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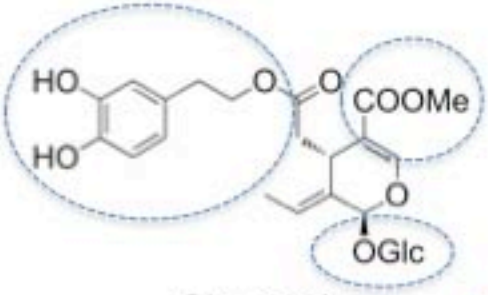
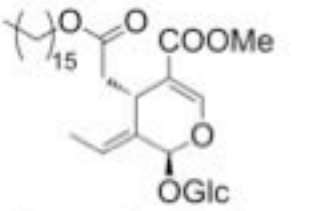
Enriched extract

Olive tree leaves



Oleuropein

52 derivatives
SAR



Cytotoxic Analogue

Active in vivo
Non Toxic

Stimulates the
immune system

New semi-synthetic analogs of oleuropein show improved anticancer activity *in vitro* and *in vivo*

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ABSTRACT

Oleuropein is a glucosylated seco-iridoid present in olive fruits and leaves. Due to its broad spectrum of biological activities, including anticancer properties, oleuropein has attracted scientific attention for the past 20 years. The promising antiproliferative activity of an olive leaf extract enriched in oleuropein against a series of human cancer cell lines, prompted us to proceed with the semi-synthesis of 51 analogs of oleuropein. Following their initial screening against the estrogen receptor negative breast cancer cell line SKBR3, 7 analogs were shown to display significant cytotoxicity and were further tested against 6 additional solid tumor-derived and leukemic cell lines. The analog with the most promising antitumor activity (**24**) was selected for more detailed studies. **24** was non-toxic to peripheral blood mononuclear cells derived from healthy blood donors when tested at concentrations close to its half maximal inhibitory concentration. *In vivo* administration of **24** in melanoma-bearing mice resulted in reducing tumor size in a dose-dependent manner and in inducing anti-melanoma-reactive immune responses. Our results suggest that analog **24,** emerging from the initial structure of oleuropein, represents a promising lead structure for further optimization.

Keywords

oleuropein; *Olea europaea*; semi-synthesis; cytotoxicity screening; in vivo; melanoma; innate immune system

1. Introduction

Numerous epidemiological studies have demonstrated low occurrence of cardiovascular incidents, neurodegenerative diseases, diabetes and cancer in the Mediterranean region [1]. This favorable outcome has been ascribed to the Mediterranean diet and mostly to the consumption of olive oil and table olives. Nevertheless, the production of olive oil and edible

olives leads to the generation of tons of by-products including leaves, olive pomace, table olive wastewaters and olive processing wastewaters. These by-products are highly toxic for the environment and in general are abounded in fields. However, they represent a source of bioactive molecules [2,3] that could be further exploited in medicinal chemistry studies or in drug development. Phytochemical investigation of olive products and by-products revealed the presence of several phenolic compounds, including oleuropein (**1)**, hydroxytyrosol, ligstroside, oleocanthal and oleacein (Figure 1) [4,5].

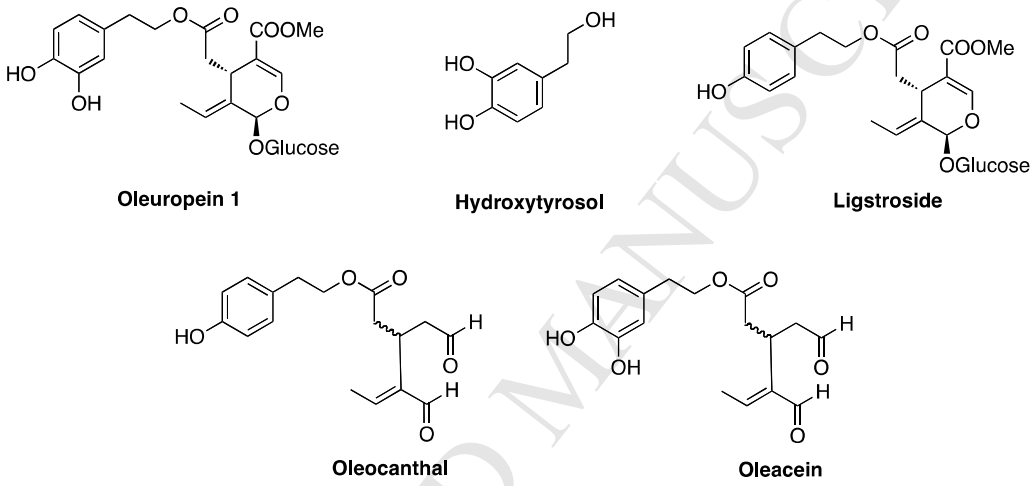


Figure 1: Chemical structures of natural phenolic compounds derived from olive products.

Among them, oleuropein **1** (Figure 1) is a phenolic seco-iridoid glucoside and the major component of leaves and unprocessed olive drupes of *Olea europaea* (Oleaceae) responsible for the bitterness of olives [4]. We recently showed that the huge amount of olive leaves collected during the olive-harvest period can be used as starting material for the efficient large-scale isolation of oleuropein **1** [6].

Many studies have reported a wide range of biological activities for oleuropein [4,7] including antioxidant [8], anti-hypertensive [9], anti-inflammatory [7], anti-atherogenic [10], antimicrobial [11] and antiviral [12]. Additional studies showed that oleuropein exerts neuroprotective [13], anti-aging [14] and skin-protecting effects [15], as well as potential

cardioprotective [16], anti-ischemic [17] and hypolipidemic activities [18]. More recent reports highlighted the *in vitro* anticancer activity of olive leaf extracts and their phenolic compounds, notably towards human breast cancer cell lines [19-23]. Specifically oleuropein **1** and hydroxytyrosol have been shown to induce cancer cell apoptosis through various mechanisms, such as *via* decreasing HIF-1α, upregulating the expression of p53 and of the cyclin-dependent inhibitor p21, and *via* down-regulating NF-kappa B and cyclin D1. Furthermore, both molecules act as inhibitors of endothelial proliferation, hindering angiogenesis [7].

Most reports conducted to date consider the phenolic part of the chemical structure of oleuropein **1** as the active moiety, however without implementing consistent structure-activity relationship (SAR) studies. Moreover, no study has focused on improving the biological activity of oleuropein **1** and reveal SARs through investigating an assembly of structurally modified derivatives. Interestingly, olive leaves are a sustainable source of oleuropein **1** and constitute an advantageous starting material for the subsequent generation of semi-synthetic analogs. In this report, we assessed the anticancer activity of an oleuropein-enriched leaf extract, already reported to possess antiparasitic properties [24]. Using oleuropein **1** extracted from this leaf extract as starting material, we further designed and synthesized 51 new semi-synthetic derivatives of oleuropein, assessed their cytotoxicity against human cancer cells *in vitro*, and the analog with the most remarkable anticancer activity was evaluated *in vivo*, in a mouse model of melanoma.

2. Results and discussion

2.1 Screening of olive leaf extract against various cancer cell lines

The enriched olive leaf extract and oleuropein **1** isolated from this extract were evaluated for their cytotoxic/cytostatic potency [(methyl-thiazol-tetrazolium) MTT dye reduction assay]. A

panel of five human cancer cell lines was selected, comprising FM3 (melanoma), HCT-116 (colon), HeLa (cervix), MCF-7 [breast, estrogen receptor alpha positive (ERa^+)] and SKBR3 [breast, ERa negative (ERa^-)]. The relative half maximal inhibitory concentrations (IC_{50}) were calculated after 72 h of exposure to the extract or oleuropein (Table 1).

Table 1. Cytotoxic evaluation of an enriched in oleuropein olive leaf extract and oleuropein isolated from the same leaf extract

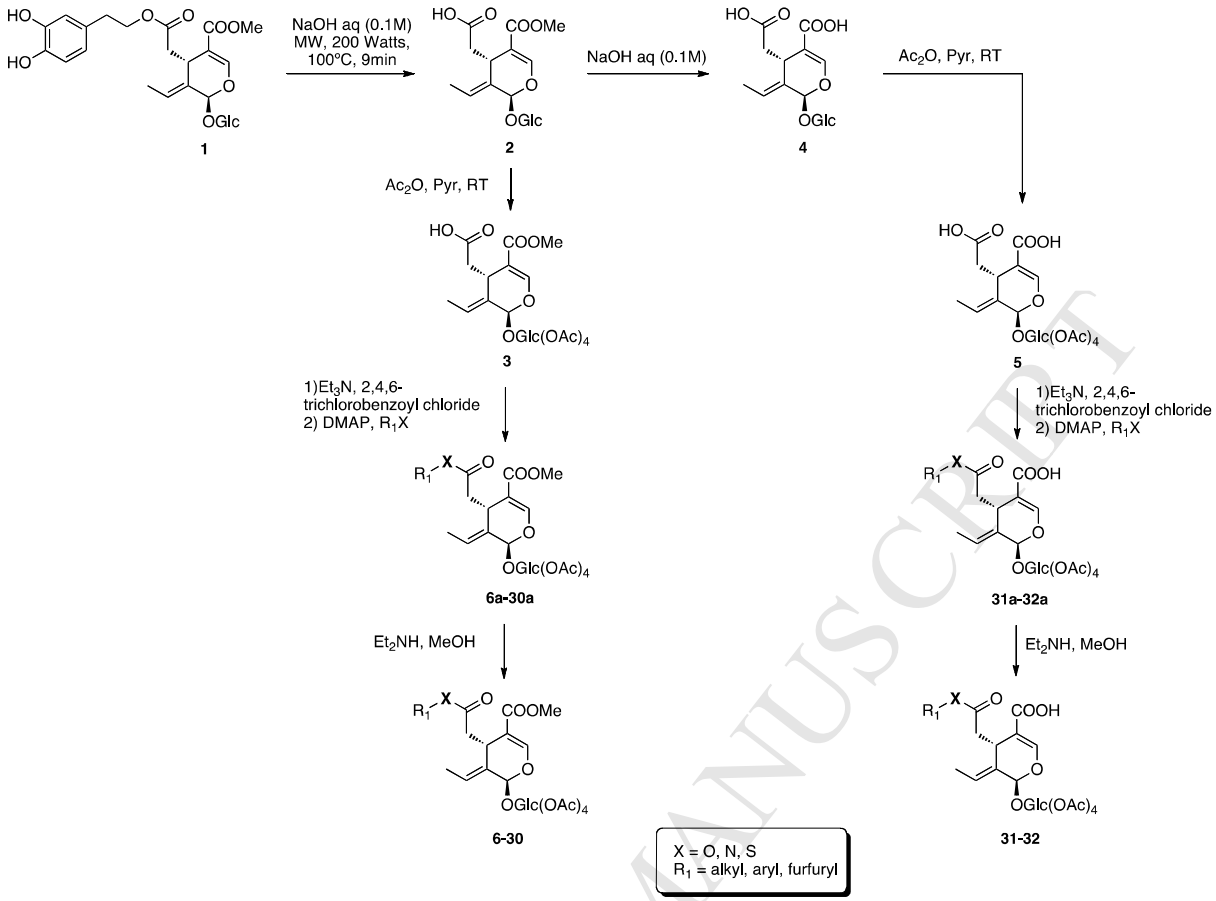
	IC_{50} ± SD (in μg/mL)[1]				
Sample	**FM3**	**HCT-116**	**HeLa**	**MCF-7**	**SKBR3**
Enriched olive leaf extract	240.00 ± 10.00	174.30 ± 8.15	165.00 ± 5.00	120.00 ± 5.00	104.30 ± 6.03
Oleuropein 1	148.30 ± 2.89 (274.53 μM)	100.00 ± 13.23 (185.12 μM)	143.30 ± 15.28 (265.28 μM)	91.67 ± 14.43 (169.70 μM)	86.67 ± 15.28 (160.44 μM)
Doxorubicin[2]	0.217 ± 0.002	0.100 ± 0.001	0.221 ± 0.004	0.220 ± 0.004	0.202 ± 0.007

[1] IC_{50} values are means ± SD from 3 independent experiments
[2] Doxorubicin was used as a positive control. Values given in μM

As shown, the olive leaf extract and oleuropein **1** were similarly cytotoxic against HeLa, MCF-7 and SKBR3 cells, with the lowest IC_{50} values recorded for SKBR3, suggesting that oleuropein **1** likely contributes to the overall cytotoxic activity of the extract. However, compared to the chemotherapeutic doxorubicin, oleuropein possessed an overall weak cytotoxicity.

2.2 Chemical synthesis of new oleuropein analogs

To our knowledge, very few reports have used **1** as raw material for the generation of semi-synthetic analogs [25]. Here, oleuropein derivatives were synthesized following a simple, yet efficient synthetic pathway, depicted in Scheme 1.

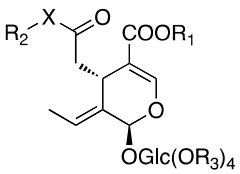


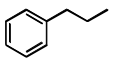
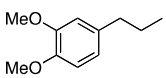
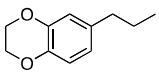
Scheme 1: Schematic representation of the procedure followed for the synthesis of oleuropein analogs.

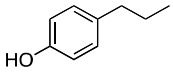
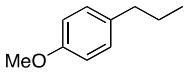
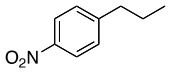
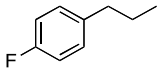
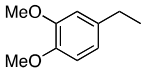
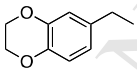
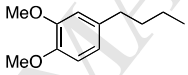
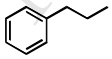
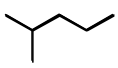
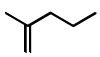
The classical conditions suggested in the literature for the selective cleavage of the hydroxytyrosol part of oleuropein involved the reaction with an aqueous solution of sodium hydroxide at room temperature over several hours [18]. In order to considerably reduce the reaction time, microwave-assisted saponification was preferred. Under these conditions, the oleoside 11-methylester (**2**) was obtained in 10 min, with a yield of 68 % when **1** was dissolved with NaOH (0.1 M). Saponification of **2** under the same conditions caused cleavage of the remaining methylester group forming the natural oleoside (**4**)**.** The latter constitutes an additional intermediate to enlarge the structural diversity of oleuropein analogs. The hydroxyl groups of the glucose moiety of **2** and **4** were then protected, hydroxyl groups were then acetylated using anhydride acetic in pyridine to generate oleoside 11-methylester tetraacetate

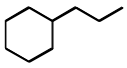
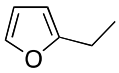
(**3**) and oleoside tetraacetate (**5**) respectively. Esterification of **3** and **5** was performed according to the Hanessian protocol [26] implying mild and non-acidic conditions. Thereby, the acid function of oleosides tetraacetate **3** and **5** was activated using the Yamaguchi reagent (2, 4, 6, trichlorobenzoylchloride) to form the corresponding anhydrides. Coupling with the different alcohols/thiols/amines was achieved one pot in presence of 4-DMAP, enabling a faster reaction with the diverse nucleophiles. The esterification yields ranged from 30% to 90%. The final selective deprotection of acetate groups was carried out using diethylamine in MeOH. However, in the case of thioesters, the corresponding deprotected compound was not obtained and deprotection led to the formation of the oleoside 7,11-methylester (**18**). Indeed, the use of diethylamine led to cleavage of the thiol part followed by methoxylation of the acid function, probably due to the increased electrophilicity of the carbonyl in position 7 when a sulfur atom is present. Overall, 51 new oleuropein analogs were synthesized, divided in two subgroups of aryl and alkyl ester derivatives (Table 2).

Table 2: Chemical structure and yield of the synthesized oleuropein **1** analogs



Comp.	X	R_1	R_2	R_3	Yield (%)
Aryl esters					
6	O	Me		H	30
6a				Ac	50
7	O	Me		H	60
7a				Ac	58
8	O	Me		H	68
8a				Ac	46

9 **9a**	O	Me		H Ac	71 23
10 **10a**	O	Me		H Ac	40 89
11 **11a**	O	Me		H Ac	43 96
12 **12a**	O	Me		H Ac	48 60
13 **13a**	O	Me		H Ac	60 40
14 **14a**	O	Me		H Ac	76 69
15 **15a**	O	Me		H Ac	74 36
16 **16a**	O	Me		H Ac	26 32
17a	S	Me		Ac	70
Alkyl Esters					
18 **18a**	O	Me	CH_3-	H Ac	57 31
19 **19a**	O	Me	CH_3CH_2-	H Ac	45 31
20 **20a**	O	Me	$CH_3CH_2CH_2$-	H Ac	85 34
21 **21a**	O	Me	$CH_3CH_2CH_2CH_2$-	H Ac	67 87
22 **22a**	O	Me		H Ac	17 47
23 **23a**	O	Me		H Ac	33 63

24 **24a**	O	Me		H Ac	28 30
25 **25a**	O	Me		H Ac	42 67
26 **26a**	O	Me		H Ac	63 67
27a	O	Me		Ac	42
28 **28a**	O	Me		H Ac	48 71
29a	S	Me	$CH_3CH_2CH_2$-	Ac	70
30 **30a**	NH	Me	$CH_3CH_2CH_2$-	H Ac	52 90
31 **31a**	O	H	CH_3-	H Ac	54 17
32 **32a**	O	H		H Ac	38 30

2.3 Some semi-synthetic oleuropein analogs effectively inhibited cancer cell proliferation

Based on previous studies highlighting the anticancer properties of **1** on breast tumors, particularly on ER^- breast cancer cell lines [27], a first screening was initiated against SKBR3 cells, including the new semi-synthetic analogs, some intermediates, and doxorubicin as a positive control. Inhibition of cell proliferation was assessed by the MTT dye reduction assay after 72 h of SKBR3 exposure to each analog and IC_{50} values were calculated (Table 3).

Table 3. Screening of oleuropein analogs against the human breast cancer cells SKBR3

Comp.	**SKBR3 IC_{50} ± SD[1] (in µM)**	**Comp.**	**SKBR3 IC_{50} ± SD[1] (in µM)**
2	>15	**19**	>15
3	>15	**19a**	>15
4	>50	**20**	>15
5	>20	**20a**	6.60 ± 0.57
6	11.02 ± 0.71	**21**	>15

6a	11.50 ± 1.06	**21a**	>15
7	>15	**22**	9.02 ± 0.71
7a	>15	**22a**	>15
8	>20	**23**	>15
8a	>15	**23a**	>15
9	>20	**24**	1.60 ± 0.42
10	>20	**24a**	3.81 ± 0.56
10a	>20	**25**	2.00 ± 0.71
11	>20	**25a**	>15
11a	>20	**26**	>15
12	>20	**26a**	>15
12a	>20	**27a**	>20
13	>15	**28**	>15
13a	>15	**28a**	>15
14	>15	**29a**	>15
14a	>15	**30**	>20
15	>15	**31**	>20
15a	>15	**31a**	>20
16	>20	**32a**	>20
16a	>20	**Oleuropein 1**	160.44 ± 1.50
17a	>15	**Hydroxytyrosol**	201.10 ± 9.18
18	>15	**Doxorubicin**	0.202 ± 0.001
18a	>15		

[1] IC_{50} values are means ± SD from 3 independent experiments

Interesting results and SAR information emerged from this first screening. Specifically and in agreement with the data shown in Table 1, oleuropein **1** and hydroxytyrosol displayed a weak cytotoxicity compared to the control compound doxorubicin. Compounds possessing methoxy (**7**, **7a**, **10**, **10a**, **14**, **14a**, **16**, **16a**), phenol (**9**), 1,4-dioxane (**8**, **8a**, **15**, 1**5a**), fluorine (**12**, **12a**) and nitro (**11**, **11a**) substitutions were less active against SKBR3 cells. All the aryl esters were also less active with the exception of compounds **6** and **6a** (IC_{50}= 11.02 and 11.50 μM, respectively), lacking the catechol moiety, which was replaced by a phenyl group. Analogs of **6** and **6a** with shortened carbon chain (**13**, **13a**) and the corresponding thioester (**17a**) were marginally cytotoxic, highlighting the importance of the nature of the ester for displaying anticancer activity. The most active molecules identified belonged to alkyl esters. The length of the carbon chain played an important role in the recorded cytotoxic potency of the semi-synthetic analogs. Thus, compounds with methyl (**18**, **18a**, **31**, **31a**), ethyl (**19**, **19a**), butyl (**21**, **21a**) or unsaturated (**23**, **23a**, **26**, **26a**, **27a, 28, 28a**) chains were less active. However,

compounds with propyl (**20a**), isopentyle (**22**) and cyclohexyle (**25**) groups displayed potent cytotoxicity, with IC_{50} values ranging between 2 and 9.02 µM. Analogs of compound **20a**, namely **29a** and **30**, were less active, confirming the prevalence of the ester upon thioester or amide for displaying cytotoxicity. The highest anticancer activity (IC_{50}= 1.60 µM) was recorded for compound **24** possessing a cetyl group (sixteen carbons). The corresponding acetylated derivative **24a** possessed also significant cytotoxicity (IC_{50}= 3.81 µM). Interestingly, the presence of a free acid in position 11 (**32a**) abrogated the activity of the compounds, highlighting the importance of the methylester in position 11. Overall, the SAR data generated could be summarized as depicted in Figure 2.

