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Short communication

Diesters of glycosylglycerols active in cancer chemoprevention

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Abstract – Enzymatic transesterification, mediated by *Pseudomonas cepacia* lipase (lipase PS), led to the pure 1,6′-diacylderivatives of 2-*O*-β-D-glucosyl-*sn*-glycerol and 2-*O*-β-D-galactosyl-*sn*-glycerol, the acyl chains being derived from short-medium length fatty acids. A study of the in vitro inhibitory effects of these diacylderivatives on Epstein–Barr virus early antigen activation induced by the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate revealed that maximum activity was reached for the hexanoyl chain and that the introduction of a second acyl chain did not significantly modify the inhibitory potential referring to the corresponding 1- or 6′-monoesters. © 2001 Éditions scientifiques et médicales Elsevier SAS

glycoglycerolipids / cancer chemoprevention / lipase / Epstein–Barr virus early antigen

1. Introduction

It was recently shown that glycoglycerolipid analogues have a promising inhibitory effect on Epstein–Barr virus early antigen (EBV-EA) activation induced by the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [1–3]. The mechanism of glycoglycerolipid inhibitory effect on tumour promoting activity is still not known, but it could be that inhibitors interfere with the TPA-induced promotional events, interacting with the receptor of tumour promoters, the protein kinase C [4, 5]. Thus, we focused our studies on in vitro and in vivo [6] structure–activity relationships in an effort to gain some insight into the action mechanism. We have shown, in particular, that synthetic analogues of both the glucose and the galactose series, that bear a short-medium length fatty acid acyl chain at the 1- or 6′-position of the glycosylglycerol skeleton, shorter than chains present in natural compounds, exhibit more pronounced activity [3, 7, 8].

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Two fatty acid acyl chains are often present in natural glycoglycerolipids [9] and this prompted us to ascertain whether the introduction of a second acyl chain into the analogues would further enhance their tumour inhibitory effect. Moreover, also the carbohydrate moiety seems to play a role in improving inhibitory activity: in fact, galactosylglycerols are often more potent than the corresponding glucosylglycerols with the same structural features [1, 7].

Thus, the enzymatic synthesis of short-medium length fatty acid diesters (from four to ten carbon atoms) of 2-*O*-β-D-glucosyl-*sn*-glycerol (**1**) and of 2-*O*-β-D-galactosyl-*sn*-glycerol (**2**), together with their in vitro inhibitory effects, is reported (*figure 1*).

2. Chemistry

It is well known that lipases are flexible biocatalysts for the acylation or deacylation of a wide range of substrates. In the case of polyhydroxylated compounds the primary hydroxyl functions are selectively involved [10]. Our studies on glycosylglycerols [11–13] have shown the strong regio- and diastereoselectivity of such enzymes towards these compounds.

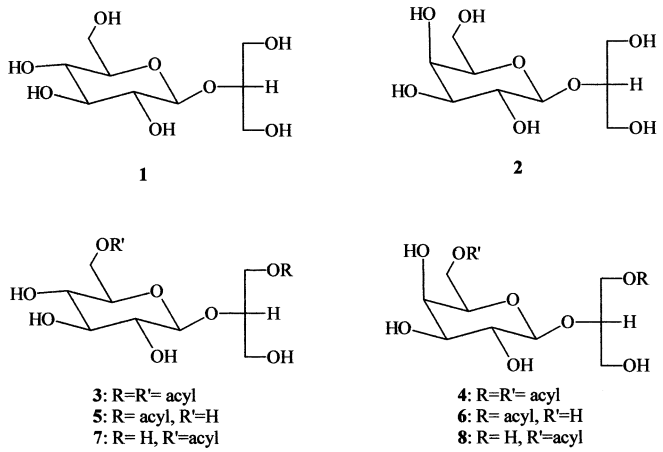

Figure 1. Structures of the tested glycosylglycerols.

By employing the selectivity of lipase PS in the acylation of 2-*O*-β-D-galactosyl-*sn*-glycerol (**2**), we recently succeeded [13] in obtaining, together with a fraction of monoacylated compounds, significant amounts of a diester fraction that was composed of a single isomer, the 1,6′-diester of 2-*O*-β-D-galactosyl-*sn*-glycerol.

After optimising the reaction conditions, we could obtain in the enzymatic transesterification only the products of double acylation at the glycerol and sugar moieties, namely the 1,6′-diacylderivatives, in very good yield for both 2-*O*-β-D-glucosyl-*sn*-glycerol (**1**) and 2-*O*-β-D-galactosyl-*sn*-glycerol (**2**). The enzymatic reactions were performed using lipase PS supported on celite as the enzyme, pyridine as the solvent and 2,2,2-trifluoroethyl *n*-alkanoates (butanoate, hexanoate, octanoate and decanoate) as acyl carriers, but with a higher acyl carrier/substrate ratio and longer reaction times than previously described [13].

