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# Biocatalysed synthesis of β-*O*-glucosides from 9-fluorenon-2-carbohydroxyesters. Part 3: IFN-inducing and anti-HSV-2 properties

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**Abstract**—In pursuing research on the antiviral, interferon (IFN)-inducing tilorone congeners, a new series of fluoren-carboxyhydroxyesters has been prepared and biologically explored. These esters have subsequently been used as sugar acceptors in the enzymatic transglycosylation reaction using the 'retaining' β-glycosidase from the archaeon *Sulfolobus solfataricus* (Ssβ-Gly).

Both aglycones (**1**–**6**) and corresponding β-glucosides (**β-glu 1**–**β-glu 6**) have been screened for cytotoxicity, interferon-stimulating and antiviral properties against HSV-2.

It was found that the addition of compounds **β-glu 5**, **β-glu 6** and **β-glu 4** to HSV-2 infected U937 cells downregulates viral replication and triggers cells to release IFN-α/β. Taken together, the results showed improved pharmacological profiles as a consequence of glycosylation. A molecular modelling study carried out on this series of compounds completed the structural characterisation of the novel compounds.
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## 1. Introduction

Worldwide, 60–95% of the population is infected by one or more viruses of the herpesviridae family. In immunocompromised patients, transplant recipient and individuals with AIDS, HSV-2 genital viruses may reactivate from their latent state and cause severe recurrent and chronic infections.[1] In addition, the intensive use of antivirals (acyclovir and its congeners) has led to the emergence of resistant strains. On the other hand, these agents have limited oral bioavailability and severe side effects.[2–4]

It is well known that endogenous interferons (IFNs) are a large family of multifunctional secreted glycoproteins (cytokines) involved in antiviral defence, cell growth regulation and immune activation.[5,6] A key mediator of innate antiviral immunity to virus infection is the alpha/beta interferon (IFN-α/β) response. Many subtypes of INF-α and IFN-β are released from infected cells and bind to a single IFN-α/β receptor. Receptor-mediated signal transduction stimulates the release of many products that interfere with viral replication.[7]

At molecular level, the IFN production by the cell can be caused by a series of interferences, among them the DNA intercalation; as a result, arranging themselves among adjacent pairs of bases, the planar aromatic

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and heteroaromatic agents alter the double helix of the DNA and consequently disturb the physiological functions of many important DNA enzymes.

Therefore, the enhancement of the release of endogenous IFN by oral administration of low-molecular weight compounds has been ardently desired.[8] Several synthetic compounds possessing IFN-inducing activity have been reported in the search for novel agents that, due to intercalation, could induce IFN production that, in turn, might stimulate antiviral and/or cytostatic effects in the cell.[9,10] Among them Tilorone, (2,7-bis[2-(diethylamino)ethoxy]-9*H*-fluoren-9-one), was the first low-molecular weight IFN-inducer orally effective in vivo against some DNA and RNA viruses.[11,12]

All these considerations prompted us to design the title agents by applying the strategy of molecular hybridation between the antiviral tilorone[13,14] and the antitumoral daunomycin,[15] two well-known DNA-intercalators.

After deeply investigating the intercalating properties, it was found that the 9-fluorenone ring prefers the minor groove of DNA, in particular adenine-timine site with 9C=O placed outwards of the complex tilorone/DNA. The opposite orientation proved to be significantly less stable. The formation of the complex occurs by electrostatic interactions between the phosphate residues and positively charged nitrogens of side chains of tilorone.[16] The altered DNA determines the IFN-inducing effect.

Instead, the four-membered ring system of anthracyclines intercalates into DNA entering from the major groove. The sugar moiety of the drug is ion-bonded to the phosphate backbone of DNA through its amino group.[17]

In applying this molecular hybridisation strategy, we decided to maintain the beneficial moieties of parent drugs, whereas those reported as responsible for side effects were ruled out. In fact the mucopolysaccharidosis, the main side effect of tilorone, is apparently due to symmetrical basic chains,[18–20] while the cardiotoxicity of anthracyclines is caused by the reduction of the tetracyclic moiety producing noxious radical species.[21]

These parts are absent in the novel fluorenic compounds.

The presence of a carbohydrate residue in the same β-configuration as in daunomycin might improve the intercalation mode, stabilising the DNA/compound complexes.[15] In addition, as in even more numerous classes of drugs, the insertion of sugar moieties might also improve solubility and pharmacokinetic properties of the potential novel agents.[22]

The first 9-fluoren-β-*O*-glycosides we have reported were obtained by chemical methods from unsubstituted 9-fluorenol and several sugars such as D- and L-arabinopyranose, D-glucopyranose.[23] As expected, these compounds displayed IFN-inducing and antiviral properties against HSV-1 and -2 viruses; their cytotoxicity was within 2% except for the L-arabinoside derivative which, in turn, possessed moderate antiproliferative activity on breast tumor cell lines.

Given the information about tilorone mode of intercalation, such preliminary and promising results could be further improved by functionalising the 9-fluorene moiety with a side chain potentially able to improve DNA-intercalation.