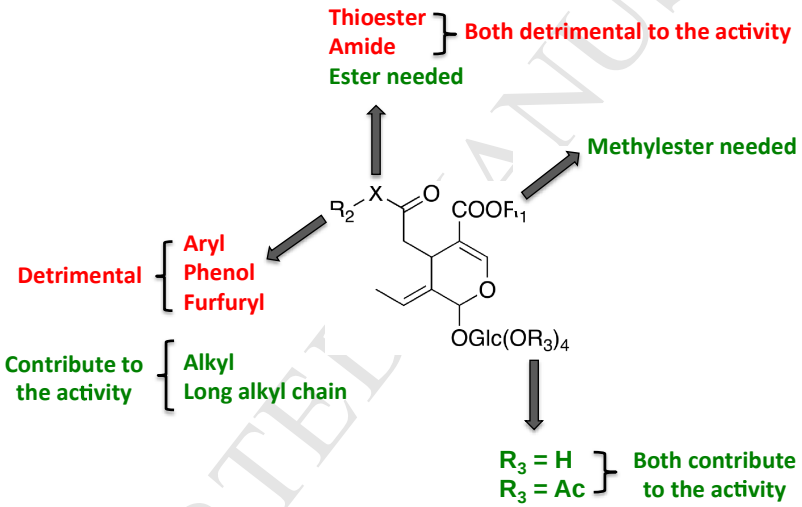


Figure 2: Summary of the SAR data of the oleuropein analogs.

7 compounds showed potent anticancer activity (Figure 3). These results suggest that the hydroxytyrosol moiety of **1** cannot be considered the active part of the molecule. Indeed, compounds **6** and **6a** possessing unsubstituted phenyl groups displayed better cytotoxicity compared to oleuropein. Moreover, the 7 active semi-synthetic analogs were mostly lipophilic and the most active did not possess aryl esters but alkyl esters with long carbon chains. Replacement of the ester in position 7 by thioester or amide was likely detrimental for the activity. The same profile was observed with the replacement of the methylester in position 11

by a free acid. Indeed, the 11-methylester could stabilize the overall structure compared to the free-acid. It is known that deglycosylation followed by 11-decarboxymethylation (chemical or enzymatic) of oleuropein could open the seco-iridoid to oleacine (Figure 1) [25,28]. Thus, similar modifications of the semi-synthetic oleuropein analogs could eventually lead, in cellulo, to inactive open metabolites.

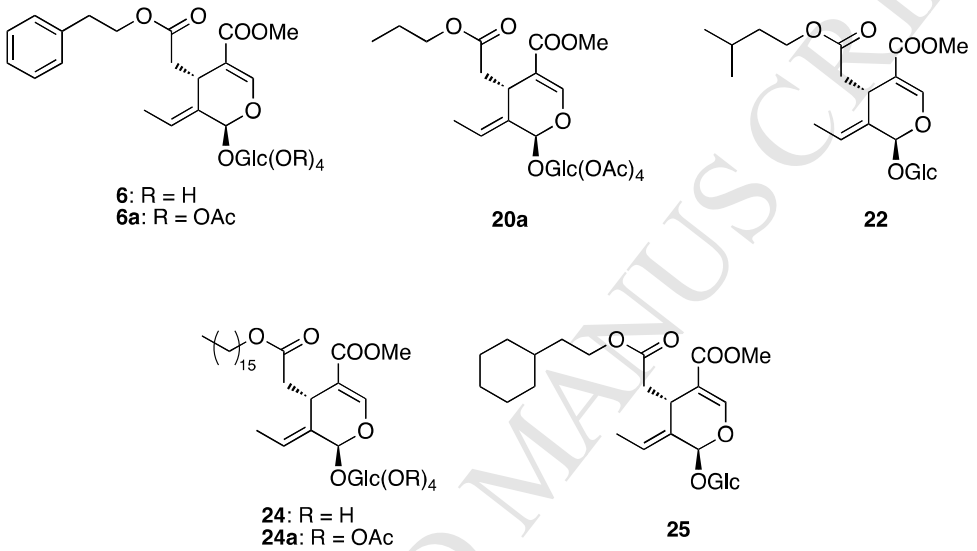


Figure 3: Chemical structure of the 7 semi-synthetic analogs of oleuropein with improved anticancer activity against SKBR3 cells.

Unexpectedly, the introduction of a flexible aliphatic ester was beneficial for improving the anticancer activity of the analogs (e.g. **25** compared to **6**). In this line, the most active molecule **24** possessed a cetyl ester, highlighting the necessity to also increase the lipophilicity of the seco-iridoid skeleton in order to improve cytotoxicity. This enhanced lipophilic character of compound **24** could have resulted in a better balance between hydrophilicity (glucose) and lipophilicity (cetyl) balance compared to **1**, eventually leading to an increased ability to cross the cancer cell membrane. However, this hypothesis should be confirmed with further experiments and identification of the cellular target.

To further evaluate the cytotoxic activity of the 7 most promising analogs, the screening was extended to the four solid tumor-derived cancer cell lines initially used to assess the activity of the oleuropein-enriched extract (Table 1) complemented with two additional leukemic cell lines, HL-60 (promyelocytic leukemia) and K562 (erythroleukemia). Doxorubicin was used as a positive control, while compound **4**, previously identified as inactive (Table 3), as a negative control. Induction of cell death was determined by the MTT dye reduction assay after 72 h of exposure to the compounds and IC_{50} values were calculated (Table 4).

Table 4: IC_{50} values (in μM) of the most active semi-synthetic analogs of oleuropein against human cancer cell lines.

	IC_{50} ± SD[1] (in μM)						
Comp.	**FM3**	**HCT-116**	**HeLa**	**MCF-7**	**SKBR3**	**HL-60**	**K562**
6	28.00 ± 2.00	25.33 ± 2.89	18.51 ± 4.04	19.33 ± 4.04	11.02 ± 0.71	10.33 ± 0.76	20.04 ± 1.20
6a	29.33 ±1.53	26.00 ± 3.60	21.02 ± 1.85	18.67 ± 2.08	11.50 ± 1.06	10.00 ± 1.00	16.03 ± 1.25
20a	29.00 ± 1.00	25.67 ± 1.53	17.51 ± 2.41	15.00 ± 2.00	6.60 ± 0.57	10.67 ± 0.58	8.50 ± 0.78
22	27.67 ± 2.52	26.67 ± 2.08	19.50 ± 2.24	21.33 ± 2.89	9.02 ± 0.71	9.83 ± 0.76	18.00 ± 0.89
24	8.83 ± 1.61	12.33 ± 2.52	2.70± 0.23	2.00 ± 0.00	1.60 ± 0.42	0.38 ± 0.04	0.70 ± 0.08
24a	27.00 ± 1.00	21.00 ± 1.00	17.00 ± 1.02	9.66 ± 0.58	3.81 ± 0.56	8.00 ± 1.00	7.00 ± 0.95
25	9.33 ± 1.53	13.67 ± 3.21	5.60 ± 0.60	5.00 ± 1.00	2.00 ± 0.71	0.48 ± 0.04	0.85 ± 0.07
1	268.82 ± 3.51	181.86 ± 2.89	275.60 ± 2.41	161.57 ± 2.08	160.44 ± 1.50	54.26 ± 4.04	64.73 ± 3.02
4	>50	>50	>50	>50	>50	>50	>50
Doxorubicin	0.217 ± 0.002	0.100 ± 0.001	0.221 ± 0.004	0.220 ± 0.004	0.202 ± 0.001	0.017 ± 0.0004	0.018 ± 0.0004

[1] IC_{50} values are means ± SD from 3 independent experiments performed

As shown in Table 4, the most promising compounds were analogs **24** (IC_{50}= 0.38-12.33 μM) and **25** (IC_{50}= 0.48-13.67 μM)**,** having a cetyl group (sixteen carbons) and a cyclohexyl ethyl group (eight carbons) in position 7 and a methyl ester in position 11, respectively. Their anticancer effect was more evident against the promyelocytic leukemic cell line HL-60, presenting the lowest recorded IC_{50} values (0.38 and 0.48 μM, respectively). Regarding these

findings, compounds **24** and **25** were further evaluated *in vitro*, prior to their administration in mice.

2.4. The semi-synthetic oleuropein analog 24 is non-toxic to normal cells

Assessing the cytotoxicity of potential drugs against cancer cell lines is substantial when selecting the most potent anticancer molecules; however, experiments of comparative toxicity on tumor and normal cells are of ultimate importance, as these will define the toxicity profile of the molecule. Theoretically, normal cells, like peripheral blood mononuclear cells (PBMCs), can withstand toxicity and higher concentrations of active compounds are required for their lysis [29]. Nevertheless, on the way to tumor target, the majority of anticancer drugs used in the clinic pass through the systemic circulation to the body, as, in principle, they are administered intravenously. PBMCs are usually the first cells encountering high concentrations of the drug and, consequently, a critical tool for evaluating drug toxicity that may cause severe adverse events, such as lymphopenia, neutropenia and susceptibility to infections [30]. Thus, PBMCs were used to determine the concentrations of **1**, **24** and **25** that induce acceptable toxicity. Freshly isolated PBMCs from healthy blood donors were cultured for 24 h with **1**, **24** and **25**, and were analyzed by flow cytometry, following annexin V/propidium iodide (PI) staining. The results showed that PBMCs incubated in plain culture medium (negative control) exhibited <2% annexin V and/or PI positivity, while, as expected, 25 μg/mL doxorubicin led >50% of PBMCs to necrosis (Figure 4).

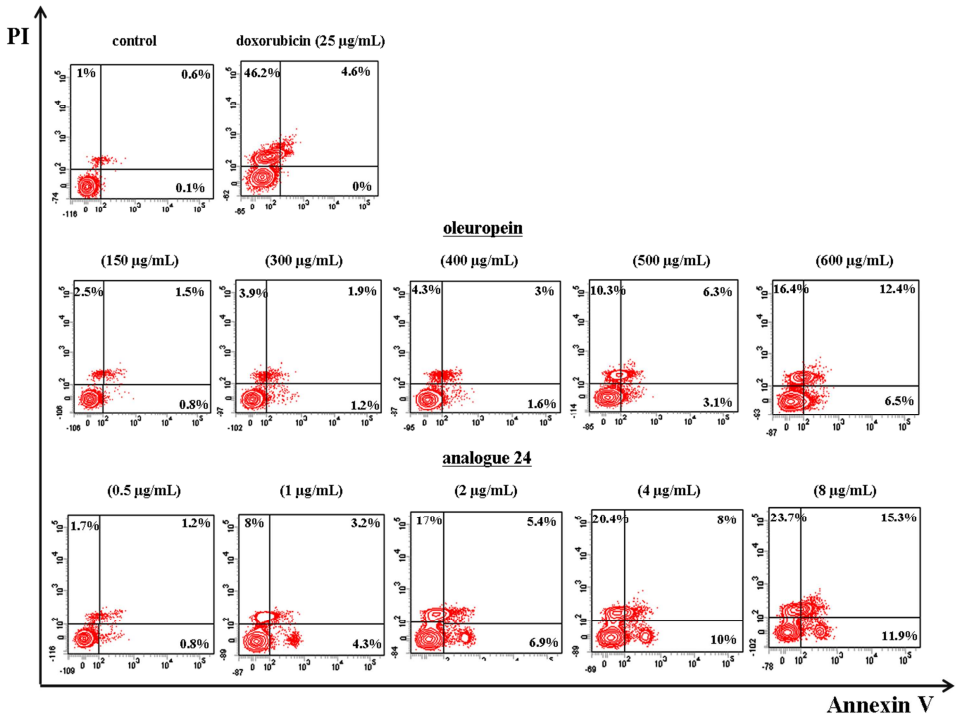


Figure 4: Toxicity assessment of oleuropein and analog 24 against peripheral blood mononuclear cells (PBMCs). After exposure to increasing concentrations of the compounds for 24 h, PBMCs were stained with annexin V (which labels apoptotic cells) and propidium iodide (PI; which stains necrotic cells), and immediately analyzed by flow cytometry. Cells in quadrants were characterized as apoptotic (annexin V+ PI-; lower right) and necrotic (PI+; upper right and upper left). Numbers in each quadrant indicate the percentage of total cells acquired. One representative experiment out of 5 performed with PBMCs from different donors is shown. Control, PBMCs incubated in culture medium.

Oleuropein **1** induced death in <10% of PBMCs at concentrations between 150 and 400 µg/mL. However, when incubated with higher concentrations (500 and 600 µg/mL), 3-6.5% of PBMCs were phenotypically characterized as apoptotic and ~17-29% acquired characteristics of necrotic cells. Based on the results of Table 4, analog **24** was tested at much lower concentrations than **1**, from 0.5 to 8 µg/mL. As shown, **24** was marginally cytotoxic at 0.5 µg/mL (0.8% apoptotic, 2.9% necrotic cells), caused low PBMC toxicity at 1 and 2 µg/mL (~16 and ~30%, respectively), while 24 h exposure of PBMCs to 4 and 8 µg/mL of **24**

resulted in driving ~28% and 39% cells to necrosis, respectively, and 10-12% to apoptosis. Although **24** was more toxic to PBMCs than **1**, at least 50% of the cells still remained viable (Fig. 3, lower left quadrant) even at the highest concentration tested. Most importantly, **1** and **24** at concentrations close to their IC_{50} against cancer cells (~200 and 3 μg/mL, respectively; Table 4) induced low PBMC toxicity, in the range of 5-15% (Figure 4). Analog **25** tested at its IC_{50} (~2 μg/mL) induced much higher cytotoxicity (>30%) to PBMCs compared to **24** (data not shown).

2.5. In vivo administration of analog 24 retarded mouse melanoma growth

Next, the seven most active oleuropein analogs were *in vitro* evaluated against the mouse melanoma cell line B16.F1 (MTT assay). As shown in Table 5, **24** remained the most cytotoxic analog towards this cell line (IC_{50}= 1.50 μM), whereas **1** presented the same profile activity as previously determined against FM3 cells (Table 4).

Table 5: IC_{50} values (in μM) of the 7 most active semi-synthetic analogs of oleuropein against the mouse melanoma cell line B16.F1.

Comp.	**B16.F1 IC_{50} (in μM)**
6	41.00 ± 1.44
6a	43.01 ± 1.12
20a	44.02 ± 1.48 [1]
22	39.12 ± 1.10
24*	1.50 ± 0.18
24a	34.05 ± 1.95
25	5.04 ± 0.31
4	>50
Oleuropein 1**	283.90 ± 1.82
Doxorubicin***	0.230 ± 0.002

* equals 0.94 μg/mL; ** equals 150.45 μg/mL; *** equals 0.130 μg/mL
[1] IC_{50} values are means ± SD from 3 independent experiments performed

Subsequently, an *in vitro* time kinetic study was performed to determine the onset of cytotoxicity induced by oleuropein and **24** on B16.F1 cells up to 72 h. To this end, B16.F1 cells were incubated for 24, 48 and 72 h with **1** and **24** at concentrations equal to their IC_{50} values (283.90 μM and 1.50 μM, respectively). The percentages of melanoma cells driven to apoptosis and necrosis were recorded by flow cytometry. Doxorubicin was used as positive control. As shown in Figure 4, B16.F1 cells incubated in plain culture medium (control) were mostly viable (>95%) throughout the 72 h of incubation, and apoptotic/necrotic cells accounted for <5% of cells. B16.F1 cells treated with 25μg/mL doxorubicin were gradually led to apoptosis/necrosis (26% at 24 h, 60.4% at 48 h and 90% at 72 h) and a very low percentage thereof (10%) remained viable after 72 h of incubation. Oleuropein **1** and **24** showed a different kinetic profile compared to doxorubicin. B16.F1 cells exposed for 24, 48 and 72 h to **1** were gradually driven to apoptosis (9.3%, 13.6% and 22.5%, respectively) and necrosis (9%, 14% and 21.8%, respectively), but the overall percentage of dying or dead cells even at 72 h accounted for less than 50% of total cells. On the contrary, **24** used at much lower concentration than **1**, rapidly induced apoptosis recorded as early as 24 h of exposure, which became more intense at 48 h, and at 72 h ~60% of B16.F1 cells were non-viable (Figure 5).

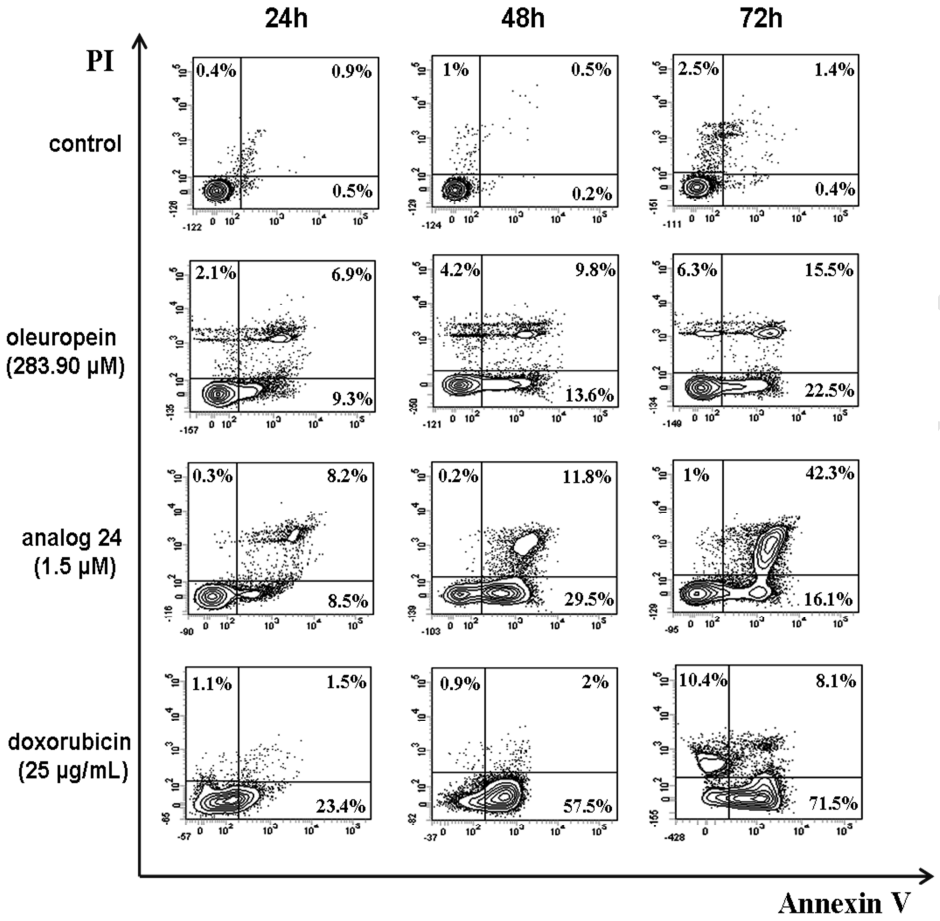


Figure 5: Flow cytometry analysis of B16.F1 cell death after exposure to 1 and analog 24. One representative experiment out of 3 performed with similar results is shown. Other details as in legend of Figure 4.