The method is especially advantageous as a series of short-medium length diacyl compounds can be obtained directly in a single step, contrary to the complexity of the time consuming multistep chemical procedure.

3. Pharmacology

Compounds **3a**–**d** and **4a**–**d** were tested for their anti-tumour-promoting activities using a short-term in vitro assay for Epstein–Barr virus activation in Raji cells induced by TPA [2]. For each compound the assays were performed in triplicate. None of the samples exhibited significant toxicity against Raji cells and their in vitro tumour inhibitory activity is shown in *table I*, in comparison with that previously described for compounds **5a**–**d**, **7a**–**d** (gluco series) [3, 8] and **6a**–**d**, **8a**–**d** (galacto series) [7]. *Figure 2* shows the diester activity in a graphical mode, reporting the percentage to positive control at different inhibitor concentrations versus acyl chain length; *figure 3* shows the data referred to the glucose series for both the 1,6′-diesters **3a**–**d** and the 1- and 6′-monoesters **5a**–**d** and **7a**–**d**; *figure 4* shows the same data referred to the galactose series. The graphic figures facilitate the comparison of the results.

4. Results and discussion

The data reported above indicate that, along with the already studied compounds, the glycoglycerolipid analogues **3a**–**d** and **4a**–**d** also exhibit significant activity. Thus, the previous findings can also be applied to the 1,6′-diesters, given that also in this case the strongest activity is related to the hexanoyl chains and is comparable to that of the most active monoesters [7]; moreover, the esters of the galactose series are a bit more potent than the corresponding glucose analogues (see *figure 2*). It was found that the introduction of a second acyl chain does not significantly modify the inhibitory potential of the corresponding 1- and 6′-monoesters, as evidenced by the data reported in *figures 3 and 4*; in conclusion, the two acyl chains appear unable to reinforce each other in their roles. However, the easy chemoenzymatic preparation and the high yields obtained in such glycosylglycerol diesters, together with their good inhibitory effect against EBV-EA activation, make these compounds ideal candidates for future use in cancer prevention strategies.

5. Experimental protocols

5.1. Chemistry

5.1.1. Materials

Pseudomonas cepacia lipase (lipase PS, specific activity 30.5 triacetin units mg^{-1} solid), a generous gift from Amano Pharmaceutical Co. (Mitsubishi Italia), was supported on celite [11] and kept overnight, under vacuum,

Table I. Inhibitory effects of **3a–d**, **4a–d**, **5a–d**, **6a–d**, **7a–d** and **8a–d**, on TPA-induced EBV-EA activation.

Compound	Concentration (mol ratio/TPA)				
	1000		500	100	10
	Percentage to positive control ± SE ($n = 3$) (% viability) [a]				
3a	0 ± 0.1 [b]	(70) [c]	24.0 ± 0.4	46.5 ± 1.3	83.0 ± 1.1
3b	0 ± 0	(70)	15.1 ± 0.5	34.6 ± 1.2	71.5 ± 1.9
3c	0 ± 0	(70)	19.2 ± 0.5	57.3 ± 1.6	90.1 ± 1.6
3d	0 ± 0	(70)	21.6 ± 1.1	58.7 ± 2.0	91.6 ± 1.6
4a	0 ± 0	(70)	21.1 ± 0.4	47.6 ± 1.2	80.5 ± 1.3
4b	0 ± 0	(70)	12.4 ± 0.2	32.3 ± 1.1	69.5 ± 1.8
4c	0 ± 0	(70)	15.2 ± 0.6	50.3 ± 1.3	83.2 ± 1.6
4d	0 ± 0	(70)	20.0 ± 1.3	56.7 ± 2.1	90.2 ± 0.9
5a [d]	0 ± 0	(60)	15.7 ± 0.4	37.2 ± 1.5	79.4 ± 2.0
5b [d]	0 ± 0	(70)	10.6 ± 0.6	30.9 ± 1.8	68.2 ± 2.5
5c [d]	0 ± 0	(70)	16.9 ± 0.7	55.0 ± 1.9	89.2 ± 2.2
5d [d]	0 ± 0	(60)	18.4 ± 1.0	56.5 ± 2.5	88.2 ± 1.9
6a [e]	0 ± 0	(70)	22.4 ± 0.4	45.1 ± 1.1	80.2 ± 1.0
6b [e]	0 ± 0	(70)	11.4 ± 0.3	32.1 ± 0.9	63.4 ± 1.3
6c [e]	0 ± 0	(70)	13.2 ± 0.6	48.5 ± 1.3	86.2 ± 0.9
6d [e]	0 ± 0	(70)	20.3 ± 0.5	55.9 ± 1.0	90.3 ± 0.5
7a [f]	0 ± 0	(70)	27.9 ± 0.8	51.3 ± 1.3	88.4 ± 1.0
7b [f]	0 ± 0	(70)	19.3 ± 0.3	37.6 ± 1.5	78.1 ± 1.8
7c [f]	0 ± 0	(70)	21.1 ± 0.4	57.2 ± 1.4	90.6 ± 1.1
7d [f]	0 ± 0	(70)	27.3 ± 0.6	62.8 ± 2.3	92.1 ± 1.2
8a [e]	0 ± 0	(70)	20.4 ± 0.8	43.6 ± 1.0	78.2 ± 1.1
8b [e]	0 ± 0	(70)	10.7 ± 0.1	30.1 ± 0.9	67.8 ± 1.2
8c [e]	0 ± 0	(70)	12.2 ± 0.7	48.3 ± 1.1	82.6 ± 0.8
8d [e]	0 ± 0	(70)	18.1 ± 1.1	54.4 ± 1.9	88.2 ± 1.1