Thus, the aglycone ring was differently functionalised in 2 or 4 positions by introducing chains containing basic heteroatoms and/or hydroxyester groups. The first series of these aglycones, the 9-fluorenon-4-carboxyamides, recently published, proved to be endowed with interesting anti-HSV-2 activity, IFN-α, IFN-γ and TNF-α inducing properties.[24]

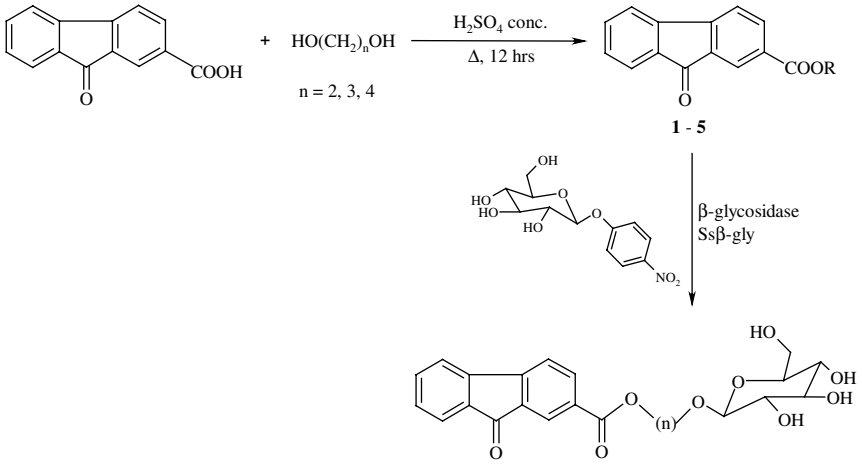
The enzymatic synthesis of carbohydrate-containing compounds is based upon the use of two enzymes: glycoside hydrolase and glycosyltransferases. The ability of Ssβ-gly, a β-glycosidase isolated from the archeon *Sulfolobus solfataricus*, in the formation of glycosidic bonds has been employed to prepare different glycosides (β-D-glucosides, β-D-galactosides and β-D-fucosides) using transglycosylation reactions with aryl donors and different acceptors. Various preparations of Ssβ-gly, such as the crude homogenate of *S. solfataricus* cells,[25] the immobilised whole microorganism or lyophilised cells and the homogeneous enzyme purified from the archaeon or in recombinant form from *E. coli*,[26] were used as catalysts for the kinetically controlled glycosylation of various aglycones.

Now, we report on the synthesis and the pharmacological evaluation of a series of fluoren-carboxyhydroxyesters (**1**–**6**) together with their corresponding β-glucosides (**β-glu 1**–**β-glu 6**). To obtain these latter, we have exploited transglycosylation reactions catalysed by the **β**-glycosidase extracted from archeon Sulfolobus sulfataricus (Ssβ-gly). Such enzymatic procedures have the advantage of warranting absolute β-stereocontrol in the products, thus improving the β-anomer yields that, by chemical methods, had been very low.

## 2. Results

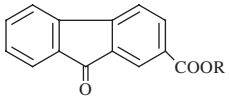
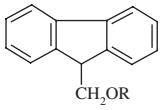
### 2.1. Synthesis of 9-oxo-9*H*-fluoren-2-hydroxyesters and fluoren-β-*O*-glucosides

The hydroxyesters **1**–**5** have been prepared in good yields by the classical Fischer esterification between 9-fluorenone-2-carboxylic acid and the appropriate diols, used in excess to avoid double esterification. The 9-oxo-9*H*-fluoren-2-carboxyesters **1**–**5** as well as the alcohol **6** were used as sugar acceptors in the transglycosylation reactions. The transglycosylation procedure was carried out using commercial *p*-nitrophenyl β-D-glucopyranoside as donor (Scheme 1).




**Scheme 1.**

**Table 1.** Structural formulas of aglycones **1**–**6** and β-glucosides (**β-glu 1**–**β-glu 6**)

| | Comp. | R | Comp. | R |
|---|---|---|---|---|
|  | **1** | $(CH_2)_2OH$ | **β-glu 1** | $(CH_2)_2$-*O*-β-Glc |
| | **2** | $(CH_2)_3OH$ | **β-glu 2** | $(CH_2)_3$-*O*-β-Glc |
| | **3** | $CH_2CHOHCH_3$ | **β-glu 3** | $CH_2CH$(-*O*-β-Glc) $CH_3$ |
|  | **4** | $(CH_2)_4OH$ | **β-glu 4** | $(CH_2)_4$-*O*-β-Glc |
| | **5** | $(CH_2)_2O(CH_2)_2OH$ | **β-glu 5** | $(CH_2)_2O(CH_2)_2$-*O*-β-Glc |
| | **6** | — | **β-glu 6** | β-Glc |

The retaining mechanism of the Ssβ-gly allowed us to obtain homochiral β-glucosides in moderate yields (15–25%)[26,27] (Table 1).

Analogously, β-9-fluorenemethyl glucoside (**β-glu 6**) from commercial 9-fluorenemethanol[28] was prepared with 15% yield.

The structure of the homochiral β-glycosides has been attributed by $^1$H NMR.