The anticancer effect of **1** and **24** was further tested in an *in vivo* therapeutic melanoma model. B16.F1 cells were subcutaneously (sc) inoculated in the right flank of syngeneic C57BL/6 mice. After 11 days, when tumors became palpable, mice were treated intraperitoneally (ip) with phosphate buffered saline (PBS, control), oleuropein (300 and 600 µg/mouse/dose; total 2.4 and 4.8 mg/mouse or 100 and 200 mg/kg) or **24** (2, 4 and 6 µg/mouse; total 16, 32 and 48 µg/mouse), monitored for 28 days and euthanized on day 29. DMSO was also used as a control producing similar results with PBS (data not shown). The dose of **1** administered was based on previous reports showing that daily oral administration

of the compound up to 200 mg/kg did not induce toxicity [16]. Here, the same total concentration of **1** was administered but divided in 8 doses and given ip, in order to administer the same concentration of oleuropein or **24** in all animals. As shown in Figure 6, control animals (injected with PBS) showed a rapid increase in tumor dimensions (average tumor volume 2.83 cm^3 on day 29). Melanoma tumor growth in mice treated with 300 μg/mouse/dose of **1** showed a similar pattern to controls, but slightly smaller tumor masses were recorded on the day of sacrifice (2.41 cm^3 on day 29). Mice treated with 600 μg/mouse/dose of **1** showed a slower tumor increase rate (2.33 cm^3 on day 29) compared to controls or mice treated with 300 μg/mouse/dose oleuropein. Therapeutic administration of **24** reduced melanoma growth rates in all three groups (2, 4 and 6 μg/mouse/dose) compared to controls and the oleuropein groups, but statistical significance ($p<0.05$) was reached only for animals treated with 4 and 6 μg of **24** *vs* control and *vs* the 300 μg/mouse/dose oleuropein group. The lower average tumor volume was recorded in mice receiving **24** at 6 μg/mouse/dose (0.84 cm^3 on day 29), which was by ~3.4-fold smaller compared to controls. These data suggest that (*i*) inhibition of melanoma growth *in vivo* is dose-dependent both for **1** and **24**; (*ii*) **1** is non-toxic, possesses a weak anticancer activity *in vivo*, and even when administered at high concentrations *in vivo* does not significantly impact on tumor growth and consequently on animal survival; and (*iii*) **24** used at 100-fold lower concentration than **1**, displayed both a potent antitumor effect and, similarly to oleuropein **1**, no obvious signs of toxicity *in vivo* were observed. Moreover, administration of **24** resulted in significant delay of melanoma tumor growth, which is usually accompanied by prolongation in animals' survival [31].

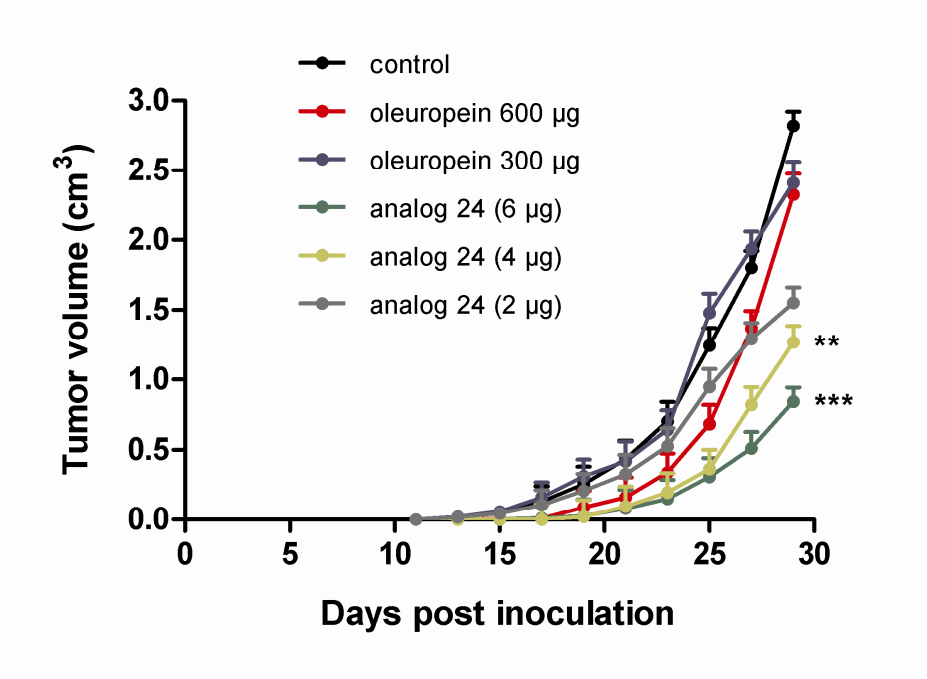


Figure 6: Oleuropein 1 and its analog 24 delay melanoma tumor growth *in vivo*. C57BL/6 mice were sc inoculated with syngeneic B16.F1 cells and therapeutically treated ip with 8 doses of oleuropein (300 and 600 µg/dose/mouse) or **24** (2-6 µg/dose/mouse) administered every other day. Control mice received PBS. Tumor growth was monitored for 29 days (B). Pooled data from 5 mice/group are shown. $^{**}p<0.01$; $^{***}p<0.001$ compared to control and oleuropein 300 µg/dose/mouse group.

Oleuropein **1** has already been used in *in vivo* tumor models, showing promising results. Specifically, when administered orally to mice that developed spontaneous tumors, oleuropein completely regressed tumors in 9-12 days [32]. Moreover, oleuropein in diet inhibited tumor growth and metastasis spreading in ovariectomised nude mice injected with MCF-7 tumor xenografts [33], while oleuropein dissolved in water reduced tumor volume and weight in mice after breast cancer xenograft [34].

2.6. Analog 24 promotes the in vivo generation of antitumor effectors

The *in vivo* reduction of tumor growth observed upon treatment with **24** prompted us to further assess whether **24** induced *in vivo* antitumor-reactive immune responses. Splenocytes were isolated from treated mice and, without additional *ex vivo* stimulation, were used as effectors against the mouse NK-sensitive YAC-1, the syngeneic B16.F1 and the LAK-sensitive WEHI 164 cells. Cell cytotoxicity was assessed by flow cytometry, based on surface expression of the characteristic degranulation marker CD107 on effector cells [35]. Our results showed that splenocytes from control mice and mice treated with 300μg/dose oleuropein were not cytotoxic against any target, whereas spleenocytes from mice treated with 600 μg/dose oleuropein were slightly, but not statistically significantly more lytic, particularly against the syngeneic B16.F1 cells [mean fluorescence intensity (MFI)s 6.6/7.5 *vs* 3.3/3.9 of controls]. Marginal cytotoxicity against YAC-1 and WEHI 164 was recorded (MFI <3). Spleen cells from mice treated with **24** (6 μg) were the most efficient in killing all target cells and especially B16.F1 (MFIs 19.3/21.2; Figure 7). The same splenocytes lysed YAC-1 (MFIs 4.9/5.1) and WEHI 164 targets (MFIs 4.9/4.1). Accordingly, splenocytes from mice treated with 2 and 4 μg of **24** effectively killed B16.F1 (MFIs 14.1/11.0 and 15.1/17.5, respectively), YAC-1 and WEHI 164 targets (Figure 7). These results suggest that **24**, in particularly when administered at the highest dose used (total 48 μg/mouse), can induce the *in vivo* expansion mainly of melanoma-reactive T cells and secondarily, stimulate non-specific immune responses, mediated by NK and LAK cells.

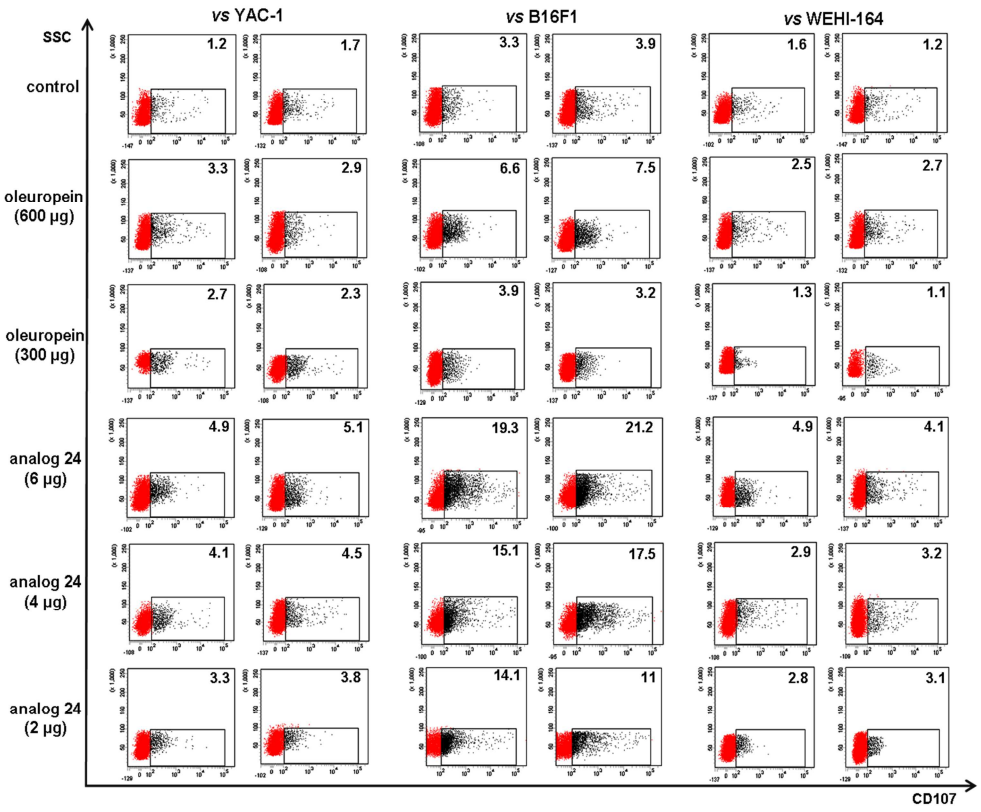


Figure 7: Oleuropein and analog 24 induce the generation of tumor-reactive immune responses *in vivo*. Mouse splenocytes isolated on day 29 were co-incubated with B16-F1, YAC-1 (NK-sensitive) and WEHI 164 (LAK-sensitive) target cells, and CD107 expression (gated events) was assessed by flow cytometry. Numbers show mean fluorescence intensity (MFI) from spleen cells of 2 representative mice per group tested individually.

3. Conclusion

Using the natural scaffold of oleuropein as starting material, we synthesized a chemical library of 51 novel oleuropein analogs with variable tri-dimensional structures using a simple and fast synthesis method. The panel of analogs was tested for its cytotoxicity against the ER^- human breast cancer SKBR3 cells, highlighting 7 structures all deprived of the hydroxytyrosol moiety. These 7 analogs (**6**, **6a**, **20a**, **22**, **24**, **24a** and **25**) exhibited improved anticancer activity against 6 additional human cancer cell lines (deriving from solid tumors and leukemias), compared to the parental molecule oleuropein. Thus, the ideal

pharmacophore leading to a satisfactory cytotoxic profile possessed a long alkyl chain in position 7. Among the analogs, **24** showed the strongest inhibitory activity, suggesting that the ester in position 11 is of major importance for its anticancer activity. **24** efficiently and selectively killed cancer cells, without causing severe toxicity in peripheral blood lymphocytes. Most importantly, **24** displayed potent *in vivo* activity against melanoma, retarding tumor growth and stimulating antitumor immune responses, by mainly inducing the *in vivo* expansion of melanoma-reactive effector T cells. Taken as a whole, based on the natural product oleuropein, we developed a promising analog and performed, for the first time, detailed SAR studies on the natural scaffold of oleuropein.

4. Experimental Section

4.1. Materials and instruments

HPLC was performed with a Thermo Finnigan HPLC system (ThermoFinnigan, San Jose, CA) connected to a Spectral System UV2000 PDA detector and an autosampler. ChromQuest 2.1 software was used for the management of data. Column: Supelco RP-18 HS C_{18}, 250 x 4.6 mm i.d., 5.0 μm (Discovery). Optical rotations were measured with a Perkin-Elmer 241 polarimeter in methanol. NMR spectra were recorded on a Bruker Avance-600 spectrometer at 600 MHz for ^{1}H NMR, at 150 MHz for ^{13}C NMR using $CDCl_3$ and CD_3OD as solvents; chemical shifts are expressed in ppm downfield to TMS. HRMS spectra were recorded on LTQ-Orbitrap spectrometer (ThermoScientific, Bremen, Germany). Isolation of oleuropein from the extract was conducted using a FCPC apparatus (Kromaton). Column chromatography were conducted using silica gel Merck [20-45 μm, or 35-70 μm (flash)].

4.2 Production of the oleuropein enriched extract from olive leaves

The *Olea europaea* var koroneiki leaves were collected in Crete (Greece), dried in a well-ventilated shady place and subsequently stored in a dark room. Before extraction, the leaves

were ground using an Allen West type SCIS grinder with a sieve of 3 mm. A quantity of 3.5 kg leaves was processed by pressurized liquid extraction with acetone. The extract was evaporated completely and washed with a mixture of CH_2Cl_2 / MeOH 98:2 (3 L). The insoluble material was separated and dried under reduced pressure, producing a yellow powder (360 g) containing 60% oleuropein [24] and analyzed using HPLC-DAD.

4.3 Isolation of oleuropein

Ten grams of the yellow powder were subjected to countercurrent chromatography, using a fast centrifugal partition chromatograph (FCPC) apparatus. The system of solvents used in this procedure were EtOAc/EtOH/H_2O 10/1/10 (6 L). The capacity of the column was 1 L, rapidity of rotation 900 rpm, and flow rate 15 mL/min. A total of 5.0 g oleuropein (purity 90%) was isolated by the above-mentioned process [6].

4.4. Chemistry

4.4 1. Synthesis of oleoside-11-methylester 2 and oleoside 3

Oleuropein **1** (1g, 1.85 mmol) was dissolved in 50 mL of 1N NaOH solution. The mixture was subjected to microwave radiation and the reaction monitored with TLC. After completion (around 10 min), a 1N HCl solution was added to reach a pH around 4-5. After removal of the solvent under vacuum, the crude mixture was subjected to column chromatography (CH_2Cl_2:MeOH 95/5 to 50/50). Overall, 324.5 mg of **2** were collected (68% yield). The analytical data of **2** were conform to the literature.

2 (750mg) was then subjected to the same procedure to give oleoside **4** (650mg, 90% yield). The analytical data of **4** were conform to the literature.

4.4.2. General procedure for the acetylation of 2 and 4

2 or **4** (1eq) was dissolved in pyridine and stirred under argon. To this solution, 35eq of acetic anhydride were added at 0^oC. After stirring for 2 hours at room temperature, the mixture was diluted with chloroform ($CHCl_3$), washed with water at 0^oC, and quenched with aqueous 1M

HCl solution, dropwise, until pH = 5. The solution was extracted and the two phases, organic and aqueous, were separated. The aqueous phase was further extracted with ethyl acetate (EtOAc). The collected organic layers were dried with anhydrous sodium sulfate (Na_2SO_4) and concentrated under reduced pressure. Purification of the product by column chromatography (CH_2Cl_2:MeOH, 99/1 to 9/1) afforded the desired product as a white foam. The analytical data of **3** and **5** were conform to the literature.

4.4.3. General procedure for esterification

To a solution of oleoside 11-methylester tetraacetate **3** or oleoside tetraacetate **5** (1 equiv.) in dichloromethane were added at 0^oC 1.2 equiv of trichlorobenzoyl chloride and 1.4 equiv triethylamine. After 2 h stirring at room temperature, the mixture was cooled to 0°C and a solution of alcohol/thiol/amine (1.5 equiv.) in dichloromethane and DMAP (1.4 equiv.) were added. After stirring for 2 h at room temperature, the mixture was quenched with a saturated aqueous solution of NH_4Cl and extracted with dichloromethane and ethyl acetate. The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. Purification by flash chromatography (cyclohexane/ethylacetate 1:1) led to the acetylated derivatives.

*4.4.3.1 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-5-(methoxycarbonyl)-4-(2-oxo-2-phenethoxyethyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**6a**)*

According to the general method of esterification with alcohols, 41.0 mg (50% yield) of **6a** were obtained, as a white solid, from 70.0 mg of **3**.

$[\alpha]_D$ -160 (c 0.05, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.70 (d, 3H, J = 6.5 Hz); 1.93 (s, 3H); 2.02-2.04 (2s, 6H); 2.08 (s, 3H); 7.48 (s, 1H); 2.43 (dd, 1H, J = 14.5 and 9.0 Hz); 2.74 (dd, 1H, J = 14.5 and 4.5 Hz); 2.93 (m, 2H); 3.78 (s, 3H); 3.79 (ddd, 1H, J = 9.5, 4.5 and 2.0 Hz); 3.99 (dd, 1H, J = 9.0 and 4.5 Hz); 4.13 (dd, 1H, J = 12.5 and 2.5 Hz); 4.21 (dt, 1H, J = 11.0 and

7.0 Hz); 4.29 (dt, 1H, *J* = 11.0 and 7.0 Hz); 4.33 (dd, 1H, *J* = 4.5 and 12.5 Hz); 5.05 (d, 1H, *J* = 8.0 Hz); 5.16 (m, 2H); 5.29 (t, 1H, *J* = 9.5 Hz); 5.73 (s, 1H); 6.00 (q, 1H, *J* = 7.0 Hz); 7.23 (dd, 1H, *J* = 8.0 and 2.0 Hz); 7.26 (t, 2H, *J* = 8.0 Hz); 7.31 (dd, 2H, *J* = 8.0 and 2.0 Hz); ^{13}C NMR ($CDCl_3$) δ 13.4, 20.5, 20.6, 26.8, 30.1, 34.9, 39.8, 51.3, 61.6, 65.0, 68.1, 70.5, 70.6, 72.1, 72.4, 93.6, 97.0, 108.6, 124.7, 126.5, 127.9, 128.4, 128.8, 137.6, 152.9, 166.6, 169.2, 169.3, 170.0, 170.4, 170.9. HRMS (ESI+) *m/z* 699.2226 (calcd for $C_{33}H_{40}O_{15}Na$: 699.2265) $[M+Na]^+$.

*4.4.3.2 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-4-(2-(3,4-dimethoxyphenethoxy)-2-oxoethyl)-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**7a**)*

According to the general method of esterification with alcohols, 52.0 mg (58% yield) of **7a** were obtained, as a white-yellow solid, from 70.0 mg of **3**.

