 1H), 6.34 (s, 1H), 6.01 (s, 1H), 4.93 (d, *J* = 10.8 Hz, 2H,CH_2Ph), 4.73 (d, *J* = 11.6 Hz, 2H, CH_2Ph), 4.66 (d, *J* = 12.0 Hz, 2H, CH_2Ph), 4.56 (d, *J* = 9.6 Hz, 2H, CH_2Ph), 4.50 (d, *J* = 15.8 Hz, 1H, CH_2Ph), 4.42 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.36 (d, *J* = 12.8 Hz, 1H, CH_2Ph), 4.23 (d, *J* = 9.6 Hz, 2H), 3.98-3.65 (m, 8H).^{13}C NMR (100 MHz, $CDCl_3$): δ 160.0, 158.4, 154.9, 151.7, 151.0, 140.9, 137.9, 137.7, 137.4, 137.3,137.1, 132.8, 132.2,131.5, 130.0, 129.9, 128.6, 128.5, 128.4, 128.1, 128.0, 127.9, 127.8, 127.6, 127.3, 126.9, 122.8, 122.4, 122.4, 122.3, 122.2, 118.3, 118.2, 115.5, 110.9, 108.5, 100.9, 100.4, 99.4, 97.2, 96.2, 81.9, 80.9, 78.6, 78.4, 76.6, 75.9, 75.2, 73.6, 73.4, 73.1, 71.6, 70.3, 68.8, 68.4. HRMS(ESI), calcd. m/z, for $C_{37}H_{31}ClO_7$, $[M+ Na]^+$, 645.1651; Found: 645.1693.

(8R,9S,10R)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-2-methyl-8,9,10,11a-tetrahydro-6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (24a/b): Yellow amorphous solid, R_f = 0.59(3:7, Ethylacetate:Hexane),^{1}H NMR (400 MHz, $CDCl_3$): δ 7.69 (s,2H), 7.35-7.16 (m, 33H ArH), 6.68 (s, 1H), 6.32 (s, 1H), 6.27 (s, 1H), 5.72 (s, 1H), 5.66 (s, 1H), 4.95 (d, *J* = 10.8 Hz, 2H, CH_2Ph), 4.92 (d, *J* = 10.8 Hz, 2H, CH_2Ph), 4.90 (d, *J* = 11.6 Hz, 2H, CH_2Ph), 4.67 (d, *J* = 8.4 Hz, 1H, CH_2Ph), 4.64 (d, *J* = 11.4 Hz, 1H, CH_2Ph), 4.60 (d, *J* = 12.4 Hz, 2H, CH_2Ph), 4.54 (d, *J* = 11.2 Hz, 1H, CH_2Ph), 4.53 (d, *J* = 10.6 Hz, 1H, CH_2Ph), 3.96-3.692 (m, 10H), 2.40 (s, 3H, CH_3), 2.38 (s, 3H, CH_3).^{13}C NMR (100 MHz, $CDCl_3$): δ 160.9 (C-4), 160.5, 159.6,159.6, 156.2, 156.0,151.6, 137.9, 137.7, 137.4, 137.0, 136.8, 135.4, 134.0, 133.4, 132.8, 128.6, 128.5,128.4, 128.3, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 127.3, 123.0, 122.5, 122.3, 116.6, 116.5, 116.1, 111.3, 109.4, 99.1, 97.2, 96.2, 96.1, 82.0, 80.9, 79.2, 79.1, 75.8, 75.1, 73.5, 73.0, 72.7, 72.5, 71.56, 70.5, 70.3, 68.5, 67.9, 22.7, 20.8 (2 × CH_3). HRMS(ESI), m/z, $C_{38}H_{34}O_7$[M+H]$^+$ 603.2377; Found : 603.2388.

(8R,9S,10R)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-2-bromo-8,9,10,11a-tetrahydro-6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (25a/b): Off white amorphous solid, R_f = 0.81 (3:7, Ethylacetate: Hexane), ^{1}H NMR (400 MHz,$CDCl_3$): δ 7.95-7.94 (m, 2H), 7.54 - 7.47 (m, 3H), 7.33-7.05 (m, 29H, ArH), 6.82 (d, *J* = 8.0 Hz, 1H), 6.73 (d, *J* = 1.2 Hz,1H), 6.77 (d, *J* = 1.2 Hz, 1H), 6.52 (s, 1H), 6.23 (d, *J* = 6.0 Hz, 1H), 5.90 (s, 1H), 4.87 (d, *J* = 9.6 Hz, 1H, CH_2Ph), 4.82 (d, *J* = 12.0 Hz, 2H, CH_2Ph), 4.62 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.57 (d, *J* = 9.2 Hz, 1H, CH_2Ph), 4.54 (d, *J* = 10.2 Hz, 1H, CH_2Ph), 4.50 (d, *J* = 9.8 Hz, 2H, CH_2Ph), 4.45 (d, *J* = 9.2 Hz, 1H, CH_2Ph), 4.38 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.30 (d, *J* = 12.4 Hz, 1H, CH_2Ph), 4.25 (d, *J* = 11.6Hz, 1H, CH_2Ph), 4.11(d, *J* = 12.0 Hz, 2H), 3.89-3.53 (m, 8H), ^{13}C NMR (100 MHz,$CDCl_3$): δ 159.9, 159.8, 158.2, 154.8, 152.1, 151.5,