[a] TPA 32 pmol, 100%.
[b] Values represent relative percentages to the positive control value (at least 500 cells were counted).
[c] Values in parentheses are viability percentages of Raji cells.
[d] See Ref. [3].
[e] See Ref. [7].
[f] See Ref. [8].

prior to use. Pyridine was distilled from calcium hydride.

The acyl carriers 2,2,2-trifluoroethyl-*n*-alkanoates (butanoate, hexanoate, octanoate and decanoate) were synthesised from the corresponding acyl chloride and 2,2,2-trifluoroethanol [12].

2-*O*-β-D-Glucosyl-*sn*-glycerol (**1**) and 2-*O*-β-D-galactosyl-*sn*-glycerol (**2**) were synthesised according to literature procedures [14, 15].

Evaporation under reduced pressure was always effected with a bath temperature below 40 °C. All the new compounds were characterised by ^{1}H-NMR analysis at 500 MHz and chemical ionisation mass spectrometry (CIMS) [12]. The elemental analyses were consistent with the theoretical ones. Optical rotations were determined in a Perkin–Elmer 241 polarimeter in chloroform solutions ($c = 1.0$) in a 1 dm cell at 20 °C. Melting points were recorded in a Büchi 510 capillary melting point apparatus and are uncorrected. Analyses of the new compounds, indicated by the symbols of the elements, were within ±0.4% of the theoretical values.

*5.1.2. General procedure for the enzymatic synthesis of 1,6′-diesters **3a–d***

2-*O*-β-D-Glucosyl-*sn*-glycerol (**1**) (0.50 g, 2 mmol) was dissolved in pyridine (10 mL) and the appropriate trifluoroethyl ester (10 mmol) and lipase PS (2.50 g) were added in the order. The mixture was stirred for 20 h at 45 °C and the reaction was stopped by filtering off the enzyme and washing with pyridine. The solvent was removed under vacuum and flash chromatography (dichloromethane–methanol from 10:1 to 8:2, v/v) of the crude product yielded pure 1,6′-diesters **3a–d**.

*5.1.2.1. 1-O-Butanoyl-2-O-(6-O-butanoyl-β-D-gluco-pyranosyl)-sn-glycerol (**3a**)*

Yield 81%; oil; $[\alpha]_D$: −21.0. MS; *m*/*z*: 412 $[M+NH_4]^+$. ^{1}H-NMR selected signals (pyridine-d_5): δ 5.02 (d, 1H, $J_{1',2'} = 8.0$ Hz, H-1′), 4.95 (dd, 1H, $J_{6'a,6'b} = 12.0$ Hz, $J_{5',6'a} = 1.0$ Hz, H-6′a), 4.70 (dd, 1H, $J_{5',6'b} = 5.5$ Hz, H-6′b), 4.68–4.63 (m, 2H, H_2-1). Anal. $C_{17}H_{30}O_{10}$ (C, H, O).