$[\alpha]_D$ -60 (c 0.05, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.71 (d, 3H, *J* = 7.0 Hz); 2.05-2.07 (3s, 9H); 2.42 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.78 (dd, 1H, *J* = 14.5 and 4.5 Hz); 2.88 (m, 2H); 3.76 (s, 3H); 3.80 (ddd, 1H, *J* = 9.5, 4.5 and 2.5 Hz); 3.85 (2s, 6H); 4.01 (dd, 1H, *J* = 9.5 and 4.5 Hz); 4.15 (dd, 1H, *J* = 12.5 and 4.5 Hz); 4.19 (dt, 1H, *J* = 11.0 and 7.0 Hz); 4.27 (dt, 1H, *J* = 11.0 and 7.0 Hz); 4.33 (dd, 1H, *J* = 12.5 and 2.5 Hz); 5.06 (d, 1H, *J* = 8.0 Hz); 5.15(dd, 1H, *J* = 8.0 and 9.5 Hz); 5.16 (t, 1H, *J* = 9.5 Hz); 5.30 (t, 1H, *J* = 9.5 Hz); 5.74 (s, 1H); 6.02 (q, 1H, *J* = 7.0 Hz); 6.75 (d, 1H, *J* = 1.5 Hz); 6.77 (dd, 1H, *J* = 8.0 and 1.5 Hz); 6.83 (d, 1H, *J* = 8.0 Hz); 7.48 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.4, 20.5, 26.8, 30.1, 30.8, 34.5, 39.9, 51.3, 55.8, 60.3, 61.6, 65.2, 68.1, 70.5, 70.6, 72.1, 72.4, 93.6, 97.0, 108.6, 111.3, 112.1, 120.8, 124.7, 128.0, 130.1, 147.7, 148.8, 152.9, 166.6, 169.2, 170.0, 170.4, 170.9, 206.8. HRMS (ESI+) *m/z* 759.2435 (calcd for $C_{35}H_{44}O_{17}Na$: 759.2476) $[M+Na]^+$.

*4.4.3.3 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-4-(2-(2-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)ethoxy)-2-oxoethyl)-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**8a**)*

According to the general method of esterification with alcohols, 42.0 mg (46% yield) of **8a** were obtained, as a white solid, from 70.0 mg of **3**.

$[\alpha]_D$ -140 (c 0.05, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.69 (d, 3H, *J* = 7.0 Hz); 1.90 (s, 3H); 2.03 (3s, 9H); 2.43 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.71 (dd, 1H, *J* = 14.5 and 4.5 Hz); 2.77 (m, 2H); 3.72 (s, 3H); 3.77 (ddd, 1H, *J* = 9.0, 5.0 and 2.5 Hz); 3.97 (dd, 1H, *J* = 12.5 and 5.0 Hz); 4.23 (m, 7H); 4.33 (dd, 1H, *J* = 12.5 and 2.5 Hz); 5.04 (d, 1H, *J* = 8.0 Hz); 5.13 (m, 2H); 5.28 (t, 1H, *J* = 9.5 Hz); 5.69 (s, 1H); 5.99 (q, 1H, *J* = 7.0 Hz); 6.64 (dd, 1H, *J* = 8.0 and 1.5 Hz); 6.70 (d, 1H, *J* = 1.5 Hz); 6.77 (d, 1H, *J* = 8.0 Hz); 7.44 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.8, 20.5, 26.8, 30.1, 34.2, 39.6, 51.0, 60.1, 61.5, 64.1, 65.0, 68.1, 70.5, 70.6, 72.1, 72.5, 93.7, 97.0, 108.5, 117.0, 117.3, 121.6, 124.5, 128.0, 130.7, 142.1, 143.3, 152.8, 166.5, 168.9, 169.0, 169.8, 170.2, 170.7. HRMS (ESI+) *m/z* 757.2271 (calcd for $C_{35}H_{42}O_{17}Na$: 757.2320) $[M+Na]^+$.

*4.4.3.4 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-4-(2-(4-hydroxyphenethoxy)-2-oxoethyl)-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate. (Ligstroside tetraacetate) (**9a**)*

According to the general method of esterification with alcohols, 14.0 mg (23%) of **9a** were obtained, as a white solid, from 50.0 mg of **3**.

$[\alpha]_D$ -450 (c 0.02, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.65 (d, 3H, *J* = 7.0 Hz); 2.07 (2s, 6H); 2.08 (s, 3H); 2.08 (s, 3H); 2.39 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.74 (dd, 1H, *J* = 14.5 and 4.5 Hz); 2.84 (m, 2H); 3.76 (s, 3H); 3.80 (ddd, 1H, *J* = 9.0, 4.5 and 2.5 Hz); 3.97 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.15 (dt, 1H, *J* = 11.0 and 7.0 Hz); 4.18 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.33-4.29 (m, 2H); 5.06 (d, 1H, *J* = 8.0 Hz); 5.18 (m, 2H); 5.31 (t, 1H, *J* = 9.5 Hz); 5.69 (s, 1H); 5.93 (q,

1H, *J* = 7.0 Hz); 6.80 (d, 2H, *J* = 8.0 Hz); 7.05 (d, 2H, *J* = 8.0 Hz); 7.47 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.5, 20.1, 20.7, 20.8, 30.2, 34.1, 39.8, 51.4, 61.7, 65.3, 68.3, 70.7, 72.2, 72.4, 93.4, 96.8, 108.8, 115.4, 124.9, 127.6, 129.7, 130.0, 153.0, 154.4, 166.7, 169.4, 169.6, 170.2, 170.7, 171.0. HRMS (ESI+) *m/z* 715.2370 (calcd for $C_{33}H_{40}O_{16}Na$: 715.2214) $[M+Na]^+$.

*4.4.3.5 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-5-(methoxycarbonyl)-4-(2-(4-methoxyphenethoxy)-2-oxoethyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**10a**)*

According to the general method of esterification with alcohols, 55.0 mg (89% yield) of **10a** were obtained, as a white solid, from 50.0 mg of **3**.

$[\alpha]_D$ -365 (c 0.02, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.71 (d, 3H, *J* = 7.0 Hz); 2.05 (3s, 6H); 2.19 (s, 3H); 2.42 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.73 (dd, 1H, *J* = 14.5 and 4.5 Hz); 2.86 (m, 2H); 3.75 (s, 3H); 3.81 (s, 3H); 3.98 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.16 (ddd, 1H, *J* = 9.0 and 4.5 and 2.5 Hz); 4.27-4.23 (m, 2H); 4.33 (dd, 1H, *J* = 12.5 and 2.5 Hz); 5.04 (d, 1H, *J* = 8.0 Hz); 5.15 (m, 2H); 5.29 (t, 1H, *J* = 9.5 Hz); 5.72 (s, 1H); 6.00 (q, 1H, *J* = 7.0 Hz); 6.85 (d, 2H, *J* = 7.0 Hz); 7.13 (d, 2H, *J* = 8.0 Hz); 7.48 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.7, 20.7, 21.0, 21.0, 21.0, 26.0, 34.5, 41.0, 52.2, 55.8, 62.6, 65.4, 69.3, 71.1, 72.0, 74.4, 104.5, 107.4, 109.4, 114.0, 114.1, 124.8, 129.7, 129.8, 130.4, 130.8, 155.2, 157.7, 168.3, 170.2, 170.2, 170.3, 170.3, 173.0. HRMS (ESI+) *m/z*, 729.2348 (calcd for $C_{34}H_{42}O_{16}Na$: 729.2371) $[M+Na]^+$.

*4.4.3.6 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-5-(methoxycarbonyl)-4-(2-(4-nitrophenethoxy)-2-oxoethyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**11a**)*

According to the general method of esterification with alcohols, 60.5 mg (96% yield) of **11a** were obtained, as a white solid, from 50.0 mg of **3**.

$[\alpha]_D$ -246 (c 0.02, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.67 (d, 3H, *J* = 7.0 Hz); 2.05 (3s, 6H); 2.07 (s, 3H); 2.42 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.73 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.05 (m, 2H);

3.75 (s, 3H); 3.80 (ddd, 1H, *J* = 9.0, 4.5 and 2.5 Hz); 3.96 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.15 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.27-4.23 (m, 2H); 4.34 (dd, 1H, *J* = 12.5 and 4.5 Hz); 4.36 (m, 1H); 5.06 (d, 1H, *J* = 8.0 Hz); 5.16 (m, 2H); 5.31 (t, 1H, *J* = 9.5 Hz); 5.70 (s, 1H); 5.99 (q, 1H, *J* = 7.0 Hz); 7.40 (d, 2H, *J* = 7.0 Hz); 7.48 (s, 1H); 8.20 (d, 2H, *J* = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 13.4, 20.4, 20.5, 20.6, 26.8, 30.1, 34.7, 39.8, 51.4, 61.7, 63.9, 68.2, 70.6, 72.2, 72.4, 76.7, 76.9, 93.4, 96.9, 108.5, 123.7, 124.7, 127.9, 128.0, 129.7, 145.5, 146.8, 153.0, 166.7, 169.3, 169.4, 170.1, 170.5, 170.9; HRMS (ESI+) *m/z* 744.2096 (calcd $C_{33}H_{39}NO_{17}Na$: 744.2116) $[M+Na]^+$.

*4.4.3.7 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-4-(2-(4-fluorophenethoxy)-2-oxoethyl)-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**12a**)*

According to the general method of esterification with alcohols, 35.9 mg (60% yield) of **12a** were obtained, as a white solid, from 50.0 mg of **3**.

$[\alpha]_D$ -365 (c 0.02, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.70 (d, 3H, *J* = 7.0 Hz); 2.06 (4s, 12H); 2.42 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.75 (dd, 1H, *J* = 14.5 and 4.5 Hz); 2.90 (m, 2H); 3.75 (s, 3H); 3.79 (ddd, 1H, *J* = 9.0, 4.5 and 2.5 Hz); 3.98 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.15 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.27-4.23 (m, 2H); 4.36 (m, 1H); 4.34 (dd, 1H, *J* = 12.5 and 4.5 Hz); 5.06 (d, 1H, *J* = 8.0 Hz); 5.15 (m, 2H); 5.30 (t, 1H, *J* = 9.5 Hz); 5.72 (s, 1H); 6.00 (q, 1H, *J* = 7.0 Hz); 7.00 (d, 2H, *J* = 8.0 Hz); 7.20 (dd, 2H, *J* = 8.0 and 5.5 Hz); 7.48 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.8, 20.7, 21.0, 21.0, 21.1, 26.2, 34.7, 41.1, 52.3, 62.7, 65.5, 69.2, 71.0, 72.0, 74.5, 104.5, 107.5, 109.6, 115.4, 115.4, 124.9, 130.8, 131.7, 131.7, 133.7, 155.3, 160.3, 168.5, 170.2, 170.3, 170.3, 170.3, 173.1. HRMS (ESI+) *m/z* 717.2153 (calcd for $C_{33}H_{39}FO_{15}Na$: 717.2171) $[M+Na]^+$.

*4.4.3.8 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-4-(2-(benzyloxy)-2-oxoethyl)-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**13a**)*

According to the general method of esterification with alcohols, 48.0 mg (40%) of **13a** were obtained, as a white solid, from 100.0 mg of **3**.

$[\alpha]_D$ -97.5 (c 0.0004, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.58 (bd, 3H, *J* = 7.0 Hz); 2.39 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.80 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.73 (s, 3H); 3.78 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 4.02 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.14 (dd, 1H, *J* = 1.5 and 12.5 Hz); 4.31 (dd, 1H, *J* = 4.5 and 12.5 Hz); 5.03 (d, 1H, *J* = 8 Hz); 5.04 (d, 1H, *J* = 12.0 Hz); 5.15 (d, 1H, *J* = 12.0 Hz); 5.15 (t, 1H, *J* = 9.5 Hz); 5.15 (m, 2H); 5.29 (t, 1H, *J* = 9.5 Hz); 5.73 (s, 1H); 5.98 (q, 1H, *J* = 7.0 Hz); 7.40-7.32 (m, 5H); 7.48 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.4, 20.5, 20.6, 26.8, 30.2, 39.9, 51.4, 66.3, 68.2, 70.7, 72.1, 72.4, 91.7, 93.7, 97.0, 108.6, 124.9, 126.9, 127.8, 128.2, 135.7, 153.0, 166.7, 169.2, 169.3, 170.1, 170.5, 170.9. HRMS (ESI+) *m/z* 685.2096, (calcd for $C_{32}H_{38}O_{15}Na$: 685.2108) $[M+Na]^+$.

*4.4.3.9 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-4-(2-((3,4-dimethoxybenzyl)oxy)-2-oxoethyl)-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**14a**)*

According to the general method of esterification with alcohols, 61.0 mg (69%) of **14a** were obtained, as a white solid, from 70.0 mg of **3**.

$[\alpha]_D$ -97.5 (c 0.0004, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.71 (dd, 3H, *J* = 7.0 and 1.5 Hz); 2.03-2.01 (4s, 12H); 2.51 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.78 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.73 (s, 3H); 3.78 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.90 (2s, 6H); 4.03 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.15 (dd, 1H, *J* = 1.5 and 12.5 Hz); 4.32 (dd, 1H, *J* = 4.5 and 12.5 Hz); 5.02 (d, 1H, *J* = 8.0 Hz); 5.05 (d, 2H, *J* = 12.0 Hz); 5.15 (t, 1H, *J* = 9.5 Hz); 5.15 (dd, 1H, *J* = 8.0 and 9.5 Hz); 5.30 (t, 1H, *J* = 9.5 Hz); 5.72 (bs, 1H); 6.01 (q, 1H, *J* = 7.0 Hz); 6.87 (d, 1H, *J* = 8.0 Hz); 6.90

(d, 1H, *J* = 2.0 Hz); 6.93 (dd, 1H, *J* = 8.0 and 2.0 Hz); 7.48 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.4, 20.6, 26.0, 27.6, 30.2, 39.9, 51.4, 53.3, 55.8, 60.3, 61.7, 66.5, 68.2, 70.7, 72.1, 72.4, 93.7, 97.0, 108.6, 110.9, 111.9, 124.8, 128.0, 128.2, 148.9, 149.0, 153.0, 166.7, 169.3, 170.1, 170.5, 170.9, 171.0. HRMS (ESI+) *m/z* 745.2305 (calcd for $C_{34}H_{42}O_{17}Na$: 745.2320) $[M+Na]^+$.

*4.4.3.10 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-4-(2-((2,3-dihydrobenzo[b][1,4]dioxin-6-yl)methoxy)-2-oxoethyl)-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**15a**)*

According to the general method of esterification with alcohols, 32.0 mg (36%) of **15a** were obtained, as a white solid, from 70.0 mg of **3**.

$[\alpha]_D$ -1000 (c 0.03, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.71 (dd, 3H, *J* = 7.0 and 1.5 Hz); 2.03-2.01 (4s, 12H); 2.51 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.78 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.73 (s, 3H); 3.78 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.90 (2s, 6H); 4.03 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.15 (dd, 1H, *J* = 1.5 and 12.5 Hz); 4.32 (dd, 1H, *J* = 4.5 and 12.5 Hz); 4.90 (d, 1H, *J* = 8.0 Hz); 5.05 (d, 2H, *J* = 12.0 Hz); 5.15 (t, 1H, *J* = 9.5 Hz); 5.15 (dd, 1H, *J* = 8.0 and 9.5 Hz); 5.30 (t, 1H, *J* = 9.5 Hz); 5.72 (bs, 1H); 5.95 (q, 1H, *J* = 7.0 Hz); 6.83 (d, 1H, *J* = 2.0 Hz); 6.85 (d, 1H, *J* = 8.0 Hz); 6.89 (d, 1H, *J* = 2.0 Hz); 7.46 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.4, 20.6, 26.0, 27.6, 30.2, 39.9, 51.4, 53.3, 55.8, 60.3, 61.7, 66.5, 68.2, 70.7, 72.1, 72.4, 93.7, 97.0, 108.6, 110.9, 111.9, 124.8, 128.0, 128.2, 148.9, 149.0, 153.0, 166.7, 169.3, 170.1, 170.5, 170.9, 171.0. HRMS (ESI+) *m/z* 743.2141 (calcd for $C_{34}H_{40}O_{17}Na$: 743.2163) $[M+Na]^+$.

*4.4.3.11 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-4-(2-(3-(3,4-dimethoxyphenyl)propoxy)-2-oxoethyl)-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**16a**)*

According to the general method of esterification with alcohols, 17.0 mg (32%) of **16a** were obtained, as a white solid, from 40.0 mg of **3**.

$[\alpha]_D$ -60 (c 0.05, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.75 (dd, 3H, *J* = 1.0 and 6.5 Hz); 1.90 (m, 2H); 2.05-2.07 (4s, 12H); 2.45 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.60 (t, 2H, *J* = 7.5 Hz); 2.76 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.73 (s, 3H); 7.48 (s, 1H); 3.76 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.85 (2s, 6H); 4.10 (m, 2H); 4.27 (dd, 1H, *J* = 12.5 and 4.5 Hz); 5.03 (d, 1H, *J* = 8 Hz); 5.11 (m, 2H); 5.27 (t, 1H, *J* = 9.5 Hz); 5.74 (s, 1H); 6.02 (q, 1H, *J* = 7.0 Hz); 6.72 (d, 1H, *J* = 2.0 Hz); 6.74 (dd, 1H, *J* = 8.0 and 2.0 Hz); 6.81 (d, 1H, *J* = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 13.4, 20.5, 26.8, 30.1, 30.8, 34.5, 39.9, 51.3, 55.8, 60.3, 61.6, 65.7, 68.2, 70.7, 72.1, 72.4, 93.7, 97.0, 108.6, 124.7, 132.2, 132.5, 133.4, 136.0, 147.2, 147.3, 148.8, 153.0, 164.1, 166.7, 169.3, 170.1, 170.6, 171.2. HRMS (ESI+) *m/z* 773.2602 (calcd for $C_{36}H_{46}O_{17}Na$: 773.2633) $[M+Na]^+$.

*4.4.3.12 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-5-(methoxycarbonyl)-4-(2-oxo-2-(phenethylthio)ethyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**17a**)*

According to the general method of esterification with alcohols, 42.0 mg (70% yield) of **17a** were obtained, as a white solid, from 100.0 mg of **3**.

$[\alpha]_D$ -160 (c 0.05, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.72 (bd, 3H, *J* = 7.0 Hz); 1.93 (s, 3H); 2.01-2.02 (4s, 12H); 2.67 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.80 (m, 2H); 2.98 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.10 (m, 2H); 3.73 (s, 3H); 3.78 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 4.04 (dd, 1H, *J* = 12.5 and 4.5 Hz); 4.14 (dd, 1H, *J* = 1.5 and 12.5 Hz); 4.32 (dd, 1H, *J* = 4.5 and 12.5 Hz); 5.05 (d, 1H, *J* = 8.0 Hz); 5.15 (t, 1H, *J* = 9.5 Hz); 5.15 (dd, 1H, *J* = 8.0 and 9.5 Hz); 5.29 (t, 1H, *J* = 9.4 Hz); 5.71 (s, 1H); 6.03 (q, 1H, *J* = 7.0 Hz); 7.22 (bd, 1H, *J* = 7.5 Hz); 7.25 (bd, 2H, *J* = 7.5 Hz); 7.32 (bdd, 2H, *J* = 7.5 Hz); 7.48 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.5, 20.5, 20.6, 30.2, 31.5, 39.9, 51.4, 58.2, 61.7, 63.9, 68.2, 70.6, 72.1, 72.4, 93.7, 97.0, 108.6, 124.7, 132.2, 132.5, 133.4, 136.1, 147.3, 148.8, 153.1, 164.1, 166.7, 169.3, 170.1, 170.6, 171.1. HRMS (ESI+) *m/z* 715.2036 (calcd for $C_{33}H_{40}O_{14}SNa$: 715.2036) $[M+Na]^+$.

*4.4.3.13 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-4-(2-methoxy-2-oxoethyl)-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**18a**)*

According to the general method of esterification with alcohols, 16.0 mg (31% yield) of **18a** were obtained from 50.0 mg of **3**, as a white solid. Analytical data of **18a** were conform to the literature [36].