It is interesting to note that although the yields are not high, fluorene derivatives with three aromatic rings can access the active site of the enzyme forming single β-anomers of each product. Obviously, the anomeric purity of the products is an advantage in correct assignment of biological activity to a single structure.

## 3. Discussion

All fluorenic derivatives were submitted to a pharmacological screening to evaluate their cytotoxicity as well as the IFN-inducent properties and the antiviral activity on Herpes simplex type 2. The results of cytotoxicity on WISH cells are collected in Table 2 and those regarding the IFN-α/β induction on human monocytic cells U937 in Table 3. The inhibition of HSV-2 replication on U937 cells is shown in Table 4. Two concentrations of compounds (10 and 100 μg/mL) have been used for all the experiments.

**Table 2.** Percentage (%) of cytotoxicity (100 μg/mL) on WISH cells

| Compound | % |
|---|---|
| **1** | 19 |
| **2** | 8 |
| **3** | 24 |
| **4** | 10 |
| **5** | 9 |
| **β-glu 1** | 0 |
| **β-glu 2** | 4 |
| **β-glu 3** | 2 |
| **β-glu 4** | 0 |
| **β-glu 5** | 0 |
| **β-glu 6** | 0 |

(a) *Cytotoxicity assays*: In Table 2 are reported the cytotoxicity data expressed as percentage of stained (dead) cells. These results demonstrate that compounds displayed different percentage of cytotoxicity. In particular, aglycones **1**, **2**, **3**, **4** and **5** at a concentration of 100 μg/mL displayed a clear cytotoxicity, that however was absent at a concentration of 10 μg/mL. The compound **6** has not been evaluated since it is insoluble in aqueous system. **β-glu 1**–**6**, at the concentrations tested, did not reveal significant cytotoxic effect. On the basis of these results, in the further experiments we tested the highest noncytotoxic concentration for each compound.

(b) *Immunomodulatory activity*: Supernatants harvested from U937 cells, pre-treated with different compounds and then infected with HSV-2 were analysed for the

**Table 3.** IFN-α/β inducent activity on U937 cells infected with HSV-2

| Compound | IFN induction (U.I./mL) | |
|---|---|---|
| | 10 μg/mL | 100 μg/mL |
| **1** | 2 | n.d. |
| **2** | 6 | n.d. |
| **3** | <1 | n.d. |
| **4** | 9 | n.d. |
| **5** | 9 | n.d. |
| **β-glu 1** | <1 | 2 |
| **β-glu 2** | 2 | 21 |
| **β-glu 3** | 3 | 16 |
| **β-glu 4** | 21 | 58* |
| **β-glu 5** | 12 | 45* |
| **β-glu 6** | 14 | 49* |
| None | 0 | |

n.d. = not determined because cytotoxic.
* $p < 0.05$.

**Table 4.** Anti-HSV-2 activity on U937 cells

| Compound | HSV-2 $\log_{10}$PFU/mL ± SD | |
|---|---|---|
| | 10 μg/mL | 100 μg/mL |
| **1** | 3.17 ± 1.4 | n.d. |
| **2** | 3.30 ± 1.3 | n.d. |
| **3** | 3.01 ± 1.4 | n.d. |
| **4** | 3.28 ± 1.5 | n.d. |
| **5** | 3.36 ± 1.8 | n.d. |
| **β-glu 1** | 3.54 ± 1.8 | 3.50 ± 1.9 |
| **β--glu 2** | 3.54 ± 1.5 | 3.07 ± 1.7 |
| **β-glu 3** | 3.54 ± 1.7 | 3.27 ± 1.9 |
| **β-glu 4** | 3.40 ± 1.4 | 2.74 ± 0.6* |
| **β-glu 5** | 3.46 ± 1.6 | 2.86 ± 1.2* |
| **β-glu 6** | 3.41 ± 1.8 | 2.82 ± 0.8* |
| Control virus HSV-2 | 3.56 ± 1.5 | |

n.d. = not determined because cytotoxic.
* $p < 0.05$ v/s control virus.

presence of IFN-α/β. Our data demonstrated that no IFN-α/β basal production was detected in untreated cells infected or not with HSV-2. Nonglycosylated compounds at the concentrations tested were not able to induce IFN-α/β. Compounds **β-glu 4**, **βglu 5** and **β-glu 6** at a concentration of 100 μg/mL induced a significant increase of IFN-α/β production compared with those obtained at a lower concentration (10 μg/mL, $p < 0.05$). Whereas, the compounds **β-glu2** and **β-glu3** have been shown to significantly produce IFN-α/β only at a concentration of 100 μg/mL.

(c) *Antiviral activity*: In Table 4, the effects of different concentrations of compounds on HSV-2 replication are reported. Our results demonstrate that compounds **β-glu 4**, **β-glu 5** and **β-glu 6** possess antiviral activity compared with control virus ($p < 0.05$). This effect was dose-dependent, in fact, a significant viral inhibition was evident at a concentration of 100 μg/mL compared with those obtained at 10 μg/mL ($p < 0.05$).

(d) *Conformational search study*: The conformational search was carried out by molecular mechanics techniques with the aim to complete the characterisation of the synthesised compounds. This study is essential for sampling the most probable conformers that will be considered for multiconformational automatic docking experiments.