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137.9, 137.7, 137.4, 137.3, 137.1, 135.6, 135.0, 128.6, 128.4, 128.1, 128.0, 127.9, 127.8, 127.7, 127.5, 127.3, 125.9, 125.4, 122.4, 122.3, 118.6, 118.4, 117.2, 117.1, 116.0, 110.9, 100.9, 100.4, 99.4, 97.2, 96.2, 81.9, 80.9, 78.6, 78.4,76.6, 75.2, 73.6, 73.4, 73.1, 71.6, 71.6, 70.3, 68.7, 68.4. HRMS(ESI) m/z, calcd. for $C_{37}H_{31}BrO_7$, $[M + Na]^+$, 689.1145; Found : 689.1197.

(8R,9S,10R)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-8,9,10,11a-terahydro-6H-2,3-benzo[g]pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (26a/b): Light yellow amorphous solid, R_f = 0.53 (3:7, Ethylacetate:Hexane), ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.52 (s, 2H), 7.88-7.84 (m, 4H, ArH), 7.69-7.59 (m, 7H, ArH), 7.48-7.30 (m, 26H, ArH), 7.18 (s, 2H), 6.98 (s, 1H), 6.90 (s, 1H), 6.73 (s, 1H), 6.64 (s, 1H), 6.39 (s,1H), 6.33 (s,1H), 4.95 (d, *J* = 10.4Hz, 2H, CH_2Ph), 4.73 (d, *J* = 12 Hz, 1H, CH_2Ph), 4.66 (d, *J* = 10.4 Hz, 2H, CH_2Ph), 4.60 (d, *J* = 13.6 Hz, 2H, CH_2Ph), 4.55 (d, *J* = 7.6 Hz, 2H, CH_2Ph), 4.50 (d, *J* = 11.6 Hz, 2H, CH_2Ph), 4.42 (d, *J* = 12 Hz, 1H, CH_2Ph), 4.36 (m, 1H), 4.22 (s, 1H), 4.01-3.63 (m, 8 H).^{13}C NMR(100 MHz,$CDCl_3$): δ 160.7, 160.4, 157.1, 156.9, 156.5,156.1, 156.0, 155.0, 149.6, 138.0,137.9, 137.8, 137.7, 137.4, 135.5, 135.4,134.8, 128.6, 128.5, 128.4, 128.3, 128.0, 127.9, 127.8, 127.6, 127.5, 127.4, 127.3, 127.1, 126.6, 124.2, 123.5, 122.9, 122.6, 122.5,118.2, 111.3, 110.0, 109.4, 109.2, 101.9,99.3, 97.2, 97.1,96.3, 96.1,82.0,80.9, 78.7, 78.6, 75.8, 75.2, 73.7, 73.5, 73.4, 73.1, 70.8, 70.5, 70.3, 69.5,68.9. HRMS(ESI), calcd. m/z, for $C_{41}H_{34}O_7$, $[M + Na]^+$661.2197; Found : 661.2232.