*4.4.3.14 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-4-(2-ethoxy-2-oxoethyl)-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**19a**)*

According to the general method of esterification with alcohols, 16.0 mg (31% yield) of **19a** were obtained from 50.0 mg of **3**, as a white solid.

$[\alpha]_D$ -375 (c 0.04, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.25 (t, 3H); 1.74 (d, 3H, J = 7.0 Hz); 2.01-2.03 (m, 9H); 2.08 (s, 3H); 2.43 (dd, 1H, J = 14.5 and 9.0 Hz); 2.76 (dd, 1H, J = 14.5 and 4.5 Hz); 3.72 (s, 3H); 3.79 (ddd, 1H, J = 9.5, 4.5 and 1.5 Hz); 4.01 (dd, 1H, J = 9.0 and 4.5 Hz); 4.06 (dt, 1H, J = 11.0 and 7.0 Hz); 4.13 (dt, 1H, J = 11.0 and 7.0 Hz); 4.14 (dd, 1H, J = 1.5 and 12.5 Hz); 4.33 (dd, 1H, J = 12.5 and 4.5 Hz); 5.05 (d, 1H, J = 8.0 Hz); 7.48 (s, 1H); 5.15 (t, 1H, J = 9.5 Hz); 5.15 (dd, 1H, J = 9.5 and 8.0 Hz); 5.74 (bs, 1H); 5.28 (t, 1H, J = 9.5 Hz); 6.03 (q, 1H, J = 7.0 Hz); ^{13}C NMR ($CDCl_3$) δ 13.5, 14.0, 20.5, 20.6, 20.8, 26.8, 30.1, 39.9, 51.3, 60.4, 61.7, 68.1, 70.6, 72.1, 72.4, 93.7, 97.0, 108.7, 128.0, 129.0, 152.9, 169.2, 170.0, 170.1, 170.2, 170.5, 171.0. HRMS (ESI+) *m/z* 623.1964 (calcd for $C_{27}H_{36}O_{15}Na$: 623.1952) $[M+Na]^+$.

*4.4.3.15 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-5-(methoxycarbonyl)-4-(2-oxo-2-propoxyethyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**20a**)*

According to the general method of esterification with alcohols, 18.0 mg (34% yield) of **20a** were obtained from 50.0 mg of **3**, as a white solid.

$[\alpha]_D$ -633.3 (c 0.03, MeOH); ^{1}H NMR ($CDCl_3$) δ 0.94 (t, 3H); 1.65 (m, 2H); 1.74 (d, 3H, *J* = 7.0 Hz); 2.01-2.03 (3s, 9H); 2.08 (s, 3H); 2.45 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.77 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.72 (s, 3H); 3.79 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.95 (dt, 1H, *J* = 11.0 and 7.0 Hz); 4.02 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.06 (dt, 1H, *J* = 11.0 and 7.0 Hz); 4.14 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.33 (dd, 1H, *J* = 12.5 and 4.5 Hz); 5.06 (d, 1H, *J* = 8.0 Hz); 5.15 (dd, 1H, *J* = 9.5 and 8.0 Hz); 5.15 (t, 1H, *J* = 9.5 Hz); 5.30 (t, 1H, *J* = 9.5 Hz); 5.74 (s, 1H); 6.03 (q, 1H, *J* = 7.0 Hz); 7.48 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 10.3, 13.7, 20.9, 21.0, 21.1, 21.2, 21.9, 26.0, 41.0, 52.3, 62.7, 66.2, 69.3, 71.1, 72.1, 74.8, 104.5, 107.4, 124.8, 129.4, 130.7, 155.2, 168.5, 169.9, 170.1, 170.2, 170.3, 173.1. HRMS (ESI+) *m/z* 637.2120 (calcd for $C_{28}H_{38}O_{15}Na$: 637.2108) $[M+Na]^+$.

4.4.3.16 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-4-(2-butoxy-2-oxoethyl)-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate ***(21a)***

According to the general method of esterification with alcohols, 45.4 mg (87% yield) of **21a** were obtained from 50.0 mg of **3**, as a white solid.

$[\alpha]_D$ -475 (c 0.04, MeOH); ^{1}H NMR ($CDCl_3$) δ 0.95 (t, 3H); 1.37 (m, 2H); 1.60 (m, 2H); 1.77 (dd, 3H, *J* = 1.0 and 7.0 Hz); 2.01-2.03 (m, 9H); 2.08 (s, 3H); 2.43 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.75 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.72 (s, 3H); 3.79 (ddd, 1H, *J* = 2.5, 4.5 and 12.5 Hz); 3.99 (dt, 1H, *J* = 6,5 and 11.0 Hz); 4.01 (dd, 1H, *J* = 9.0 and 5.0 Hz); 4.09 (dt, 1H, *J* = 6,5 and 11.0 Hz); 4.13 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.33 (dd, 1H, *J* = 12.5 and 4.5 Hz); 5.05 (d, 1H, *J* = 8 Hz); 5.14 (dd, 1H, *J* = 9.5 and 8 Hz); 5.14 (t, 1H, *J* = 9.5 Hz); 5.29 (t, 1H, *J* = 9.5 Hz); 5.74 (s, 1H); 6.03 (q, 1H, *J* = 7.0 Hz); 7.48 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.5, 13.6, 19.0, 20.5, 20.6, 30.2, 30.5, 40.0, 51.4, 61.7, 64.4, 68.2, 70.7, 72.1, 72.5, 93.6, 97.0, 108.7,

124.7, 128.0, 152.9, 166.7, 169.3, 169.4, 170.1, 170.5. HRMS (ESI+) *m/z* 651.2271, (calcd for $C_{29}H_{40}O_{15}Na$: 651.2265) $[M+Na]^+$

*4.4.3.17 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-4-(2-(isopentyloxy)-2-oxoethyl)-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**22a**)*

According to the general method of esterification with alcohols, 36.9 mg (47% yield) of **22a** were obtained, as a white solid, from 70.0 mg of **3**.

$[\alpha]_D$ -103.3 (c = $0.3.10^{-3}$, MeOH), ^{1}H NMR ($CDCl_3$) δ 0.88-0.93 (m, 6H); 1.49 (dt, 2H, *J*= 6.5 and 11.0 Hz); 1.67 (m, 1H); 1.76 (bd, 3H, *J* = 7.0 Hz); 2.03-2.05 (3s, 9H, Ac); 2.10 (s, 3H, Ac); 2.42 (dd, 1H, *J*= 14.5 and 9.0 Hz); 2.73 (dd, 1H, *J*= 14.5 and 4.5 Hz); 3.74 (s, 3H, OMe); 3,78 (ddd, 1H, *J*= 9.5, 5.0 Hz and 2.0 Hz); 3.97-4.04 (m, 2H); 4.08-4.15 (m, 2H); 4.33 (dd, 1H *J*= 12.5 and 5.0 Hz); 5.04 (d, 1H, *J*= 8.0 Hz); 5.13 (dd, 1H, *J* = 9.5 and 8 Hz); 5.13 (t, 1H, *J* = 9.5 Hz); 5.28 (t, 1H, *J*= 9.5 Hz); 5.73 (s, 1H); 6.02 (q, 1H, *J* = 7.0 Hz); 7.47 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.4, 20.4, 20.6, 22.2, 22.3, 24.9, 30.1, 37.1, 39.9, 51.3, 61.7, 63.1, 68.2, 70.6, 72.1, 72.4, 93.6, 97.0, 108.7, 124.6, 128.1, 152.9, 166.6, 169,2, 169.3, 170.0, 170.5, 171.1. HRMS (ESI+) *m/z* 665.2419 (calcd for $C_{30}H_{42}O_{15}Na$: 665.2421) $[M+Na]^+$.

*4.4.3.18 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-5-(methoxycarbonyl)-4-(2-((3-methylbut-3-en-1-yl)oxy)-2-oxoethyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**23a**)*

According to the general method of esterification with alcohols, 34.0 mg (63% yield) of **23a** were obtained, as a white solid, from 50.0 mg of **3**.

$[\alpha]_D$ -100 (c = $0.2.10^{-3}$, MeOH), ^{1}H NMR ($CDCl_3$): δ 1.76 (m, 6H); 2.03-2.05 (3s, 9H, Ac); 2.10 (s, 3H, Ac); 2.32 (m , 2H); 2.43 (dd, 1H, *J*= 14.5 and 9 Hz); 2.74 (dd, 1H, *J*= 14.5 and 4.5 Hz); 3.74 (s, 3H, OMe); 3,78 (ddd, 1H, *J*= 9.5, 5.0 and 2.0 Hz); 4.00 (dd, 1H, *J*= 12.5 and 2.5 Hz); 3.97-4.04 (m, 2H); 4.08-4.15 (m, 2H); 4.33 (dd, 1H, *J*= 12.5 and 5.0 Hz); 4.73 (s,

1H); 4.81 (s, 1H); 5.05 (d, 1H, *J*= 8.0 Hz); 5.14 (dd, 1H, *J* = 9.5 and 8 Hz); 5.14 (t, 1H, *J* = 9.5 Hz); 5.29 (t, 1H, *J*= 9.5 Hz); 5.73 (s, 1H); 6.03 (q, 1H, *J*= 7.0 Hz); 7.48 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.5, 20.5, 20.60, 22.4, 30.1, 36.5, 39.8, 51.4, 61.7, 62.8, 68.2, 70.6, 72.1, 72.4, 93.6, 97.0, 108.7, 112.3, 124.7, 128.0, 141.4, 152.9, 166.7, 169.3, 170.1, 170.5, 170.8. HRMS (ESI+) *m/z* 663.2269 (calcd for $C_{30}H_{40}O_{15}Na$: 663.2265) $[M+Na]^+$.

*4.4.3.19 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-4-(2-(hexadecyloxy)-2-oxoethyl)-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**24a**)*

According to the general method of esterification with alcohols, 20.0 mg (30% yield) of **24a** were obtained, as a white solid, from 50.0 mg of **3**.

$[\alpha]_D$ -633.3 (c 0.03, MeOH); ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 3H); 1.30 (s, 26H); 1.60 (quin, 2H); 1.76 (d, 3H, *J* = 6.5 Hz); 2.01-2.03 (m, 9H); 2.08 (s, 3H); 2.45 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.74 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.72 (s, 3H); 3.78 (ddd, 1H, *J* = 2.5, 4.5 and 12.5 Hz); 3.98 (dd, 1H, *J* = 9.0 and 5.0 Hz); 3.98 (dt, 1H, *J* = 6,5 and 11.0 Hz); 4.06 (dt, 1H, *J* = 6,5 and 11.0 Hz); 4.13 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.33 (dd, 1H, *J* = 12.5 and 4.5 Hz); 5.05 (d, 1H, *J* = 8.0 Hz); 5.14 (dd, 1H, *J* = 9.5 and 8 Hz); 5.14 (t, 1H, *J* = 9.5 Hz); 5.29 (t, 1H, *J* = 9.5 Hz); 5.73 (bs, 1H); 6.02 (q, 1H, *J* = 7.0 Hz); 7.47 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.4, 14.0, 20.4, 20.5, 20.6, 22.6, 25.7, 26.8, 28.4, 29.1, 29.2, 29.4, 29.5, 29.6, 30.1, 31.8, 39.9, 51.3, 61.7, 64.7, 68.2, 70.6, 72.1, 72.4, 93.7, 97.0, 108.6, 124.6, 128.1, 152.9, 166.7, 169.2, 169.3, 170.1, 170.5, 171.1. HRMS (ESI+) *m/z* 819.4131 (calcd for $C_{41}H_{64}O_{15}Na$: 819.4143) $[M+Na]^+$.

*4.4.3.20 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-4-(2-(2-cyclohexylethoxy)-2-oxoethyl)-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**25a**)*

According to the general method of esterification with alcohols, 54.2 mg (67% yield) of **25a** were obtained, as a white solid, from 70.0 mg of **3**.

$[\alpha]_D$ -100 (c = $0.2.10^{-3}$, MeOH), ^{1}H NMR ($CDCl_3$) δ 1.1-1.5 (m, 11H); 1.77 (dd, 3H, *J* = 1.0 and 6.5 Hz); 2.03-2.05 (3s, 9H, Ac); 2.10 (s, 3H, Ac); 2.44 (dd, 1H, *J* = 14.5 and 9 Hz;); 2.74 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.74 (s, 3H, OMe); 3,79 (ddd, 1H, *J* = 9.5, 5.0 and 2 Hz); 4.00 (dt, 1H, *J* = 4,5 and 11.0 Hz); 4.03 (t, 1H, *J* = 7.0 Hz); 4.12 (t, 1H, *J* = 7.0 Hz); 4.13 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.34 (dd, 1H, *J* = 12.5 and 5.0 Hz); 5.05 (d, 1H, *J* = 8.0 Hz); 5.14 (dd, 1H, *J* = 9.5 and 8 Hz); 5.14 (t, 1H, *J* = 9.5 Hz); 5.29 (t, 1H, *J* = 9.5 Hz); 5.74 (s, 1H); 6.03 (q, 1H, *J* = 7.0 Hz); 7.49 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.5, 20.5, 20.60, 30.1, 33.0, 33.1, 34.4, 35.8, 39.9, 51.4, 61.7, 62.8, 68.2, 70.6, 72.1, 72.4, 93.6, 97.0, 108.7, 124.6, 128.1, 152.9, 166.7, 169.3, 169.4, 170.5, 171.2. HRMS (ESI+) *m/z* 705.2720 (calcd for $C_{33}H_{46}O_{15}Na$: 705.2734) $[M+Na]^+$.

*4.4.3.21 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-5-(methoxycarbonyl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**26a**)*

According to the general method of esterification with alcohols, 52.0 mg (67% yield) of **26a** were obtained from 70.0 mg of **3**, as a white solid.

$[\alpha]_D$ -375 (c 0.04, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.77 (dd, 3H, *J* = 1.0 and 6.5 Hz); 2.01-2.03 (m, 9H); 2.08 (s, 3H); 2.48 (m, 1H); 2.49 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.80 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.72 (s, 3H); 3,78 (ddd, 1H, *J* = 9.5, 5.0 and 2.0 Hz); 3.99 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.14 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.32 (dd, 1H, *J* = 12.5 and 4.5 Hz); 4.65 (m, 2H); 5.04 (d, 1H, *J* = 8.0 Hz); 5.13 (dd, 1H, *J* = 9.5 and 8 Hz); 5.13 (t, 1H, *J* = 9.5 Hz); 5.28 (t, 1H, *J* = 9.5 Hz); 5.72 (s, 1H); 6.03 (q, 1H, *J* = 7.0 Hz); 7.48 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.6, 20.5, 20.6, 20.7, 30.1, 39.5, 51.4, 51.9, 61.7, 68.2, 70.6, 72.1, 72.4, 75.0, 93.70, 97.03, 108.4, 125.0, 127.7, 153.1, 166.6, 169.2, 169.3, 170.1. HRMS (ESI+) *m/z* 633.1795 (calcd for $C_{28}H_{34}O_{15}Na$: 633.1795) $[M+Na]^+$.

*4.4.3.22 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-4-(2-(allyloxy)-2-oxoethyl)-3-ethylidene-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**27a**)*

According to the general method of esterification with alcohols, 22.0 mg (42% yield) of **27a** were obtained, as a white solid, from 50.0 mg of **3**.

$[\alpha]_D$ -825 (c 0.04, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.77 (d, 3H, J = 7.0 Hz); 2.11-2.05 (2s, 9H); 2.19 (s, 3H); 2.48 (dd, 1H, J = 14.5 and 9.0 Hz); 2.79 (dd, 1H, J = 14.5 and 4.5 Hz); 3.75 (s, 3H); 3.79 (ddd, 1H, J = 9.5, 5.0 and 2.0 Hz); 4.03 (dd, 1H, J = 9.0 and 4.5 Hz); 4.13 (dd, 1H, J = 12.5 and 2.5 Hz); 4.33 (dd, 1H, J = 12.5 and 4.5 Hz); 4.52 (m, 1H); 4.58 (m, 1H); 5.05 (d, 1H, J = 8.0 Hz); 5.15 (m, 2H); 5.23 (dd, 2H, J = 1.0 and 12.0 Hz); 5.29 (t, 1H, J = 9.5 Hz); 5.74 (s, 1H); 5.91 (m, 1H); 6.03 (q, 1H, J = 7.0 Hz); 7.49 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.7, 21.3, 21.5, 21,6, 21.7, 26.0, 41.4, 52.3, 62.7, 65.9, 69.3, 71.1, 72.1, 74.8, 104.3, 107.5, 118.2, 124.8, 128.0, 130.7, 132.1, 155.2, 168.5, 169.9, 170.1, 170.2, 170.3, 173.1. HRMS (ESI+) *m/z* 635.2088 (calcd for $C_{28}H_{36}O_{15}Na$: 635.1952) $[M+Na]^+$.

*4.4.3.23 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-4-(2-(furan-2-ylmethoxy)-2-oxoethyl)-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**28a**)*

According to the general method of esterification with alcohols, 57.0 mg (71% yield) of **28a** were obtained, as a white solid, from 70.0 mg of **3**.

$[\alpha]_D$ -850 (c 0.06, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.78 (d,d 3H, J = 7.0 and 1.5 Hz); 1.97-2.06 (3s, 9H, Ac); 2.13 (s, 3H, Ac); 2.48 (dd, 1H, J = 14.5 and 9.0 Hz); 2.78 (dd, 1H, J = 14.5 and 4.5 Hz); 3.65 (s, 3H); 3.79 (ddd, 1H, J = 9.5, 4.5 and 2.0 Hz); 4.01 (dd, 1H, J = 4.5 and 9.0 Hz); 4.15 (dd, 1H, J = 12.5 and 2.0 Hz); 4.33 (dd, 1H, J = 12.5 and 4.5 Hz); 5.03 (m, 1H); 5.04 (d, 1H, J = 8.0 Hz); 5.15 (dd, 1H, J = 9.5 and 8.0 Hz); 5.15 (t, 1H, J = 9.5 Hz); 5.30 (t, 1H, J = 9.5 Hz); 5.72 (bs, 1H); 6.01 (q, 1H, J = 7.0 Hz); 6.38 (dd, 1H, J = 3.5 and 2.0 Hz);

6.41 (bd, 1H, *J* = 3.5 Hz); 7.43 (dd, 1H, *J* = 1.5 and 0.5 Hz); 7.47 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.4, 20.5, 20.6, 21.0, 26.8, 30.0, 39.7, 51.3, 58.0, 60.7, 68.2, 70.6, 72.1, 72.4, 93.7, 97.0, 108.6, 110.5, 110.7, 124.9, 127.9, 143.1, 149.2, 153.0, 166.7, 169.3, 170.1, 170.5, 170.6. HRMS (ESI+) *m/z* 675.1896 **(**calcd for $C_{30}H_{36}O_{16}Na$: 675.1901) $[M+Na]^+$.

*4.4.3.24 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-5-(methoxycarbonyl)-4-(2-oxo-2-(propylthio)ethyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**29a**)*

According to the general method of esterification with thiols, 42.0 mg (70% yield) of **29a** were obtained, as a yellow solid, from 50.0 mg of **3**.