Since compound **3** contains one asymmetric centre, only the *R* enantiomer has been considered. Its derivative **β-glu 3**, due to the presence of the sugar moiety, shows two diastereoisomers obtained from the *R* and *S* configuration of the chiral carbon atom located at the β position of the side chain. In this case, both stereoisomers have been modeled.

In detail, after building the 3D structures of each compound, we proceeded with the exploration of the internal degrees of freedom by Monte Carlo (MC) randomisation of any rotatable bond. 15,000 or 50,000 conformations, respectively, for hydroxy and β-glucosidic esters, were generated and energy minimised with the AMBER* force field using the united atoms notation and the implicit model of solvation GB/SA water[29] as implemented in MacroModel ver. 7.2.[30] The convergence in the conformational search was evaluated for each molecule using the averaged number of duplicate conformers (AND). Usually, an AND value equal or higher than 2 indicates a good conformational space exploration. In Table 5, the MC results are summarised.

As reported in Table 5, the presence of the sugar moiety clearly reduced the exposition of the fluorene moiety to the solvent. This feature can be important in the intercalative recognition of such a moiety into the DNA and will be extensively investigated in a future work. In Figure 1, the comparison of the energy global minimum conformers of all compounds is shown.

The higher exposition to the solvent of the hydroxy esters fluorene moiety with respect to β-glucoside deriva-

**Table 5.** Conformational search conditions of the Monte Carlo simulations and statistics of the results in terms of energy minimum conformations

| Compound | RB[a] | MC ite[b] | NCONF[c] | AND[d] |
|---|---|---|---|---|
| **1** | 4 | 15,000 | 28 | 487.3 |
| **2** | 5 | 15,000 | 77 | 183.1 |
| **3** | 4 | 15,000 | 56 | 245.4 |
| **4** | 6 | 15,000 | 234 | 61.3 |
| **5** | 7 | 15,000 | 673 | 21.5 |
| **6** | 2 | 15,000 | 15 | 858.9 |
| **β-glu 1** | 13 | 50,000 | 6123 | 4.7 |
| **β-glu 2** | 14 | 50,000 | 8175 | 3.7 |
| **β-glu 3R** | 13 | 50,000 | 2572 | 7.6 |
| **β-glu 3S** | 13 | 50,000 | 4001 | 5.4 |
| **β-glu 4** | 15 | 50,000 | 10,000 | 2.6 |
| **β-glu 5** | 16 | 50,000 | 10,000 | 2.0 |
| **β-glu 6** | 11 | 50,000 | 3021 | 10.2 |

[a] Number of rotatable bonds.
[b] Monte Carlo iterations.
[c] Number of conformers within 11.95 kcal/mol above the global minimum.
[d] Averaged number of duplicates with RMS (root mean square) deviation computed on the atomic coordinates lower than 0.25 Å and with energy difference lower than 1 kcal/mol.