(8R,9S,10R)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-2-fluoro-8,9,10,11a-tetrahydro-6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (27a/b): Yellow amorphous solid, R_f = 0.65(3:7, Ethylacetate: Hexane), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.87-7.82 (m, 3H), 7.39- 7.21 (m, 21H,ArH), 7.12 (s, 5H, ArH), 7.00-6.98 (m, 6H, ArH), 6.85 (s, 1H), 6.77 (s, 1H), 6.55 (s,1H), 6.26 (d, *J* = 6.8 Hz, 1H), 5.94 (s, 1H), 4.87 (d, *J* = 11.2 Hz, 2H, CH_2Ph), 4.68 (s, 1H, CH_2Ph), 4.63 (d, *J* = 12.0 Hz, 2H, CH_2Ph), 4.56 (d, *J* = 12.8 Hz, 2H, CH_2Ph), 4.50 (d, *J* = 8.0 Hz, 2H, CH_2Ph), 4.44 (d, *J* = 12.8 Hz, 1H, CH_2Ph), 4.35 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.30 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.16 (d, *J* = 7.6 Hz, 2H), 3.94-3.57 (m, 8H). ^{13}C NMR(100 MHz, $CDCl_3$): δ 160.5, 160.3, 160.2, 159.2, 156.2, 155.8, 154.6, 138.2, 138.0, 137.7, 137.4, 137.1, 137.2, 128.6, 128.4, 128.0, 127.9, 127.4, 126.9, 125.4, 124.8, 122.6, 121.3, 112.6, 111.0, 104.2, 99.3, 98.8, 97.1, 96.2, 81.9, 80.9, 78.6, 78.4, 75.8, 75.2, 73.7, 73.5, 73.3, 73.1, 71.6, 70.2, 68.8, 68.5. HRMS(ESI), calcd., m/z $C_{37}H_{31}FO_7$ $[M+ Na]^+$, 629.1946; Found : 629.196**7.**

(8R,9S,10R)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-2,4-dichloro-8,9,10,11a-tetrahydro-6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (28a/b): Yellow amorphous solid, R_f = 0.71 (3:7, Ethylacetate:Hexane), ^{1}H NMR (400MHz,$CDCl_3$): δ 7.80 (d, *J* = 2.4Hz, 2H), 7.59 (d, *J* = 8.8 Hz, 2H), 7.53 (d, *J* = 8.8 Hz, 1H), 7.45-7.29 (m, 25H, ArH), 7.15-7.13 (m, 3H), 6.90 (s, 1H), 6.80 (s, 1H), 6.60 (s, 1H), 6.31 (s, 1H), 5.99 (s, 1H), 4.95 (d, *J* = 10.4 Hz, 1H, CH_2Ph), 4.90 (d, *J* = 12.4 Hz, 2H, CH_2Ph), 4.71 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.59 (d, *J* = 12.8 Hz, 2H, CH_2Ph), 4.52 (d, *J* = 10.4 Hz, 2H, CH_2Ph), 4.47 (d, *J* = 11.6 Hz, 2H, CH_2Ph), 4.39 (d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.33 (d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.18 (m, 2H), 3.94-3.62 (m, 8H).^{13}C NMR (100 MHz,$CDCl_3$): δ 158.8, 154.4, 153.6, 147.1, 137.9, 137.3, 137.2, 132.7, 132.1, 131.4, 129.6, 128.7, 128.6, 128.4, 128.1, 128.0, 127.9, 127.8, 127.5, 127.3, 122.6, 122.0, 121.4, 120.9, 120.4, 116.5, 110.7, 99.6, 97.1, 96.3, 81.8, 81.0, 78.5, 78.4, 75.9, 75.2, 73.7, 73.6, 73.4, 73.2, 71.6, 70.4, 68.7, 68.4, HRMS(ESI), calcd. m/z for $C_{37}H_{30}Cl_2O_7$, $[M + Na]^+$ 679.1261; Found: 679.1264.

(8R,9S,10R)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-3,4-dimethoxy-8,9,10,11a-tetrahydro-6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one(29a/b): Yellow amorphous solid, R_f = 0.62(3:7,Ethylacetate:Hexane).^{1}H NMR (400 MHz, $CDCl_3$):δ8.09 (d, *J* = 7.6Hz 1H, H-8), 7.62 (s, 1H), 7.64-7.60 (m, 2H), 7.40-7.27(m, 25H, ArH), 7.15 (s, 3H), 6.90(d, *J* = 8.8 Hz, 2H), 6.83(s, 1H), 6.55(s, 1H), 6.33(s, 1H), 5.95(s, 1H),4.97-4.88(m, 2H, CH_2Ph), 4.69(d, *J* = 11.2 Hz, 2H, CH_2Ph), 4.65(d, *J* = 9.5 Hz,2H,CH_2Ph), 4.61-4.58(m, 1H, CH_2Ph), 4.53(d, *J* = 12.0Hz, 2H, CH_2Ph), 4.49(d, *J* = 8.0 Hz, 2H, CH_2Ph), 4.44(d, *J* = 11.6 Hz, 1H, CH_2Ph), 4.18(d, *J* = 9.2 Hz, 2H), 3.98(s, 6H, 2 × OCH_3), 3.94(s, 6H, 2 × OCH_3), 3.83 - 3.57(m, 8H).^{13}C NMR(100 MHz, $CDCl_3$):δ 160.5,160.3, 159.9, 158.9, 157.3,156.5, 156.2, 156.0, 155.6, 138.1, 138.0, 137.7,137.3, 136.1, 128.5, 128.4, 128.3, 128.0, 127.9, 127.7, 127.6, 125.8, 123.2, 120.2, 118.7, 118.1, 111.4, 108.8, 99.1, 98.1, 97.2, 96.1, 81.9, 80.9, 78.7, 78.5, 75.7, 75.1, 73.3, 73.0, 72.3, 71.5, 70.1, 68.9, 68.6, 68.2, 67.7, 61.6, 56.4. HRMS (ESI), calcd.,m/z for $C_{39}H_{36}O_9$, $[M+H]^+$ 649.2439; Found : 649.2424.