$[\alpha]_D$ -116 (c 0.03, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.72 (d, 3H, *J* = 6.5 Hz); 2.06-2.05 (3s, 9H); 2.08 (s, 3H); 2.66 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.85 (m, 2H); 2.98 (dd, 1H, *J* = 14.5 and 3.5 Hz); 3.10 (td, 2H); 3.76 (s, 3H); 3.79 (ddd, 1H); 4.04 (dd, 1H, *J* = 9.0 and 3.5 Hz); 4.13 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.31 (dd, 1H, *J* = 12.5 and 4.5 Hz); 5.05 (d, 1H, *J* = 8.0 Hz); 5.14 (dd, 1H, *J* = 9.5 and 8 Hz); 5.14 (t, 1H, *J* = 9.5 Hz); 5.29 (t, 1H, *J* = 9.5 Hz); 5.72 (s, 1H); 6.03 (q, 1H, *J* = 7.0 Hz); 7.24 (m, 3H); 7.32 (dd, 2H, *J* = 8.0 and 1.5 Hz); 7.49 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.6, 20.5, 20.6, 26.8, 30.5, 30.8, 35.6, 35.7, 48.6, 51.4, 61.7, 68.1, 70.6, 72.1, 72.4, 93.7, 97.0, 108.5, 125.0, 126.4, 127.4, 128.4, 128.5, 139.8, 153.1, 166.6, 169.3, 169.4, 170.1, 170.5, 196.3. HRMS (ESI+) *m/z* 653.1874 (calcd for $C_{28}H_{38}SO_{14}Na$: 653.1880) $[M+Na]^+$.

*4.4.3.25 (2S,3S,4R,5S,6R)-2-(acetoxymethyl)-6-(((E)-3-ethylidene-5-(methoxycarbonyl)-4-(2-oxo-2-(propylamino)ethyl)-3,4-dihydro-2H-pyran-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**30a**)*

According to the general method of esterification with amines, 45.0 mg (90% yield) of **30a** were obtained, as a yellow solid, from 30mg of **3**.

$[\alpha]_D$ -630 (c 0.03, MeOH); ^{1}H NMR ($CDCl_3$) δ 0.93 (m, 3H); 1.51 (m, 2H); 1.75 (d, 3H, *J* = 6.5 Hz); 2.06-2.05 (2s, 9H); 2.12 (s, 3H); 2.23 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.60 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.15 (m, 1H); 3.21 (m, 1H); 3.77 (s, 3H); 3.92 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.01 (ddd, 1H, *J* = 9.0, 4.5 and 2.5 Hz); 4.18 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.33 (dd, 1H, *J* = 12.5 and 4.5 Hz); 5.04 (d, 1H, *J* = 8.0 Hz); 5.14 (dd, 1H, *J* = 9.5 and 8.0 Hz); 5.14 (t, 1H, *J* = 9.5 Hz); 5.29 (t, 1H, *J* = 9.5 Hz); 5.73 (s, 1H); 6.05 (q, 1H, *J* = 7.0 Hz); 7.49 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 11.2, 13.7, 20.7, 20.9, 21.0, 21.1, 23.1, 26.6, 39.4, 42.5, 52.3, 62.7, 69.4, 71.1, 72.1, 74.9, 104.5, 107.0, 109.4, 124.8, 130.8, 155.3, 168.5, 169.9, 170.1, 170.2, 170.3, 173.4. HRMS (ESI+) *m/z* 636.2250 (calcd for $C_{28}H_{39}NO_{14}Na$: 636.2268) $[M+Na]^+$.

*4.4.3.26 (E)-3-ethylidene-4-(2-methoxy-2-oxoethyl)-2-(((2R,3S,4R,5S,6S)-3,4,5-triacetoxy-6-(acetoxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylic acid (**31a**)*

According to the general method of esterification with alcohols, 285 mg (17% yield) of **31a**, were obtained, as a white solid, from 1.11g of **5**.

$[\alpha]_D$ -850 (c 0.06, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.78 (d, 3H, *J* = 6.5 Hz); 1.97-2.06 (3s, 9H); 2.13 (s, 3H); 2.47 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.80 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.65 (s, 3H); 3.78-3.81 (dd, *J* = 4.5 and 9.0 Hz, 1H); 4.00 (ddd, 1H, *J* = 9.5, 4.5 and 2.0 Hz); 4.14 (dd, 1H, *J* = 12.5 and 2.0 Hz); 4.33 (dd, 1H, *J* = 12.5 and 4.5 Hz); 5.06 (d, 1H, *J* = 8.0 Hz); 5.15 (dd, 1H, *J* = 9.5 and 8 Hz); 5.15 (t, 1H, *J* = 9.5 Hz); 5.30 (t, 1H, *J* = 9.5 Hz); 5.76 (s, 1H); 6.06 (q, 1H, *J* = 7.0 Hz); 7.60 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.4, 20.5, 20.6, 21.0, 21.1, 29.9, 40.7, 51.5, 61.7, 70.6, 72.1, 72.4, 77.2, 93.8, 97.0, 119.5, 125.1, 130.8, 155.0, 169.3, 170.0, 170.1, 170.2, 170.3, 171.3. HRMS (ESI+) *m/z* 595.1620 (calcd for $C_{25}H_{32}O_{15}Na$: 595.1639) $[M+Na]^+$.

*4.4.3.27 (E)-3-ethylidene-4-(2-(hexadecyloxy)-2-oxoethyl)-2-(((2R,3S,4R,5S,6S)-3,4,5-triacetoxy-6-(acetoxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylic acid (**32a**)*

According to the general method of esterification with alcohols, 6.0 mg (30% yield) of **32a**, were obtained, as a white solid, from 14.0 mg of **5**.

$[\alpha]_D$ -250 (c 0.06, MeOH); ^{1}H NMR ($CDCl_3$) δ 0.88 (m, 3H); 1.30 (s, 26H); 1.62 (q, 2H); 1.76 (d, 3H, *J* = 7.0 Hz); 2.06-1.97 (m, 9H); 2.07 (s, 3H); 2.35 (m, 1H); 2.45 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.77 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.80 (ddd, 1H, *J* = 9.5, 4.5 and 2.0 Hz); 4.00 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.07 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.14 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.34 (dd, 1H, *J* = 12.5 and 4.5 Hz); 5.06 (d, 1H, *J* = 8.0 Hz); 5.16 (dd, 1H, *J* = 9.5 and 8 Hz); 5.16 (t, 1H, *J* = 9.5 Hz); 5.31 (t, 1H, *J* = 9.5 Hz); 5.76 (s, 1H); 6.05 (q, 1H, *J* = 7.0 Hz); 7.57 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.7, 14.1, 20.7, 21.2, 21.6, 21.9, 22.8, 25.8, 28.4, 28.9, 29.3, 29.6, 37.8, 41.7, 62.7, 65.2, 69.3, 71.1, 72.1, 74.8, 101.4, 104.5, 124.8, 128.6, 129.1, 153.9, 168.7, 169.2, 169.8, 170.2, 170.5, 173.1. HRMS (ESI+) *m/z* 805.4159 (calcd for $C_{40}H_{62}O_{15}Na$: 805.3986) $[M+Na]^+$.

4.4.4. General procedure for deprotection

To a solution of the previous acetylated derivatives in methanol at 0°C under argon was added 5 equiv of diethylamine. After stirring 6 h at room temperature, the mixture was diluted with methanol and quenched with a small amount of silica gel. After evaporation of the solvents under reduced pressure, the crude material was directly submitted to flash chromatography on silica gel (dichloromethane/methanol 95:5) affording the final deprotected compound.

*4.4.4.1 (E)-methyl 3-ethylidene-4-(2-oxo-2-phenethoxyethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**6**)*

According to the general method of deprotection, 9.0 mg (30% yield) of **6**, were obtained, as a white solid, from 40.0 mg of **6a**.

$[\alpha]_D$ -350 (c 0.06, MeOH); ^{1}H NMR (D_2O) δ; 1.53 (d, 3H, *J* = 7.0 Hz); 2.43 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.62 (dd, 1H, *J* = 14.5 and 4.5 Hz); 2.89 (m, 2H); 3.39 (t, 1H, *J* = 9.5 Hz); 3.39

(dd, 1H, *J* = 8.0 and 9.5 Hz); 3.41 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.47 (t, 1H, *J* = 9.0 Hz); 3.64 (s, 3H); 3.64 (dd, 1H, *J* = 12.5 and 4.5 Hz); 3.85 (m, 1H); 3.85 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.17 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.28 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.81 (d, 1H, *J* = 8.0 Hz); 5.73 (s, 1H); 5.98 (q, 1H, *J* = 7.0 Hz); 7.24 (dd, 1H, *J* = 8.0 and 2.0 Hz); 7.26 (t, 2H, *J* = 8.0 Hz); 7.31 (dd, 2H, *J* = 8.0 and 1.5 Hz); 7.47 (s, 1H); ^{13}C NMR (D_2O) δ 11.6, 29.4, 33.3, 39.0, 51.1, 59.7, 65.3, 68.8, 71.9, 74.9, 75.6, 94.2, 96.4, 98.6, 109.4, 119.9, 124.5, 125.9, 128.0, 128.3, 130.8, 139.1, 153.8, 166.3, 172.5. HRMS (ESI+) *m/z* 531.1831 (calcd for $C_{25}H_{32}O_{11}Na$: 531.1842) $[M+Na]^+$.

*4.4.4.2 (E)-methyl 4-(2-(3,4-dimethoxyphenethoxy)-2-oxoethyl)-3-ethylidene-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate - Lucidumoside D (**7**)*

Method A: According to the general method of deprotection, 23.0 mg (60% yield) of **7** were obtained, as a white solid, from 50.0 mg of **7a**.

Method B: Another method used to prepare the **7** is starting with **1** as raw material.

200.0 mg of **1** was dissolved in 6 mL of toluene and 4 mL MeOH. 2mL of TMSCHN2 were then carefully added dropwise and the reaction mixture was let to stir for 30-50 minutes at room temperature. Purification of the crude product by column chromatography on silica gel, gave 85.0 mg (40% yield) of **7**, as a white solid, from 200.0 mg of **1**.

$[\alpha]_D$ -80 (c 0.05, MeOH); ^{1}H NMR (D_2O) δ 1.71 (d, 3H, *J* = 7.0 Hz); 2.42 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.63 (dd, 1H, *J* = 14.5 and 4.5 Hz); 2.84 (m, 2H); 3.37 (t, 1H, *J* = 9.5 Hz); 3.37 (dd, 1H, *J* = 8.0 and 9.5 Hz); 3.42 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.48 (t, 1H, *J* = 9.0 Hz); 3.64 (s, 3H); 3.66 (dd, 1H, *J* = 12.5 and 4.5 Hz); 3.79 (2s, 6H); 3.82 (dd, 1H *J* = 9.0 and 4.5 Hz); 3.84 (dd, 1H, J = 12.5 and 2.5 Hz); 4.16 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.30 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.83 (d, 1H, *J* = 8.0 Hz); 5.75 (s, 1H); 5.95 (q, 1H, *J* = 7.0 Hz); 6.84 (dd, 1H, *J* = 8.0 and 1.5 Hz); 6.92 (d, 1H, *J* = 1.5 Hz); 6.96 (d, 1H, *J* = 8.0 Hz); 7.46 (s, 1H); ^{13}C

NMR (D_2O) δ 13.4, 30.1, 33.6, 39.8, 51.3, 55.8, 60.6, 65.2, 68.1, 70.5, 70.6, 72.1, 72.4, 93.6, 97.0, 108.6, 111.3, 112.1, 120.8, 124.7, 128.0, 130.1, 147.7, 148.8, 152.9, 170.9. HRMS (ESI+) *m/z* 591.2048 (calcd for $C_{27}H_{36}O_{13}Na$: 591.2054) $[M+Na]^+$.

*4.4.4.3 (E)-methyl 4-(2-(2-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)ethoxy)-2-oxoethyl)-3-ethylidene-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**8**)*

According to the general method of deprotection, 19.6 mg of **8** (68% yield) were obtained, as a white solid, from 37.0 mg of **8a**.

$[\alpha]_D$ -866 (c 0.03, MeOH); ^{1}H NMR (D_2O) δ 1.66 (d, 3H, *J* = 7.0 Hz); 2.22 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.56 (dd, 1H, *J* = 14.5 and 4.5 Hz); 2.82 (m, 2H); 3.38 (t, 1H, *J* = 9.5 Hz); 3.42 (m, 2H); 3.51 (d, 1H, *J* = 8.0 Hz); 3.54 (dd, 1H, *J* = 12.5 and 5.0 Hz); 3.65 (s, 3H); 3.79 (dd, 1H, *J* = 12.5 and 2.5 Hz); 3.82 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.02 (m, 1H); 4.12 (m, 2H); 4.15 (m, 4H); 4.82 (d, 1H, *J* = 8.0 Hz); 5.74 (s, 1H); 5.95 (q, 1H, *J* = 7.0 Hz); 6.74 (dd, 1H, *J* = 8 and 1.5 Hz); 6.79 (d, 1H, *J* = 1.5 Hz); 6.88 (d, 1H, *J* = 8.0 Hz); 7.42 (s, 1H); ^{13}C NMR (D_2O) 13.8, 26.0, 34.8, 41.0, 52.3, 62.2, 64.2, 65.3, 71.5, 74.2, 76.9, 81.5, 107.7, 108.2, 109.4, 112.6, 114.6, 121.0, 124.8, 130.8, 136.4, 147.9, 148.6, 155.2, 168.5, 173.1. HRMS (ESI+) *m/z* 589.1896 (calcd for $C_{27}H_{34}O_{13}Na$: 589.1897) $[M+Na]^+$.

*4.4.4.4 (E)-methyl 3-ethylidene-4-(2-(4-hydroxyphenethoxy)-2-oxoethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate - Ligstroside (**9**)*

According to the general method of deprotection, 45.0 mg (71% yield) of **9** were obtained, as a white solid, from 90.0 mg of **9a**.

$[\alpha]_D$ -162 (c 0.06, MeOH); ^{1}H NMR (D_2O) δ 1.49 (d, 3H, *J* = 7.0 Hz); 2.37 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.58 (dd, 1H, *J* = 14.5 and 4.5 Hz); 2.78 (m, 2H); 3.37 (t, 1H, *J* = 9.5 Hz); 3.37 (dd, 1H, *J* = 8.0 and 9.5 Hz); 3.41 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.48 (t, 1H, *J* = 9.5 Hz);

3.65 (dd, 1H, *J* = 12.5 and 4.5 Hz); 3.65 (s, 3H); 3.82 (dd, 1H, *J* = 9.0 and 4.5 Hz); 3.82 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.08 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.22 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.81 (d, 1H, *J* = 8.0 Hz); 5.70 (s, 1H); 5.96 (q, 1H, *J* = 7.0 Hz); 6.77 (d, 2H, *J* = 8.5 Hz); 7.07 (d, 2H, *J* = 8.5 Hz); 7.44 (s, 1H); ^{13}C NMR (D_2O) δ 12.5, 30.2, 33.1, 39.8, 51.8, 60.6, 66.2, 69.3, 69.4, 72.5, 75.6, 94.6, 99.4, 107.9, 115.4, 124.9, 128.1, 130.1, 130.2, 154.1, 154.4, 168.9, 174.0. HRMS (ESI+) *m/z* 547.1813 (calcd for $C_{25}H_{32}O_{12}Na$: 547.1791) $[M+Na]^+$.

*4.4.4.5 (E)-methyl 3-ethylidene-4-(2-(4-methoxyphenethoxy)-2-oxoethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**10**)*

According to the general method of esterification with alcohols, 16.6 mg (40% yield) of **10** were obtained, as a white solid, from 55.0 mg of **10a**.

$[\alpha]_D$ -245 (c 0.06, MeOH); ^{1}H NMR (D_2O) δ 1.50 (d, 3H, *J* = 7.0 Hz); 2.45 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.63 (dd, 1H, *J* = 14.5 and 4.5 Hz); 2.78 (m, 2H); 3.37 (t, 1H, *J* = 9.5 Hz); 3.37 (dd, 1H, *J* = 8.0 and 9.5 Hz); 3.41 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.48 (t, 1H, *J* = 9.5 Hz); 3.65 (dd, 1H, *J* = 12.5 and 4.5 Hz); 3.65 (s, 3H); 3.72 (dd, 1H, *J*= 9.0 and 4.5 Hz); 3.78 (s, 3H); 3.84 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.14 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.29 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.89 (d, 1H, *J* = 8.0 Hz); 5.75 (s, 1H); 5.95 (q, 1H, *J* = 7.0 Hz); 6.93 (d, 2H, *J* = 8.0 Hz); 7.20 (d, 2H, *J* = 8.0 Hz); 7.46 (s, 1H); ^{13}C NMR (D_2O) δ 12.1, 29.5, 35.3, 41.0, 52.1, 55.4, 62.0, 66.3, 69.8, 72.5, 74.9, 76.5, 95.1, 96.5, 98.8, 115.9, 115.9, 125.8, 128.6, 128.8, 130.3, 130.6, 156.8, 157.2, 166.8, 172.6. HRMS (ESI+) *m/z* 561.1929 (calcd for $C_{26}H_{34}O_{12}Na$: 561.1948) $[M+Na]^+$.

*4.4.4.6 (E)-methyl 3-ethylidene-4-(2-(4-nitrophenethoxy)-2-oxoethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**11**)*

According to the general method of deprotection, 20.0 mg (43% yield) of **11** were obtained, as a white solid, from 50.0 mg of **11a**.

$[\alpha]_D$ -90 (c 0.04, MeOH); ^{1}H NMR (D_2O) δ 1.44 (d, 3H, *J* = 7.0 Hz; 2.45 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.65 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.05 (m, 2H); 3.37 (t, 1H, *J* = 9.5 Hz); 3.37 (dd, 1H, *J* = 8.0 and 9.5 Hz); 3.41 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.48 (t, 1H, *J* = 9.5 Hz); 3.65 (dd, 1H, *J* = 12.5 and 4.5 Hz); 3.65 (s, 3H); 3.67 (dd, 1H, *J* = 9.0 and 4.5 Hz); 3.86 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.22 (dt, 1H, *J* = 6.5 and 11.0); 4.41 (dt, 1H, *J* = 6.5 and 11.0); 4.83 (d, 1H, *J* = 8.0 Hz); 5.76 (s, 1H); 5.85 (q, 1H, *J* = 7.0 Hz); 6.93 (d, 2H, *J* = 8.0 Hz); 7.46 (d, 2H, *J* = 8.0 Hz); 7.46 (s, 1H); 8.20 (d, 2H, *J* = 8.0 Hz); ^{13}C NMR (D_2O) δ 12.8, 19.8, 25.3, 32.6, 51.1, 59.5, 63.6, 69.8, 72.5, 74.2, 77.5, 93.8, 97.1, 105.4, 119.9, 120.0, 122.8, 126.7, 140.3, 142.1, 142.8, 142.8, 148.8, 166.3, 172.5. HRMS (ESI+) *m/z* 576.1677 (calcd for $C_{25}H_{31}NO_{13}Na$: 576.1693) $[M+Na]^+$.

*4.4.4.7 (E)-methyl 3-ethylidene-4-(2-(4-fluorophenethoxy)-2-oxoethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**12**)*

According to the general method of deprotection, 13.0 mg (48% yield) of **12** were obtained, as a white solid, from 35 mg of **12a**.

$[\alpha]_D$ -245 (c 0.06, MeOH); ^{1}H NMR (D_2O) δ 1.51 (d, 3H, *J* = 7.0 Hz); 2.45 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.63 (dd, 1H, *J* = 14.5 and 4.5 Hz); 2.78 (m, 2H); 3.37 (t, 1H, *J* = 9.5 Hz); 3.37 (dd, 1H, *J* = 8.0 and 9.5 Hz); 3.41 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.48 (t, 1H, *J* = 9.5 Hz); 3.70 (dd, 1H, *J* = 12.5 and 4.5 Hz); 3.70 (s, 3H); 3.85 (dd, 1H, *J* = 9.0 and 4.5 Hz); 3.85 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.16 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.30 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.84 (d, 1H, *J* = 8.0 Hz); 5.76 (s, 1H); 5.97 (q, 1H, *J* = 7.0 Hz); 7.04 (d, 2H, *J* = 8.0 Hz); 7.24 (dd, 2H, *J* = 8.0 and 5.5 Hz); 7.46 (s, 1H); ^{13}C NMR (D_2O) δ 12.5, 19.6, 31.7, 37.5, 51.1, 59.2, 61.2, 69.7, 72.1, 73.9, 77.1, 93.5, 97.0, 104.7, 113.9, 114.0, 122.8, 126.6, 129.7, 129.7,

129.8, 131.8, 155.8, 166.0, 172.2. HRMS (ESI+) *m/z* 549.11726 (calcd for $C_{25}H_{31}FO_{11}Na$: 549.1748) $[M+Na]^+$.