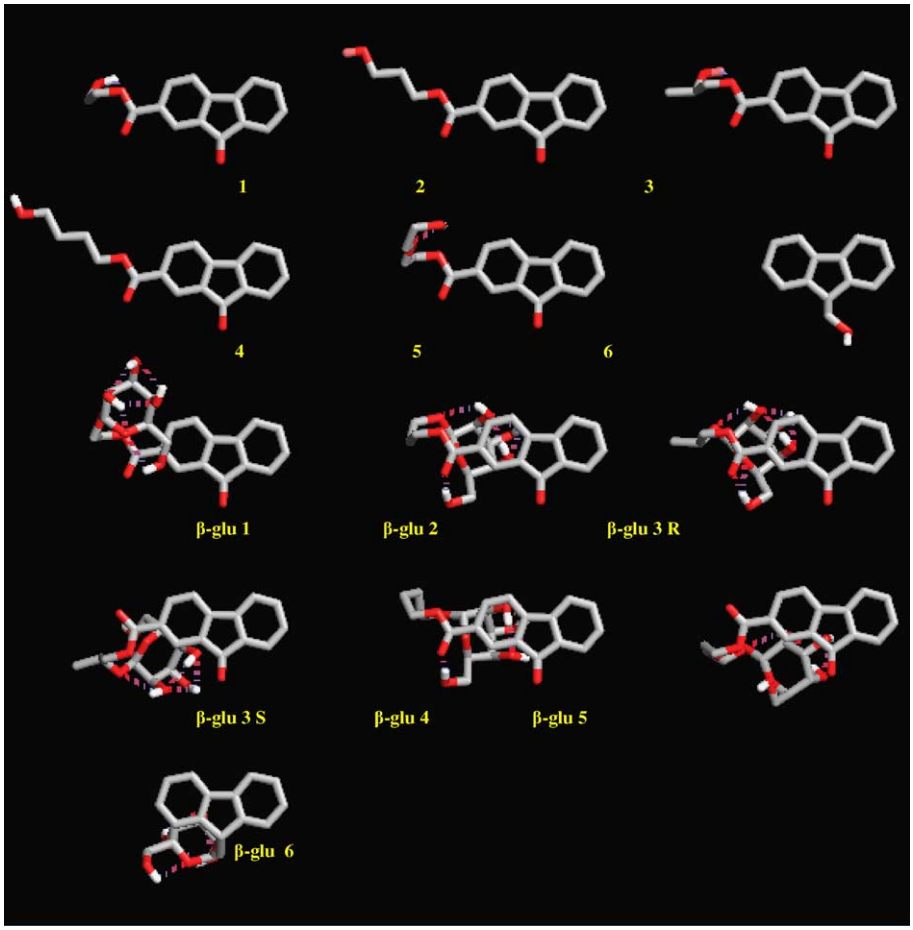



**Figure 1.** Energy global minimum conformers of compounds (polytube models) obtained in the Monte Carlo conformational search. Hydrogen bonds are depicted as dashed red lines.

tive is clear. This can be addressed to the larger steric hindrance of the sugar and to the short spacer between this moiety and that fluorene, which spatially locates the glucoside portion very close to the tricyclic nucleus.

Hydroxy esters showed a very low occurrence of intramolecular hydrogen bond, actually these interactions have been observed only for compounds **1**, **3** and **5**. In all these cases, only one hydrogen bond has been indicated between the terminal alcoholic function (the donor) and the vicinal $sp^3$ oxygen (the acceptor). Conversely, β-glucoside derivatives showed several intramolecular hydrogen bonds at level of the sugar. Only compounds **β-glu 2**, **β-glu 3R** and **β-glu 4** revealed, also, one hydrogen bond between the sugar primary alcoholic function and the spacer carbonyl oxygen hiding these two moieties.

## 4. Conclusion

The understanding of the immunopathogenesis of herpes virus infection is a major prerequisite for rationally improving therapeutic strategies, developing immunotherapeutics and antiviral drugs. As in other virus infections, the individual course of HSV infection depends on both host and viral factors.

As might be expected, both the cellular and humoral aspect of the immune response are involved. Interferons are important in limiting the initial infection and natural killer cells are also involved at this stage. The virus can also spread from one cell to another without entering the extracellular space and coming in contact with humoral antibodies. This means that cell-mediated responses are pivotal in controlling herpes infections. HSV-2 infections initiate in the genital epithelium and rapidly spread into sensory nerves innervating the mucosa. Innate immune responses, including the type I interferon (IFN-α/β) response, are the host's first line of defence against viruses during this period of acute infection. IFN-α synthesis by cells within hours of virus infection prepares neighbouring uninfected cells to enter a protective antiviral state. Several studies demonstrate the importance of IFN-α in restraining acute HSV infection.[31,32] Furthermore, HSV has evolved mechanisms for evading the IFN-α response.[33–36]

On the basis of these findings, we analysed the IFN-α/β inducent and anti-HSV-2 properties of aglycones and β-*O*-glycosides. We have shown that compounds **β-glu 4**, **β-glu 5** and **β-glu 6** possess an appreciable antiviral activity compared with compounds **β-glu 1**, **β-glu 2** and **β-glu 3**. Moreover, compounds **β-glu 4**, **β-glu 5** and **β-glu 6** at a concentration of 100 μg/mL induced a significant

increase of IFN-α/β production compared with other compounds, and this effect was dose-dependent.

These findings demonstrate the importance of glycosylation, which have profound effects on the capacity of compounds to trigger U937 cells to release IFN-α/β production and, at the same time, to hinder virus replication.

The molecular modelling work completed the structural characterisation of the novel compounds. Further experiments, currently in progress in our computational laboratory, are oriented toward the docking simulations of the novel compounds with different DNA duplex structures. Together with other experimental direct DNA intercalation studies, they will give more information about the mechanism of action of the series presented in this work and will be the object of a future communication.

## 5. Experimental

### 5.1. General chemistry

$^1$H NMR and $^{13}$C NMR spectra were recorded on a Varian 300 spectrometer at 300 and 75.6 MHz, respectively, using the residual $CHCl_3$ peak at 7.26 ppm for $^1$H and the central peak of $CDCl_3$ at 77 ppm for $^{13}$C as reference lines. Coupling constants (*J*) are given in Hz. Melting points are uncorrected. TLC controls were carried out on precoated silica gel plates (F 254 Merck).

GC–MS spectra were recorded with Carlo Erba QMD 100 spectrometer. Elemental analyses were ±0.4% of theoretical values and were performed by CHNS-O Elementary Analyzer 2004 serie II Perkin Elmer.

### 5.2. General synthesis of hydroxyalkyl 9-oxo-2-fluorenecarboxylates (1–5)

An excess of appropriate glycol was added to 9-fluorenon-2-carboxylic acid (2.23 mmol) with catalytic amount of $H_2SO_4$. The mixture was stirred at room temperature for 12 h (TLC monitoring), then the solution was evaporated under vacuum, the residue dissolved in $CH_2Cl_2$, washed with distilled water ($3 \times 15$ mL), saturated solution of $NaHCO_3$ and finally distilled water. The organic layer was dried over anhydrous $Na_2SO_4$, then evaporated under vacuum. The residue was purified by silica gel chromatography ($CHCl_3$–$CH_3OH$ 9:1).