(8R,9S,10R)-8,9-bis(benzyloxy)-10-((benzyloxy)methyl)-1,3-dimethoxy-8,9,10,11a-tetrahydro-6H-pyrano[3',2':5,6]pyrano[3,2-c]benzopyran-6-one (30a/b): Yellow amorphous solid R_f = 0.50 (3:7, Ethylacetate:Hexane), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42-7.16 (m, 30H, ArH), 7.17-7.16 (m, 2H), 6.88 (s, 1H), 6.80 (s, 1H), 6.48 (s,1H), 6.43 (s, 1H, ArH), 6.29 (m, 1H, ArH), 5.93 (s, 1H, H-11a), 4.91 (dd, *J* = 12 Hz and *J* = 11.2 Hz, 2H, CH_2Ph), 4.67(d, *J* = 12.0 Hz, 1H, CH_2Ph), 4.61(d, *J* = 8.4 Hz, 2H, CH_2Ph), 4.56 (d, *J* = 12.4 Hz, 2H, CH_2Ph), 4.51 (d, *J* = 11.2 Hz, 2H, CH_2Ph), 4.38 (s, 1H, CH_2Ph,), 4.34 (d, *J* = 12.0 Hz, 2H, CH_2Ph), 4.16 (s, 2H), 4.01 (m, 2H), 3.90-3.84 (m, 6H, 2 × OCH_3), 3.77 - 3.72(m, 6H, 2 × OCH_3), 3.77 - 3.59 (m, 8H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.0, 156.9, 145.4, 138.2, 138.0, 137.8, 137.6, 137.3, 137.0, 128.5, 128.4, 128.2, 128.0, 127.9, 127.8, 123.5, 98.8, 96.0, 93.4, 82.0, 80.9, 78.8, 75.0, 73.4, 73.1, 72.8, 71.4, 69.9, 69.1, 63.9, 56.3, 55.7. HRMS(ESI), calcd, m/z,for $C_{39}H_{36}O_9$, $[M + H]^+$ 649.2439 Found: 649.2474.

Cell Culture

The human breast cancer cell line MCF-7, MDA-MB-231 and liver cancer cell line HepG2 and normal human embryonic kidney cells HEK293 were obtained from the American Type Culture Collection (ATCC, USA).The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, St. Louis, USA) containing with L-glutamine, supplemented with 10% heat inactivated FBS (Fetal Bovine Serum, Gibco, USA) and 1% penicillin-streptomycin (Gibco USA)in humidified CO_2 incubator at 37ºC. The cells were maintained as monolayer in 25 cm^2 culture flasks (T-25) and the medium was changed 10-12 hours prior to experiment. The cells used for each experiment were of less than 10 passage number.

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MTT assay

MTT cell viability assay was performed as per standard protocols (MTT Cell Proliferation Assay ATCC® 30-1010K). Briefly, MCF-7, MDA-MB-231 and HepG2 cells were trypsinized (Trypsin-EDTA, Invitrogen) and seeded at a density of 10,000 cells per well in complete media in 96-well flat-bottomed plate, followed by incubation at 37 °C for 20-24 hours. The cells were then treated with all synthesized carbohydrate fused pyrano[3,2-c]pyranones (n= 20) along with different substituted 4-hydroxycoumarin precursors (n = 10) for 48 hours at different concentrations.. Following incubation, 10µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Merck, USA) at a concentration of 5mg/mL in phosphate buffer saline(PBS) was added to each well and incubated for 2-3 hours until a purple precipitate is visible. The formazan crystals formed were dissolved by adding 100 µL DMSO and the plate was incubated for 15 minutes with gentle shaking. The cell viability was evaluated by measuring absorbance at 570nm and a reference wavelength of 630nm by using MicroplateReader (Varioskan, ThermoFishcer Scientific). IC_{50} values were determined by plotting graph with percent growth inhibition vs concentrations of compounds. Similar method was followed for MTT assay against normal cell line (HEK 293).