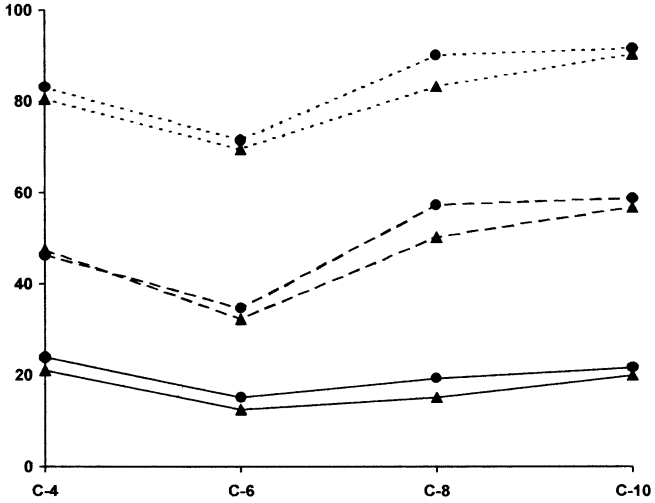

Figure 2. Inhibitory effects of **3a**–**d** (●) and **4a**–**d** (▲) on TPA-induced EBV-EA (Epstein–Barr virus early antigen) activation: percentage to positive control at different inhibitor concentrations (- - - -, 10 mol ratio/TPA; – – –, 100 mol ratio/TPA; —, 500 mol ratio/TPA) versus the acyl chain length.

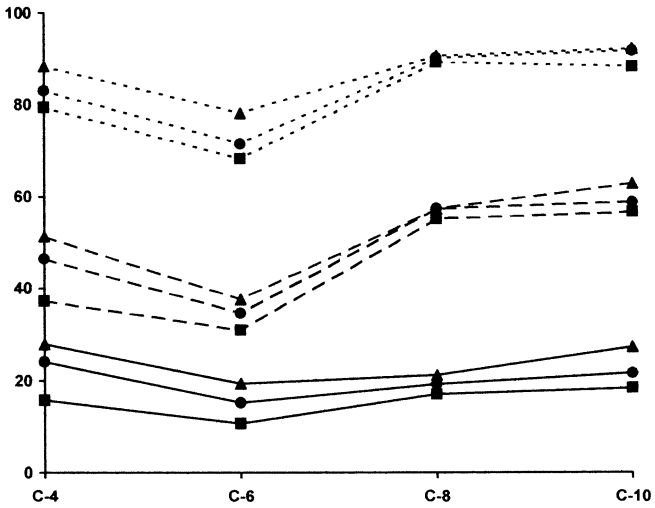

Figure 3. Inhibitory effects of **3a**–**d** (●), **5a**–**d** (■) and **7a**–**d** (▲) on TPA-induced EBV-EA (Epstein–Barr virus early antigen) activation: percentage to positive control at different inhibitor concentrations (- - - -, 10 mol ratio/TPA; – – –, 100 mol ratio/TPA; —, 500 mol ratio/TPA) versus the acyl chain length.

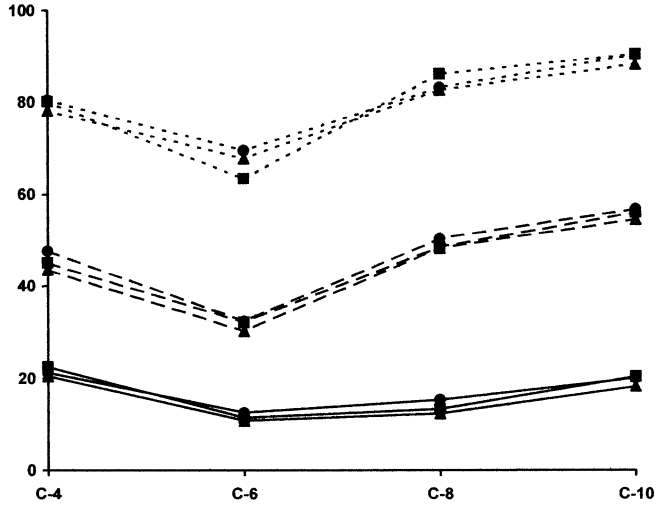

Figure 4. Inhibitory effects of **4a**–**d** (●), **6a**–**d** (■) and **8a**–**d** (▲) on TPA-induced EBV-EA (Epstein–Barr virus early antigen) activation: percentage to positive control at different inhibitor concentrations (- - - -, 10 mol ratio/TPA; – – –, 100 mol ratio/TPA; —, 500 mol ratio/TPA) versus the acyl chain length.