**5.2.1. 2-Hydroxyethyl 9-oxo-9*H*-2-fluorenecarboxylate (1).** 80% yield; mp = 155–158 °C; MW = 268.27. $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 8.32–7.39 (m, 7H, ArH); 4.51 (t, 2H, COO*CH*$_2$, *J* = 4.8 Hz); 4.01 (t, 2H, *CH*$_2$OH, *J* = 4.8 Hz). $^{13}$C NMR ($CDCl_3$) 198.4 (COO); 166.3 (9C=O); 148.82, 144.21, 136.65, 135.32, 134.13, 131.45, 130.51, 125.42, 124.81, 121.52, 121.41, 120.43 (aromatic); 66.8 (COO*CH*$_2$); 61.8 (*CH*$_2$OH). Anal. Calcd for $C_{16}H_{12}O_4$: C, 71.64; H, 4.51; O, 23.85. Found: C, 71.43; H, 4.80.

**5.2.2. 3-Hydroxypropyl 9-oxo-9*H*-2-fluorenecarboxylate (2).** 73% yield; mp = 125–127 °C; MW = 282.30. $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 8.25–7.38 (m, 7H, ArH); 4.50 (t, 2H, COO*CH*$_2$, *J* = 6.2 Hz); 3.80 (t, 2H, *CH*$_2$OH, *J* = 5.7 Hz); 2.03 (m, 2H, COOCH$_2$*CH*$_2$). $^{13}$C NMR ($CDCl_3$) 197.92 (COO); 165.90 (9C=O); 148.92, 144.11, 136.42, 135.19, 134.32, 131.73, 130.42, 125.32, 124.91, 121.31, 121.27, 120.35 (aromatic); 62.42 (COO*CH*$_2$); 59.31 (CH$_2$OH); 32.06 (2-CH$_2$). GC–MS: *m*/*z* (%); 282 ($M^{+\cdot}$, 10), 225 (74), 207 (47), 179 (27), 151 (70), 57 (100). Anal. Calcd for $C_{17}H_{14}O_4$: C, 72.33; H, 5.00; O, 22.67. Found: C, 72.13; H, 5.20.

**5.2.3. 2-Hydroxy-2-methylethyl 9-oxo-9*H*-2-fluorenecarboxylate (3).** 74% yield; mp = 128–130 °C; MW = 282.30. $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 8.28–7.32 (m, 7H, ArH); 4.37 (m, 2H, COO*CH*$_2$); 4.23 (m, 1H, *CH*OH); 1.32 (d, 3H, CH$_3$). $^{13}$C NMR ($CDCl_3$) 197.84 (COO); 165.42 (9C=O); 148.81, 144.12, 136.98, 135.21, 134.28, 131.53, 130.18, 125.11, 124.95, 121.21, 121.34, 120.38 (aromatic); 66.45 (COO*C*H$_2$); 65.13 (CHOH); 19.82 (CH$_3$). GC–MS: *m*/*z* (%); 282 ($M^{+\cdot}$, 12), 238 (5), 225 (45), 207 (100), 179 (24), 151 (57). Anal. Calcd for $C_{17}H_{14}O_4$: C, 72.33; H, 5.00; O, 22.67. Found: C, 71.66; H, 5.05.

**5.2.4. 4-Hydroxy-1-butyl 9-oxo-9*H*-2-fluorenecarboxylate (4).** 82% yield; mp = 115–116 °C; MW = 296.33. $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 8.29–7.38 (m, 7H, ArH); 4.39 (t, 2H, COO*CH*$_2$, *J* = 6.3 Hz); 3.74 (t, 2H, *CH*$_2$OH, *J* = 5.1 Hz); 1.90 (m, 2H, COOCH$_2$*CH*$_2$); 1.75 (m, 2H, COOCH$_2$CH$_2$*CH*$_2$). $^{13}$C NMR ($CDCl_3$) 198.22 (COO); 166.93 (9C=O); 148.78, 144.31, 136.42, 135.37, 134.21, 131.64, 130.34, 125.22, 124.77, 121.44, 121.31, 120.22 (aromatic); 65.49 COO*CH*$_2$); 62.66 (CH$_2$OH); 29.47 (2-CH$_2$); 25.49 (3-CH$_2$). GC–MS: *m*/*z* (%); 296 ($M^{+\cdot}$, 6), 224 (52), 207 (57), 179 (26), 151 (65), 71 (100). Anal. Calcd for $C_{18}H_{16}O_4$: C, 72.96; H, 5.44; O, 21.60. Found: C, 72.79; H, 5.49.