Cell Proliferation Assay

MCF-7 cells were seeded in 6 well plates at a density of ~3 X 10^5 cells per well and allowed to attach for 24 hours. Subsequently the cells were treated with compounds **12, 13** and **14**at 50µM concentration and further incubated for 48 hours. For trypan blue staining, 1 part of 0.4% trypan blue solution (Sigma-Merck, USA) is mixed with 1 part of diluted cell suspension and incubated for 2-4 minutes at room temperature. The total numbers of viable cells (unstained) were counted by hemocytometer (Rohem, India) at 10 X.

Clonogenic Assay for cell survival

MCF-7 cells were seeded in 6 well plates at a density of ~3 X 10^5 cells per well and allowed to attach for 24 hours. The cells were treated with compounds **12, 13** and **14** at 50 µM concentration and further incubated for 24 hours. Following incubation, the adherent cells were washed with incomplete media (without FBS), detached by trypsinization and centrifuged at 800 × g for 5 minutes. The pellet was further resuspended in complete DMEM media and single cell suspensions were obtained by pipetting. The number of cells in each compound are counted carefully using a hemocytometer (Rohem, India) and diluted such that appropriate cell numbers are seeded into petri dishes (in duplicates). The plating efficiency was calculated by counting the number of colonies obtained per plate with respect to number of cells plated. The percent survival was determined for each test condition with respect to control.

Cell cycle and morphology analysis

MCF-7 cells were seeded in 6-well plates at a density of 3 × 10^5 cells per well in complete DMEM, and treated with compounds **12, 13** and **14** at 50 µM concentrations for 48 hours. The untreated cells were taken as negative control. Etoposide treated cells (5 µM) were taken as positive control for the experiments. The morphological changes were monitored at 24 hours and 48 hours and the imaging was done using an inverted light microscope (Olympus, PA, USA). For cell cycle analysis, the cells were seeded in 6-well plates at 3 × 10^5 cells in complete media and treated with these compounds at 25 µM and 50 µM for 48 hours. Following incubation, the adherent cells were washed with incomplete media (without FBS), detached by trypsinization and centrifuged at 800 × g for 5 minutes. The floating cells in each well were collected from supernatant by centrifuging at 1000 × g for 10 minutes. The cells were washed twice with PBS and re-suspended in PBS-chilled methanol solution (1:9 ratio) at 4 °C overnight. The cells were then washed with PBS, and treated with 20µg/mL RNase (Sigma-Aldrich, St. Louis, MO, USA), for 30 minutes at 37 °C. Before analysis, the cells were stained with propidium iodide (10µg/mL, Sigma-Aldrich, USA) and incubated at room temperature in dark for 20 minutes. The cells were finally re-suspended in 200µl of PBS and cell cycle phases of 10000 cells was analyzed by flow cytometer (Becton Dickinson, CA, USA).The population of cells in each phase was determined by using the BD Diva software.

Wound Healing assay

MCF-7 cells were seeded in 6-well plates at a density of ~4 X 10^5 cells per well. After 24 hours of seeding, the cells were treated with 25µM and 50 µM concentrations of compounds. The cells were scratched through the center of the plate using microtip and imaged at different time points, 24 h and 48 h. The wound healing was monitored by counting five fields for each treatment and distance of wound was measured using Catymage software.

Immunofluorescence assay

MCF-7 cells were seeded on glass cover slips in complete DMEM media and allowed to grow for one cycle. The cells were incubated with 50 µM of compounds **12, 13** and **14** along with nocodazole (positive control) and incubated for 48 hours. The cells were washed with cold 1X PBS and fixed with chilled methanol for 30 minutes. Cells were then blocked in 5% bovine serum albumin (BSA) in PBS and then immunostained with tubulin α polyclonal rabbit antibodies (Immunotag) for 1 hour at a dilution of 1:500, followed by incubation in Alexa 594 anti-rabbit secondary antibody at room temperature for 1 hour. The cover slips were washed with PBS and mounted with antifade reagent with DAPI and visualized under fluorescence microscope (Olympus, USA) at 40X for tubulin staining.

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Uptake Assay

MCF-7 cells were seeded on glass cover slips in complete DMEM media and allowed to grow for one cycle. The cells were treated with 50 µM concentration of compounds **12, 13, 14** and **5** for 0-4 hours. The coverslips were washed with 1X PBS and put on glass slides. Autofluorescence was observed by fluorescence microscopy (Olympus, USA) at 60X objective in UV filter.

Conflicts of interest

Authors declare no conflict of interest

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://.

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