*4.4.4.8 (E)-methyl 4-(2-(benzyloxy)-2-oxoethyl)-3-ethylidene-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**13**)*

According to the general method of deprotection, 18.0 mg (60% yield) of **13** were obtained, as a white solid, from 40.0 mg of **13a**.

$[\alpha]_D$ -200 (c 0.004, MeOH); ^{1}H NMR (D_2O) δ 1.51 (d, 3H, *J* = 7.0 Hz); 2.62 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.78 (dd, *J* = 14.5 and 4.5 Hz, 1H); 3.41 (t, 1H, *J* = 9.5 Hz); 3.41 (dd, 1H, *J* = 8.0 and 9.5 Hz); 3.41 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.48 (t, 1H, *J* = 9.5 Hz); 3.65 (s, 3H); 3.69 (dd, 1H, *J* = 12.5 and 4.5 Hz); 3.80 (dd, 1H, *J* = 12.5 and 2.5 Hz); 3.93 (dd, 1H, *J* = 9.0 and 4.5 Hz); 3.82 (dd, 1H, *J* = 12.5 and 2.5 Hz); 4.73 (d, 1H, *J* = 8.0 Hz); 5.01 (d, 1H, *J* = 12 Hz); 5.13 (d, 1H, *J* = 12 Hz); 5.71 (s, 1H); 6.00 (q, 1H, *J* = 7.0 Hz); 7.37 (m, 5H); 7.44 (s, 1H); ^{13}C NMR (D_2O) δ 12.1, 20.8, 37.9, 48.6, 61.0, 65.7, 67.0, 69.8, 75.7, 78.3, 93.8, 96.1, 101.5, 122.5, 125.9, 126.7, 126.7, 127.3, 128.1, 128.2, 133.5, 154.8, 166.0, 169.8. HRMS (ESI+) *m/z* 517.1683 (calcd for $C_{24}H_{30}O_{11}Na$: 517.1686) $[M+Na]^+$.

*4.4.4.9 (E)-methyl 4-(2-((3,4-dimethoxybenzyl)oxy)-2-oxoethyl)-3-ethylidene-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**14**)*

According to the general method of deprotection, 29.0 mg (76% yield) of **14** were obtained, as a white solid, from 50.0 mg of **14a**.

$[\alpha]_D$ -350 (c 0.004, MeOH); ^{1}H NMR (D_2O) δ 1.51 (d, 3H, *J* = 7.0 Hz); 2.62 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.64 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.41 (t, 1H, *J* = 9.5 Hz); 3.41 (dd, 1H, *J* = 8.0 and 9.5 Hz); 3.41 (ddd, 1H,*J* = 9.5, 4.5 and 1.5 Hz); 3.48 (t, 1H, *J* = 9.5 Hz); 3.65 (s, 3H); 3.69 (dd, 1H, *J* = 12.5 and 4.5 Hz); 3.78 (d, 1H, *J* = 8.0 Hz); 3.79 (2s, 6H); 3.80 (dd, 1H, *J* =

12.5 and 2.5 Hz); 3.82 (dd, 1H, *J* = 12.5 and 2.5 Hz); 3.93 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.92 (d, 1H, *J* = 12 Hz); 5.06 (d, 1H, *J* = 12 Hz); 5.67 (s, 1H); 5.96 (q, 1H, *J* = 7.0 Hz); 6.98 (m, 5H); 7.44 (s, 1H); ^{13}C NMR (D_2O) δ 12.1, 20.8, 38.8, 50.8, 53.1, 53.1, 60.1, 65.3, 66.8, 69.4, 73.5, 78.0, 93.3, 95.8, 107.8, 109.7, 110.4, 120.5, 123.5, 123.7, 127.5, 145.9, 147.9, 152.6, 166.5, 170.0.. HRMS (ESI+) *m/z* 577.1897 (calcd for $C_{26}H_{34}O_{13}Na$: 577.1890) $[M+Na]^+$.

*4.4.4.10 (E)-methyl 4-(2-((2,3-dihydrobenzo[b][1,4]dioxin-6-yl)methoxy)-2-oxoethyl)-3-ethylidene-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**15**)*

According to the general method of deprotection, 17.0 mg (74% yield) of **15** were obtained, as a white solid, from 30.0 mg of **15a**.

$[\alpha]_D$ -94 (c 0.006, MeOH); ^{1}H NMR (D_2O) δ 1.51 (d, 3H, *J* = 7.0 Hz); 2.62 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.64 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.41 (t, 1H, *J* = 9.5 Hz); 3.41 (dd, 1H, *J* = 8.0 and 9.5 Hz); 3.41 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.43 (t, 1H, *J* = 9.5 Hz); 3.60 (dd, 1H. *J* = 12.5 and 4.5 Hz); 3.62 (dd, 1H, *J* = 12.5 and 2.5 Hz); 3.62 (s, 3H); 3.78 (d, 1H, *J* = 8.0 Hz); 3.93 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.84 (d, 1H, *J* = 12.0 Hz); 5.04 (d, 1H, *J* = 12.0 Hz); 5.57 (s, 1H); 5.97 (q, 1H, *J* = 7.0 Hz); 6.88 (m, 3H); 7.40 (s, 1H); ^{13}C NMR (D_2O) δ 13.7, 30.2, 34.1, 39.7, 58.8, 61.5, 64.1, 64.1, 64.9, 71.3, 74.1, 76.8, 79.7, 97.0, 108.2, 109.6, 116.2, 116.9, 120.6, 123.5, 127.8, 130.2, 144.5, 146.3, 152.0, 167.8, 170.8. HRMS (ESI+) *m/z* 575.1741 (calcd for $C_{26}H_{32}O_{13}Na$: 575.1741) $[M+Na]^+$.

*4.4.4.11 (E)-methyl 4-(2-(3-(3,4-dimethoxyphenyl)propoxy)-2-oxoethyl)-3-ethylidene-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**16**)*

According to the general method of deprotection, 6.0 mg (26% yield) of **16** were obtained, as a white solid, from 30.0 mg of **16a**.

$[\alpha]_D$ -87 (c 0.005, MeOH); ^{1}H NMR (D_2O) δ 1.60 (d, 3H, *J* = 7.0 Hz); 1.90 (m, 3H); 2.50 (dd, 1H, *J* = 14.5 and 4.5 Hz); 2.60 (m, 3H); 3.37 (t, 1H, *J* = 9.5 Hz); 3.37 (dd, 1H, *J* = 8.0 and 9.5 Hz); 3.42 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.48 (t, 1H, *J* = 9.0 Hz); 3.64 (s, 3H); 3.66 (dd, 1H, *J* = 12.5 and 4.5 Hz); 3.79 (2s, 6H); 3.84 (dd, 1H, *J* = 12.5 and 2.5 Hz); 3.90 (dd, 1H, *J* = 9.0 and 4.5 Hz); 3.90 (dt, 1H, *J* = 6.5 and 11.0); 4.10 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.83 (d, 1H, *J* = 8.0 Hz); 5.83 (s, 1H); 6.05 (q, 1H, *J* = 7.0 Hz); 6.80 (dd, 1H, *J* = 8.0 and 2.0 Hz); 6.88 (d, 1H, *J* = 2.0 Hz); 6.93 (d, 1H, *J* = 8.0 Hz); 7.54 (s, 1H); ^{13}C NMR (D_2O) δ 13.6, 27.9, 39.7, 52.1, 56.0, 62.0, 71.3, 74.0, 76.4, 76.7, 78.7, 81.5, 107.5, 108.2, 109.4, 124.6, 129.0, 154.6, 166.9, 172.8. HRMS (ESI+) *m/z* 605.2192 (calcd for $C_{28}H_{38}O_{13}Na$: 605.2210) $[M+Na]^+$.

*4.4.4.12 (E)-methyl 3-ethylidene-4-(2-methoxy-2-oxoethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**18**)*

According to the general method of deprotection, 6.1 mg (57% yield) of **18** were obtained from 15.0 mg of **18a**, as a white solid. The analytical data of **18** were conform to the literature [36].

*4.4.3.13 (E)-methyl 4-(2-ethoxy-2-oxoethyl)-3-ethylidene-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**19**)*

According to the general method of deprotection, 4.0 mg (45% yield) of **19** were obtained, as a white solid, from 11.0 mg of **19a**.

$[\alpha]_D$ -525 (c 0.04, MeOH); ^{1}H NMR (D_2O) δ 1.17 (t, 3H, *J* = 7.0 Hz); 1.65 (d, 3H, *J* = 7.0 Hz); 2.35 (dd, 1H, *J* = 13.5 and 9.5 Hz); 2.69 (dd, 1H, *J* = 13.5 and 5.0 Hz); 3.35 (t, 1H, *J* = 9.5 Hz); 3.37 (dd, 1H, *J* = 9.5 and 8.0 Hz); 3.43 (ddd, 1H, *J* = 9.5 and 6.0 and 2.0 Hz); 3.47 (t, 1H, *J* = 9.5 Hz); 3.67 (dd, 1H, *J* = 12.5 and 6.0 Hz); 3.68 (s, 3H); 3.84 (dd, 1H, *J* = 12.5 and 2.0 Hz); 3.94 (dd, 1H, *J* = 9.0 and 5.0 Hz); 4.02 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.07 (dt, 1H, *J*

= 7.0 and 11.0 Hz); 4.87 (d, 1H, *J* = 8.0 Hz); 5.87 (s, 1H); 6.04 (q, 1H, *J* = 7.0 Hz); 7.52 (s, 1H); ^{13}C NMR (D_2O) δ 13.7, 14.1, 26.0, 41.0, 52.3, 61.3, 62.2, 71.5, 74.2, 76.8, 81.5, 107.7, 108.2, 109.4, 124.8, 130.8, 155.2, 168.5, 173.1. HRMS (ESI+) *m/z* 455.1503 (calcd for $C_{19}H_{28}O_{11}Na$: 455.1529) $[M+Na]^+$.

*4.4.4.14 (E)-methyl 3-ethylidene-4-(2-oxo-2-propoxyethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**20**)*

According to the general method of deprotection, 9.0 mg (85% yield) of **20** were obtained, as a white solid, from 14.0 mg of **20a**.

$[\alpha]_D$ -784 (c 0.065, MeOH); ^{1}H NMR (D_2O) δ 0.83 (t, 3H, *J* = 7.0 Hz); 1.17 (t, 2H, *J* = 7.0 Hz); 1.66 (d, 3H, *J* = 7.0 Hz); 2.47 (dd, 1H, *J* = 13.5 and 9.0 Hz); 2.69 (dd, 1H, *J* = 13.5 and 5.0 Hz); 3.38 (dd, 1H, *J* = 9.5 and 8.0 Hz); 3.38 (t, 1H, *J* = 9.5 Hz); 3.43 (ddd, 1H, *J* = 9.5, 6.0 and 2.0 Hz); 3.47 (t, 1H, *J* = 9.5 Hz); 3.65 (dd, 1H, *J* = 12.5 and 6.0 Hz); 3.69 (s, 3H); 3.83 (dd, 1H, *J* = 12.5 and 2.0 Hz); 3.91 (dt, 1H, *J* = 6.5 and 11.0 Hz); 3.94 (dd, 1H, *J* = 9.0 and 5.0 Hz); 4.00 (dt, 1H, *J* = 7.0 and 11.0 Hz); 4.87 (d, 1H, *J* = 8.0 Hz); 5.86 (s, 1H); 6.04 (q, 1H, *J* = 7.0 Hz); 7.53 (s, 1H); ^{13}C NMR (D_2O) δ 10.3, 13.7, 21.9, 26.0, 41.4, 52.3, 62.3, 66.2, 71.5, 74.2, 76.8, 81.5, 107.7, 108.2, 109.4, 124.8, 130.8, 155.3, 168.5, 173.1. HRMS (ESI+) *m/z* 469.1665 (calcd for $C_{20}H_{30}O_{11}Na$: 469.1686) $[M+Na]^+$.

*4.4.4.15 (E)-methyl 4-(2-butoxy-2-oxoethyl)-3-ethylidene-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**21**)*

According to the general method of deprotection, 36 mg (67% yield) of **21** were obtained, as a white solid, from 45.0 mg of **21a**.

$[\alpha]_D$ -52.8 (c 0.007, MeOH); ^{1}H NMR (D_2O) δ 0.83 (t, 3H, *J* = 7.5 Hz); 1.22 (m, 2H); 1.55 (m, 2H); 1.68 (d, 3H, *J* = 7.0 Hz); 2.47 (dd, 1H, *J* = 13.5 and 9.0 Hz); 2.70 (dd, 1H, *J* = 13.5

and 5.0 Hz); 3.38 (dd, 1H, *J* = 9.5 and 8.0 Hz); 3.38 (t, 1H, *J* = 9.5 Hz); 3.45 (ddd, 1H, *J* = 9.5, 6.0 and 2.0 Hz); 3.49 (t, 1H, *J* = 9.5 Hz); 3.67 (dd, 1H, *J* = 12.5 and 6.0 Hz); 3.69 (s, 3H); 3.86 (dd, 1H, *J* = 12.5 and 2.0 Hz); 3.96 (dd, 1H, *J* = 9.0 and 5.0 Hz); 3.98 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.08 (dt, 1H, *J* = 7.0 and 11.0 Hz); 4.87 (d, 1H, *J* = 8.0 Hz); 5.86 (s, 1H); 6.04 (q, 1H, *J* = 7.0 Hz); 7.53 (s, 1H); ^{13}C NMR (D_2O) δ 10.3, 13.7, 21.9, 26.0, 41.4, 52.3, 62.3, 66.2, 71.5, 74.2, 76.8, 81.5, 107.7, 108.2, 109.4, 124.8, 130.8, 155.3, 168.5, 173.1; HRMS (ESI+) *m/z* 483.1846 (calcd for $C_{21}H_{32}O_{11}Na$: 483.1842) $[M+Na]^+$.

*4.4.4.16 (E)-methyl 3-ethylidene-4-(2-(isopentyloxy)-2-oxoethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**22**)*

According to the general method of deprotection, 7.1 mg (17% yield) of **22** were obtained, as a white solid, from 37.0 mg of **21a**.

$[\alpha]_D$ -75 (c 0.007, MeOH); ^{1}H NMR (D_2O) δ 0.83 (m, 6H); 1.47 (m, 2H); 1.60 (m, 1H); 1.68 (d, 3H, *J* = 7.0 Hz); 2.50 (dd, 1H, *J* = 13.5 and 9.0 Hz); 2.69 (dd, 1H, *J* = 13.5 and 5.0 Hz); 3.38 (dd, 1H, *J* = 9.5 and 8.0 Hz); 3.38 (t, 1H, *J* = 9.5 Hz); 3.43 (ddd, 1H, *J* = 9.5, 6.0 and 2.0 Hz); 3.49 (t, 1H, *J* = 9.5 Hz); 3.67 (dd, 1H, *J* = 12.5 and 6.0 Hz); 3.69 (s, 3H); 3.86 (dd, 1H, *J* = 12.5 and 2.0 Hz); 3.96 (dd, 1H, *J* = 9.0 and 5.0 Hz); 4.00 (dt, 1H, *J* = 6.5 and 11.0 Hz); 4.11 (dt, 1H, *J* = 7.0 and 11.0 Hz); 4.88 (d, 1H, *J* = 8.0 Hz); 5.87 (s, 1H); 6.06 (q, 1H ,*J* = 7.0 Hz); 7.54 (s, 1H); ^{13}C NMR (D_2O) δ 12.7, 21.5, 21.6, 24.3, 30.3, 36.4, 39.9, 51.8, 60.6, 64.5, 69.4, 72.6, 75.6, 76.3, 94.8, 99.8, 108.3, 128.7, 125.2, 154.9. HRMS (ESI+) *m/z* 497.1964 (calcd for $C_{22}H_{34}O_{11}Na$: 497.1999) $[M+Na]^+$.

*4.4.4.17 (E)-methyl 3-ethylidene-4-(2-((3-methylbut-3-en-1-yl)oxy)-2-oxoethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**23**)*

According to the general method of deprotection, 7.3 mg (33% yield) of **22** were obtained, as a white solid, from 30.0 mg of **23a**.

$[\alpha]_D$ -76.6 (c 0.3, MeOH); ^{1}H NMR (D_2O) δ 1.68 (dd, 3H, *J* = 1.5 and 7.0 Hz); 1.70 (bs, 2H); 2.32 (m, 2H); 2.50 (dd, 1H, *J* = 13.5 and 9.0 Hz); 2.69 (dd, 1H, *J* = 13.5 and 5.0 Hz); 3.38 (dd, 1H, *J* = 9.5 and 8.0 Hz); 3.38 (t, 1H, *J* = 9.5 Hz); 3.45 (ddd, 1H, *J* = 9.5, 6.0 and 2.0 Hz); 3.49 (t, 1H, *J* = 9.5 Hz); 3.67 (dd, 1H, *J* = 12.5 and 6.0 Hz); 3.69 (s, 3H); 3.86 (dd, 1H, *J* = 12.5 and 2.0 Hz); 3.95 (dd, 1H, *J* = 9.0 and 5.0 Hz); 4.11 (dt, 1H, *J* = 6.5 Hz, and 11.0 Hz); 4.20 (dt, 1H, *J* = 7.0 and 11.0 Hz); 4.73 (bs, 1H); 4.81 (bs, 1H); 4.88 (d, 1H, *J* = 8.0 Hz); 5.86 (s, 1H); 6.06 (q, 1H. *J* = 7.0 Hz); 7.54 (s, 1H); ^{13}C NMR (D_2O) δ 12.7, 21.5, 21.6, 24.3, 30.3, 36.4, 39.9, 51.8, 60.6, 64.5, 69.4, 72.6, 75.6, 76.3, 94.8, 99.8, 108.3, 125.2, 128.7, 154.9. HRMS (ESI+) *m/z* 495.1843 (calcd for $C_{22}H_{32}O_{11}Na$: 495.1842) $[M+Na]^+$.

*4.4.4.18 (E)-methyl 3-ethylidene-4-(2-(hexadecyloxy)-2-oxoethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**24**)*

According to the general method of deprotection, 4.0 mg (28% yield) of **24**, were obtained, as a white solid, from 21.0 mg of **24a**.

$[\alpha]_D$ -790 (c 0.04, MeOH); ^{1}H NMR ($CDCl_3$) δ 7.45 (s, 1H); 6.03 (q, 1H, *J* = 7.0 Hz); 5.77 (s, 1H); 4.80 (d, 1H, *J* = 8.0 Hz); 4.01 (dt, 1H, *J* = 7.0 and 11.0 Hz); 3.93 (dd, 1H, *J* = 9.0 and 5.0 Hz); 3.89 (dt, 1H, *J* = 6.5 and 11.0 Hz); 3.83 (dd, 1H, *J* = 12.5 and 2.0 Hz); 3.72 (dd, 1H, *J* = 12.5 and 6.0 Hz); 3.69 (s, 3H); 3.58 (t, 1H, *J* = 9.5 Hz); 3.47 (t, 1H, *J* = 9.5 Hz); 3.47 (dd, 1H, *J* = 9.5 and 8.0 Hz); 3.40 (ddd, 1H, *J* = 9.5, 6.0 and 2.0 Hz); 2.75 (dd, 1H, *J* = 13.5 and 5.0 Hz); 2.35 (dd, 1H, *J* = 13.5 and 9.0 Hz); 1.68 (d, 3H, *J* = 7.0 Hz); 1.25 (s, 28H); 0.84 (t, 3H, *J* = 6.8 Hz); ^{13}C NMR ($CDCl_3$) δ 13.7, 14.1, 22.7, 25.8, 26.0, 28.9, 29.3, 29.6, 31.9, 41.0, 52.3, 62.2, 65.2, 71.6, 74.2, 76.8, 81.5, 107.7, 108.2, 109.4, 124.8, 130.8, 155.2, 168.5, 173.1; HRMS (ESI+) *m/z* 651.3691 (calcd for $C_{33}H_{56}O_{11}Na$: 651.3720) $[M+Na]^+$.