**5.2.5. 3-Oxa-5-hydroxy-1-penthyl 9-oxo-9*H*-2-fluorenecarboxylate (5).** 76% yield; mp = 131–133 °C; MW = 312.33. $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 8.33–7.39 (m, 7H, ArH); 4.52 (t, 2H, COO*CH*$_2$, *J* = 5.1 Hz); 3.86 (t, 2H, *CH*$_2$OH, *J* = 4.5 Hz); 3.78 (t, 2H, COOCH$_2$*CH*$_2$, *J* = 5.1 Hz); 3.67 (t, 2H, O*CH*$_2$CH$_2$OH, *J* = 4.5 Hz). $^{13}$C NMR ($CDCl_3$) 198.32 (COO); 166.20 (9C=O); 148.81, 144.41, 136.62, 135.35, 134.12, 131.34, 130.7, 125.4, 124.8, 121.5, 121.6, 120.4 (aromatic); 72.71 (COO*CH*$_2$); 69.41 (CH$_2$OH); 64.63 (2-CH$_2$); 62.08 (3-CH$_2$). Anal. Calcd for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16; O, 25.62. Found: C, 6.40; H, 5.02.

### 5.3. Enzymatic reaction

The glycosyl-donor, *p*-nitrophenyl β-D-glucopyranoside 100 mg, and the acceptor, initially in molar ratio 1:5 were dissolved in 50 mM acetate buffer, pH 5.0 (5–10 mL using 10–30% acetonitrile as cosolvent) and the purified enzyme Ssβ-gly (20 μL, 4 mg/mL, 416 U/mL) or the crude homogenate (1 mL for 100 mg of acceptor) was added. The reaction was started at 70 °C under stirring in a sealed vial. The monitoring of the reaction is

secured by TLC; additional aliquots of donor were added at the end of the first one to increase the productivity of reaction. The reaction is stopped at total donor consumption, rotary evaporated and the glycoside purified by reverse phase column chromatography, or silica gel chromatography, or preparative TLC. The identity of the product was established by NMR spectroscopy of pure glycosides or acetylated derivatives.

**5.3.1. *O*-β-[2-(9-Oxo-9*H*-2-fluorenecarbonyloxy)-1-ethyl] glucoside (β-glu 1).** 18% yield; mp = 137–140 °C; MW = 430.42. $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 8.59–7.30 (m, 7H, ArH); 5.05–4.91 (m, 3H, H-2, H-3, H-4); 4.59 (m, 2H, COO*CH*$_2$); 4.43 (d, 1H, H-1, $J$ = 6.6 Hz ax/ax); 4.21 (m, 2H, COOCH$_2$*CH*$_2$); 3.93–3.31 (m, 3H, H-5, H-6, H-6′). Anal. Calcd for $C_{22}H_{22}O_9$: C, 61.39; H, 5.15. Found: C, 61.35; H, 5.22.

**5.3.2. *O*-β-[3-(9-Oxo-9*H*-2-fluorenecarbonyloxy)-1-propyl] glucoside (β-glu 2).** 21% yield; mp = 135–136 °C; MW = 444.44. $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 8.27–7.38 (m, 7H, ArH); 5.21–5.01 (m, 3H, H-2, H-3, H-4); 4.54 (d, 1H, H-1, $J$ = 6.0 Hz ax/ax); 4.39 (m, 2H, COO*CH*$_2$); 4.25–4.15 (m, 2H, H-6, H-6′); 4.04 (m, 2H, *CH*$_2$O); 3.70 (m, 3H COOCH$_2$*CH*$_2$, H-5). Anal. Calcd for $C_{23}H_{24}O_9$: C, 62.16; H, 5.44. Found: C, 61.97; H, 5.5.

**5.3.3. *O*-β-[2-Methyl-2-(9-oxo-9*H*-2-fluorenecarbonyloxy)-1-ethyl] glucoside (β-glu 3).** 13% yield; mp = 141–143 °C; MW = 444.44. $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 8.38–7.38 (m, 7H, ArH); 5.23–4.98 (m, 3H, H-2, H-3, H-4); 4.54 (d, 1H, H-1, $J$ = 6.0 Hz ax/ax); 4.45 (d, 2H, COO*CH*$_2$, $J$ = 7.5 Hz); 4.33 (dd, 1H, H-6, $J_{AX}$ = 3.9 Hz, $J_{AB}$ = 12.3 Hz); 4.25 (m, 1H, COOCH$_2$*CHO*); 3.96 (dd, 1H, H-6′, $J_{BX}$ = 2.4 Hz, $J_{BA}$ = 12.3 Hz); 3.69 (m, 1H, H-5); 1.34 (d, 3H, CH$_3$, $J$ = 6.3 Hz). Anal. Calcd for $C_{23}H_{24}O_9$: C, 62.16; H, 5.44. Found: C, 62.29; H, 5.23.

**5.3.4. *O*-β-[4-(9-Oxo-9*H*-2-fluorenecarbonyloxy)-1-butyl] glucoside (β-glu 4).** 25% yield; mp = 127–131 °C; MW = 458.47. $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 8.29–7.38 (m, 7H, ArH); 4.92–4.65 (m, 3H, H-2, H-3, H-4); 4.31 (t, 2H, COO*CH*$_2$, $J$ = 6.3 Hz); 4.20 (d, 1H, H-1, $J$ = 6.1 Hz ax/ax); 3.99–3.80 (m, 1H, H-5); 3.76–3.55 (m, 4H, H-6, H-6′, *CH*$_2$O-β-glu); 1.82 (m, 2H, COOCH$_2$*CH*$_2$); 1.69 (m, 2H, COOCH$_2$CH$_2$*CH*$_2$). Anal. Calcd for $C_{24}H_{26}O_9$: C, 62.88; H, 5.72. Found: C, 62.56; H, 5.55.