*5.1.2.2. 1-O-Hexanoyl-2-O-(6-O-hexanoyl-β-D-gluco-pyranosyl)-sn-glycerol (**3b**)*

Yield 85%; m.p.: 46–48 °C (diisopropyl ether); $[\alpha]_D$: −21.3. MS; *m*/*z*: 468 $[M+NH_4]^+$. ^{1}H-NMR selected signals (pyridine-d_5): δ 5.03 (d, 1H, $J_{1',2'} = 8.0$ Hz, H-1′), 4.98 (dd, 1H, $J_{6'a,6'b} = 12.0$ Hz, $J_{5',6'a} = 1.0$ Hz, H-6′a), 4.72 (dd, 1H, $J_{5',6'b} = 5.5$ Hz, H-6′b), 4.70–4.66 (m, 2H, H_2-1). Anal. $C_{21}H_{38}O_{10}$ (C, H, O).

*5.1.2.3. 1-O-Octanoyl-2-O-(6-O-octanoyl-β-D-gluco-pyranosyl)-sn-glycerol (**3c**)*

Yield 83%; m.p.: 70–72 °C (ethyl acetate); $[\alpha]_D$: −20.0. MS; *m*/*z*: 524 $[M+NH_4]^+$. ^{1}H-NMR selected signals (pyridine-d_5): δ 5.05 (d, 1H, $J_{1',2'} = 8.0$ Hz, H-1′), 4.99 (dd, 1H, $J_{6'a,6'b} = 12.0$ Hz, $J_{5',6'a} = 1.0$ Hz, H-6′a), 4.73 (dd, 1H, $J_{5',6'b} = 5.5$ Hz, H-6′b), 4.71–4.66 (m, 2H, H_2-1). Anal. $C_{25}H_{46}O_{10}$ (C, H, O).

*5.1.2.4. 1-O-Decanoyl-2-O-(6-O-decanoyl-β-D-gluco-pyranosyl)-sn-glycerol (**3d**)*

Yield 88%; m.p.: 78–80 °C (ethyl acetate); $[\alpha]_D$: −19.0. MS; *m*/*z*: 580 $[M+NH_4]^+$. ^{1}H-NMR selected sig-

nals (pyridine-d_5): δ 5.05 (d, 1H, $J_{1',2'} = 8.0$ Hz, H-1′), 4.99 (dd, 1H, $J_{6'a,6'b} = 12.0$ Hz, $J_{5',6'a} = 1.0$ Hz, H-6′a), 4.73 (dd, 1H, $J_{5',6'b} = 5.5$ Hz, H-6′b), 4.71–4.66 (m, 2H, H_2-1). Anal. $C_{29}H_{54}O_{10}$ (C, H, O).

*5.1.3. Configuration assignment at C-2 for **3a–d***

1-*O*-Butanoyl-2-*O*-β-D-glucosyl-*sn*-glycerol [11] (0.14 mmol) was dissolved in pyridine (1.0 mL), 2,2,2-trifluoroethylbutanoate (0.123 g, 0.70 mmol) and lipase PS (0.225 g) were added in the order and the mixture was stirred at 45 °C for 8 h. The reaction, worked-up as described above for the synthesis of **3a–d**, gave, in 70% yield, a compound identical to the 1,6′-dibutanoate **3a** obtained by the direct enzymatic procedure from **1**. Starting from 1-*O*-hexanoyl, -octanoyl, and -decanoyl-2-*O*-β-D-glucosyl-*sn*-glycerol [3, 12] the same procedure resulted in the l,6′-diesters **3b–d**.

*5.1.4. General procedure for the enzymatic synthesis of the 1,6′-diesters **4a–d***

Applying the procedure described for the preparation of the 1,6′-diesters **3a–d** to 2-*O*-β-D-galactosyl-*sn*-glycerol (**2**) (0.50 g, 2 mmol) leads, in 10 h, to an 80–88% yield of the pure 1,6′-diesters **4a–d** [13]. Their physicochemical properties, ^{1}H-NMR and mass spectra were consistent with literature data [13].

5.2. Pharmacology

5.2.1. Short-term in vitro bioassay for anti-tumour promoters

Epstein–Barr Virus (EBV) is known to be activated by tumour promoters to produce viral early antigens (EA), and an evaluation of its inhibition is often used as a primary screening for in vitro anti-tumour-promoting activities [16]. The inhibitory effect of compounds **3a–d** and **4a–d** was assayed using a short-term in vitro assay for EBV-EA activation in Raji cells induced by the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), as described in Refs. [17, 18]. The assays for each compound were performed in triplicate. No sample exhibited significant toxicity against Raji cells. The viability of the cells was assayed against treated cells using the Trypan blue staining method. The results are reported in *table I*.

Acknowledgements

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