*4.4.4.19 (E)-methyl 4-(2-(2-cyclohexylethoxy)-2-oxoethyl)-3-ethylidene-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**25**)*

According to the general method of deprotection, 16.0 mg (42% yield) of **25**, were obtained, as a white solid, from 50.0 mg of **25a**.

$[\alpha]_D$ -790 (c 0.04, MeOH); ^{1}H NMR (D_2O) δ 0.93 (m, 2H); 1.28-1.13 (m, 4H); 1.35 (m, 1H); 1.68 (d, 3H, *J* = 7.0 Hz); 1.72 (m, 7H); 2.41 (dd, 1H, *J* = 13.5 and 5.0 Hz); 2.78 (dd, 1H, *J* = 13.5 and 9.0 Hz); 3.45 (ddd, 1H, *J* = 9.5, 6.0 and 2.0 Hz); 3.55 (dd, 1H, *J* = 9.5 and 8.0 Hz); 3.55 (t, 1H, *J* = 9.5 Hz); 3.58 (t, 1H, *J* = 9.5 Hz); 3.69 (s, 3H); 3.80 (dd, 1H, *J* = 12.5 and 6.0 Hz); 3.88 (dd, 1H, *J* = 12.5 and 2.0 Hz); 3.98 (dt, 1H, *J* = 6.5 and 11.0 Hz); 3.98 (dd, 1H, *J* = 9.0 and 5.0 Hz); 4.06 (dt, 1H, *J* = 7.0 and 11.0 Hz); 4.85 (d, 1H, *J* = 8.0 Hz); 5.80 (s, 1H); 6.08 (q, 1H, *J* = 7.0 Hz); 7.50 (s, 1H); ^{13}C NMR (D_2O) δ 13.4, 26.0, 26.3, 33.0, 33.1, 34.4, 35.7, 40.3, 51.4, 61.6, 63.1, 69.8, 73.1, 76.1, 94.7, 99.9, 108.2, 109.4, 124.2, 128.6, 153.6, 166.7, 171.7. HRMS (ESI+) *m/z* 537.2272 (calcd for $C_{25}H_{38}O_{11}Na$: 537.2312) $[M+Na]^+$.

*4.4.4.20 (E)-methyl 3-ethylidene-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**26**)*

According to the general method of deprotection, 23.0 mg (63% yield) of **26** were obtained, as a white solid, from 50.0 mg of **26a**.

$[\alpha]_D$ -29 (c 0.04, MeOH); ^{1}H NMR (D_2O) δ 1.66 (d, 3H, *J* = 7.0 Hz); 2.42 (dd, 1H, *J* = 13.5 and 9.5 Hz); 2.53 (dd, 1H, *J* = 13.5 and 5.0 Hz); 2.87 (bs, 1H); 3.35 (t, 1H, *J* = 9.5 Hz); 3.37 (dd, 1H, *J* = 9.5 and 8.0 Hz); 3.43 (ddd, 1H, *J* = 9.5, 6.0 and 2.0 Hz); 3.47 (t, 1H, *J* = 9.5 Hz); 3.67 (dd, 1H, *J* = 12.5 and 6.0 Hz); 3.68 (s, 3H); 3.91 (dd, 1H, *J* = 12.5 and 2.0 Hz); 3.95 (m, 1H); 4.85 (d, 1H, *J* = 8.0 Hz); 5.84 (s, 1H); 6.03 (q, 1H, *J* = 7.0 Hz); 7.51 (s, 1H); ^{13}C NMR (D_2O) δ 12.6, 30.3, 39.8, 51.8, 52.3, 52.6, 60.5, 69.3, 72.5, 75.6, 76.2, 94.7, 94.8, 99.5, 108.1,

124.9, 128.1, 154.6, 169.0, 174.6. HRMS (ESI+) *m/z* 465.1367 (calcd for $C_{20}H_{26}O_{11}Na$: 465.1373) $[M+Na]^+$.

*4.4.4.21 (E)-methyl 3-ethylidene-4-(2-(furan-2-ylmethoxy)-2-oxoethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**28**)*

According to the general method of deprotection, 18.0 mg (48% yield) of **28**, were obtained, as a white solid, from 50.0 mg of **28a**.

$[\alpha]_D$ -97.5 (c 0.0004, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.63 (d, 3H, *J* = 7.0 Hz); 2.58 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.70 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.39 (t, 1H, *J* = 9.5 Hz); 3.39 (dd, 1H, *J* = 8.0 and 9.5 Hz); 3.46 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.51 (t, 1H, *J* = 9.5 Hz); 3.69 (dd, 1H, *J* = 12.5 and 4.5 Hz); 3.70 (s, 3H); 3.87 (dd, 1H, *J* = 12.5 and 2.5 Hz); 3.95 (dd, 1H, *J* = 9.0 and 4.5 Hz); 4.84 (d, *J* = 8.0 Hz, 1H); 5.02 (d, 1H, *J* = 13 Hz); 5.12 (d, 1H, *J* = 13 Hz); 5.79 (s, 1H); 6.04 (q, 1H, *J* = 7.0 Hz); 6.45 (dd, 1H, *J* = 1.5 and 3.0 Hz); 6.51 (d, 1H, *J* = 3.0 Hz); 7.51 (s, 1H); 7.53 (d, 1H, *J* = 1.5 Hz); ^{13}C NMR (D_2O) δ 12.6, 30.2, 39.7, 51.8, 58.7, 60.6, 69.4, 72.5, 75.6, 76.3, 94.8, 99.5, 108.3, 110.8, 111.3, 125.1, 127.4, 143.9, 149.2, 154.4, 169.3, 173.4. HRMS (ESI+) *m/z* 507.1479 (calcd for $C_{22}H_{28}O_{12}Na$: 507.1478) $[M+Na]^+$.

*4.4.4.22 (E)-methyl 3-ethylidene-4-(2-oxo-2-(propylamino)ethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylate (**30**)*

According to the general method of deprotection, 17.0 mg (52% yield) of **30**, were obtained, as a yellow solid, from 45.0 mg of **30a**.

$[\alpha]_D$ -784 (c 0.065, MeOH); ^{1}H NMR (D_2O) δ 0.81 (t, 3H, *J* = 7.4 Hz); 1.40 (sex, 2H, *J* = 7.1 Hz); 1.64 (d, 3H, *J* = 6.5 Hz); 2.22 (dd, 1H, *J* = 13.5 and 6.0 Hz); 2.58 (dd, 1H, *J* = 13.5 and 2.2 Hz); 3.07 (m, 2H); 3.38-3.36 (2xt, 2H, *J* = 9.2 Hz); 3.45 (dd, 1H, *J* = 6.0 and 2.2 Hz); 3.49 (t, 1H, *J* = 9.2 Hz); 3.66 (dd, 1H, *J* = 12.5 and 6.0 Hz); 3.70 (s, 3H); 3.88 (dd, 1H, *J* = 12.4

and 2.2 Hz); 3.91 (ddd, 1H, *J* = 9.7, 6.0 and 2.2 Hz); 5.88 (s, 1H); 6.07 (q, 1H, *J* = 6.5 Hz); 7.53 (s, 1H); ^{13}C NMR (D_2O) δ 10.4, 12.7, 21.6, 30.8, 41.3, 42.2, 51.8, 60.7, 69.4, 72.6, 75.5, 76.4, 94.6, 99.5, 108.4, 124.9, 127.8, 154.3, 169.1, 173.1. HRMS (ESI+) m/z 468.1827 (calcd for $C_{20}H_{31}NO_{10}Na$: 468.1846) $[M+Na]^+$.

*4.4.4.23 (E)-3-ethylidene-4-(2-methoxy-2-oxoethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylic acid (**31**)*

According to the general method of deprotection, 7.6 mg (54% yield) of **31**, were obtained, as a white solid, from 193.0 mg of **31a**.

$[\alpha]_D$ -700 (c 0.03, MeOH); ^{1}H NMR (D_2O) δ 1.67 (d, 3H, *J* = 7.0 Hz); 2.42 (dd, 1H, *J* = 13.5 and 9.0 Hz); 2.73 (dd, 1H, *J* = 13.5 and 5.0 Hz); 3.38 (dd, 1H, *J* = 9.5 and 8.0 Hz); 3.39 (t, 1H, *J* = 9.5 Hz); 3.45 (ddd, 1H, *J* = 9.5, 6.0 and 2.0 Hz); 7. 49 (s, 1H); 6.03 (q, 1H, *J* = 7.0 Hz); 5.79 (s, 1H); 4.89 (d, 1H, *J* = 8.0 Hz); 3.94 (dd, 1H, *J* = 9.0 and 5.0 Hz); 3.87 (dd, 1H, *J* = 12.5 and 2.0 Hz); 3.68 (dd, 1H, *J* = 12.5 and 6.0 Hz); 3.62 (s, 3H); 3.50 (t, 1H, *J* = 9.5 Hz); ^{13}C NMR (D_2O) δ 12.7, 31.5, 40.1, 52.3, 69.5, 72.7, 75.7, 76.3, 94.4, 99.6, 113.7, 124.1, 129.6, 150.2, 173.9, 174.9. HRMS (ESI+) *m/z* 427.1302 (calcd for $C_{17}H_{24}O_{11}Na$: 427.1216) $[M+Na]^+$.

*4.4.4.24 (E)-3-ethylidene-4-(2-(hexadecyloxy)-2-oxoethyl)-2-(((2R,3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-3,4-dihydro-2H-pyran-5-carboxylic acid (**32**)*

According to the general method of deprotection, 3.0 mg (38% yield) of **32**, were obtained, as a white solid, from 10.0 mg of **32a**.

$[\alpha]_D$ -688 (c 0.03, MeOH), ^{1}HNMR (D_2O) δ 0.88 (m, 3H); 1.33 (m, 26H); 1.65 (m, 2H); 1.73 (d, 3H, *J* = 7.0 Hz); 2.46 (dd, 1H, *J* = 14.5 and 9.0 Hz); 2.79 (dd, 1H, *J* = 14.5 and 4.5 Hz); 3.42 (ddd, 1H, *J* = 9.5, 4.5 and 1.5 Hz); 3.49 (t, 1H, *J* = 9.5 Hz); 3.49 (dd, 1H, *J* = 12.5 and 4.5 Hz); 3.61 (t, 1H, *J*= 9.5 Hz); 3.73 (dd, 1H, *J*= 12.5 and 4.5 Hz); 3.84 (dd, 1H, *J*= 12.5 and

2.0 Hz); 4.01 (m, 1H); 4.08 (m, 2H); 4.82 (d, 1H, *J*= 8.0 Hz); 5.82 (s, 1H); 6.03 (q, 1H, *J*= 7.0 Hz); 7.64 (s, 1H); ^{13}CNMR (D_2O) δ 29.3, 29.6, 37.8, 41.5, 62.5, 65.2, 71.3, 74.2, 76.0, 81.4, 107.7, 107.8, 119.9, 124.7, 129.5, 154.0, 168.5, 173.1. HRMS (ESI+) *m/z* 637.3772 (calcd for $C_{32}H_{54}O_{11}Na$: 637.3564) $[M+Na]^+$.

4.5. Biological evaluation of the anticancer effect of oleuropein and its synthetic analogs

4.5.1. Cells and cell cultures

The human cancer cell lines FM3 (melanoma), HCT-116 (colon), HeLa (cervix), MCF-7 [breast; ER+, progesterone receptor (PR) +/-, HER2-], SKBR3 (breast; ER-, PR-, HER2+), HL-60 (promyelocytic leukemia) and K562 (chronic myelogenous leukemia), as well as the mouse cancer cell lines B16.F1 (melanoma), YAC-1 (lymphoma) and WEHI 164 (fibrosarcoma), were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Lonza Ltd, Switzerland), supplemented with 10% heat inactivated fetal bovine serum (Lonza), 10 mM Hepes (Lonza), 50 mM mercaptoethanol (Sigma-Aldrich Chemical Co., St Louis, MO, USA), 10^2 U/mL penicillin/streptomycin (Lonza) and 5 mg/mL gentamycin (Lonza) (referred to thereafter as culture medium), at 37°C in a humidified 5% CO_2 incubator. Cells were grown as monolayer and passaged by trypsinization every 2-3 days. PBMCs were isolated from heparinized venous blood of healthy blood donors after Ficoll-Hypaque (Lonza) gradient centrifugation, as previously described [31]. Prior to blood withdrawal, all donors gave their informed consent according to the regulations approved by the 2nd Peripheral Blood Transfusion Unit and Hemorophilic "Laikon" General Hospital Institutional Review Board, Athens, Greece. All the work has been carried out in accordance with Declaration of Helsinki for experiments involving humans.

4.5.2. In vitro cytotoxicity assay

The cytotoxic activity of the compounds was determined by the MTT dye reduction assay. Briefly, cancer cells were seeded into 96-well flat bottom microplates (Greiner Bio-One

GmbH, Germany; $5x10^3$cells/mL; 200 mL/well). Adherent cell lines were allowed to adhere overnight before adding the compounds, whereas cell lines growing in suspension were seeded the day of the experiment. Stock concentrations of the oleuropein-enriched leaf extract, oleuropein **1**, its synthetic analogs and of the chemotherapeutic drug doxorubicin (positive control) were made in DMSO (except oleoside which was diluted in dH_2O) at 1 mM and stored at -80°C. On the day of each experiment, stock solutions were serially diluted in culture medium (400-10 μg/mL for the leaf extract, 400-25 μM for oleuropein, 50-0.08 μM for the analogs and 10-0.001 μM for doxorubicin) and added to the microplates, which were further incubated at 37°C for 72 h. Four hours before the incubation expiry, 100 μL of MTT (Sigma) diluted in PBS(Lonza; 1 mg/mL) was added to each well. Optical density (OD) was determined at 545 nm with reference filter set at 690 nm, using a microplate reader (Denley WeScan, Finland). IC_{50} was calculated according to the formula: 100(A0-A)/A0=50, where A and A0 are ODs of exposed and control wells, respectively.

4.5.3. Flow cytometry analysis

Annexin V and PI staining was performed using the FITC Annexin V apoptosis detection kit with PI (Biolegend, Germany). PBMCs (10^6 cells/mL) were incubated in 6-well plates (Greiner) for 24 h at 37°C with oleuropein **1** (150-600 μg/μL), its analogs 24 and 25 (0.5-8 μg/mL), 25 μg/mL doxorubicin (positive control) or in plain culture medium (negative control). B16.F1 cells were incubated in 6-well plates for 24, 48 and 72 h at 37°C with concentrations corresponding to the IC_{50} values of the aforementioned compounds. For analysis of cell death, 10^6 cells were resuspended in 1 mL annexin V-binding buffer and 100 μL of each solution was stained with 5 μL annexin V-FITC and 10 μL PI. After 15 min of incubation at 25°C in the dark, 400 μL of annexin V-binding buffer was added to each tube and samples were analyzed in a FACSCanto II, using FACSDiva software (BD Biosciences,

Germany). Annexin V-/PI- cells were defined as viable, annexin V+/PI- as apoptotic, and PI+ as necrotic.

4.5.4. In vivo melanoma mouse model

Male C57BL/6 mice (6-8 week old) were purchased from the Jackson Laboratory (Bar Harbor, USA) and housed in a specific pathogen-free, temperature- and humidity-regulated unit (21±2°C; 55±10%, respectively), with a 12/12 h light/dark cycle. Mice were housed in filter top cages (IVC) (1284 L, H-Temp™, Techniplast, Varese, Italy), at a stocking density of 4–5 mice per cage [overall dimensions of caging (L×W×H): 365×207×140 mm, floor area=530 cm^2]. Cages were kept in the same animal room with air supply, 15 ACH and a light intensity of 300 lux measured one meter above the floor in the middle of the room. All mice had *ad libitum* access to filtered tap water in drinking bottles and a vacuum-packed pelleted rodent chow that contained 18,5% protein, 5,5% fat, 4,5% fiber, 6% ash (4RF22, Mucedola, Milan, Italy). The bedding in each cage comprised of ~250 grams of autoclaved corncob bedding (Rehofix MK 2000, J. Rettenmaier & So, Rosenberg, Germany). The cages were cleaned and autoclaved once a week. Animal handling and experimentation were in accordance with Presidential Decree 56/2013 and the Directive 2010/63/EU of the Council of Europe on Animal Welfare and were approved by the Ethics and Biosafety Committee, Subcommittee on Ethics, Faculty of Biology, National and Kapodistrian University of Athens. For generating sc tumors, a suspension of 10^5 viable B16.F1 cells in 200 μL PBS was injected in the right flank of each mouse on day 0. When tumors became palpable (day 11), animals were randomly assigned in 6 experimental groups (5 animals/group) and ip received: PBS (control group; 200 μL), oleuropein **1** (300 and 600 μg/dose/mouse diluted in 200 μL PBS) or analog **24** (2, 4 and 6 μg/dose/mouse diluted in 200 μL PBS) every other day (total: 8 doses) up to day 29 post-tumor inoculation. Tumor growth was monitored every two days, measured

by a digital caliper and tumor volume (cm^3) was calculated according to the formula: 1/2 (length × $width^2$).

4.5.5. Ex vivo cytotoxicity assessment

Spleens were aseptically removed from mice and splenocytes were isolated after extensive washing and red blood cell lysis, as previously described [31]. Cell cytotoxicity was evaluated based on the detection of CD107a and CD107b exposed on cell surface, as a result of effector cell degranulation [35]. Splenocytes (10^5 cells/well; 200 μL/well) were co-cultured in 96-well U bottom microplates (Greiner) with the target cells B16.F1 (syngeneic cells), YAC-1 (sensitive to lysis by NK cells) and WEHI 164 (sensitive to lysis by LAK cells), at an effector to target (E:T) ratio of 100:1, at 37°C in 5% CO_2. FITC-conjugated anti-CD107a and anti-CD107b monoclonal antibodies (25 μL/mL) and monensin (6 μL/mL; all from BD Biosciences) were added in each well. Monensin (Golgi-Stop) was used to prevent acidification of endocytic vesicles, to avoid degradation of reinternalized CD107 proteins from the cell surface, thus allowing for their labeling and visualization. Cells were harvested 6 h later and MFI of CD107+ cells was determined using a FACSCanto II flow cytometer.

4.5.6. Statistical analysis

The results were expressed as means ± SD. GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) was used for data analysis. Differences among groups of mice were corrected with Bonferroni's post-test. $P<0.05$ was considered significant.

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New semi-synthetic analogs of oleuropein show improved anticancer activity *in vitro* and *in vivo*

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HIGHLIGHTS

- New semi-synthetic derivatives of oleuropein have been synthesized
- Extensive SAR study has been performed to identify the structural determinants to display cytotoxicity
- One analogue displayed cytotoxic activity at the micromolar levels on several cancer cell lines
- The identified analogue is non-toxic and active in vivo
- The active compound stimulates the immune system of the mouse to fight against the oncogenic cells.