**5.3.5. *O*-β-[3-Oxa-5-(9-oxo-9*H*-2-fluorenecarbonyloxy)-1-pentyl] glucoside (β-glu 5).** 23% yield; mp = 129–131 °C; MW = 461.45. $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 8.20–7.35 (m, 7H, ArH); 4.90–4.67 (m, 3H, H-2, H-3, H-4); 4.41 (t, 2H, COO*CH*$_2$, $J$ = 3.4 Hz); 4.20 (d, 1H, H-1, $J$ = 6.0 Hz ax/ax); 3.80 (m, 2H, *CH*$_2$O-β-glu); 3.74 (m, 2H, COOCH$_2$*CH*$_2$); 3.56 (m, 2H, *CH*$_2$CH$_2$O-β-glu); 3.50–3.10 (m, 3H, H-5, H-6, H-6′). Anal. Calcd for $C_{23}H_{25}O_9$: C, 59.87; H, 5.46. Found: C, 59.63; H, 5.57.

**5.3.6. β-9-Fluorenemethyl glucoside (β-glu 6).** 15% yield; mp = 119–121 °C; MW = 358.39. Anal. Calcd for $C_{20}H_{22}O_6$: C, 67.03; H, 6.19. Found: C, 67.50; H, 6.35. $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 7.77–7.26 (m, 8H, ArH); 4.46 (d, 1H, H-1, $J$ = 6.9 Hz ax/ax); 4.28–4.17 (m, 3H, H-2, H-3, H-4); 3.91–3.66 (m, 3H, H-5, H-6, H-6′); 3.44–3.28 (m, 3H, *CH*$_2$O, H-9-fluo).

### 5.4. U937 cell culture and differentiation

The human monocytic cell line U937 was kindly provided by Prof. J. P. Liautard, INSERM, University of Montpellier II, France.

Cells were maintained in culture at 37 °C in 5% $CO_2$ atmosphere in RPMI 1640 medium supplemented with 50 μM 2-mercaptoethanol, 1 mM pyruvate, 1 mM non-essential aminoacids, 1 mM HEPES, gentamycin (50 mg/mL) and 10% heat inactivated foetal calf serum (FCS) (all reagents from Seromer, Milan, Italy). Cells ($3 \times 10^5$ per well) were differentiated for 72 h with 100 nM 1α,25-dihydroxyvitamin $D_3$ (VD) (Calbiochem, Milan) plus 100 nM all-trans retinoic acid (RA) (Sigma, Milan).[38] RA and VD were dissolved in absolute ethanol and stored at −70 °C at an initial concentration of $10^{-3}$ M. Further dilutions were performed in RPMI 1640 medium. The final concentration of ethanol had no effect on cell growth and differentiation.

After 72 h, differentiation was complete and most cells were adherent. Differentiation was confirmed by growth inhibition, morphological changes and increased phagocytosis of *C. albicans*. Differentiated U937 cells were treated with compounds at different concentrations (10 or 100 μg/mL) and infected with HSV-2 at a multiplicity of infection (MOI) 0.1 for 24 h at 37 °C in 5% $CO_2$, then the plates were frozen and thawed three times in order to release the intracellular virus. Cell lysates and supernatants were kept at −80 °C until virus titration and IFN-α/β assay.

### 5.5. Cytotoxicity test

All the compounds were diluted in dimethylsulfoxide (DMSO) Hybri-Max for cell culture (Sigma, Milan, Italy) at a concentration of 1 mg/100 μL, further dilutions were performed in RPMI-1640. The final concentration of DMSO had no effects on cell viability. Cytotoxicity was performed using trypan blue exclusion test. The results were expressed as percentage of stained (dead) cells.

### 5.6. Virus

HSV-type 2 was used throughout the study. HSV-2 infection was propagated on WISH cell lines. Viral stocks were prepared by pelletting infected cells exhibiting cytopathic effect and freezing aliquots at −80 °C. The virus titre was assessed on WISH cells and expressed as $\log_{10}$ plaque forming unit (PFU) per mL. The input multiplicity of infection (MOI), used in all the experiments, was 0.1 PFU/cell.

### 5.7. Interferon assay

Supernatants from U937 cells, in different experimental conditions, were collected and analysed for the presence

of IFN-α/β. Wish cells and vesicular stomatitis virus (VSV) were used to measure the levels of interferon α/β in cell supernatants. Recombinant human IFN-α (Bender Medsystems, Milan, Italy) was used as standard and all IFN titres were corrected against standard IFN as previously described.[37] One IFN unit was defined as the amount giving 50% inhibition of cytopathic effect induced by VSV and expressed as International Unit (I.U.).

### 5.8. Limulus test

Culture media and reagents tested for the presence of endotoxin by E-Toxate kit (Sigma, Milan, Italy) were found to contain $\leqslant$10 pg of endotoxin per mL.

### 5.9. Statistical evaluation

Data were analysed by one way analysis of variance (ANOVA) and the Student–Newman–Keults test.

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