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Facile Biocatalytic Access to 9-Fluorenylmethyl Polyglycosides: Evaluation of Antiviral Activity on Immunocompetent Cells

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The biological activities of a series of mono- and oligosaccharides (β-xylosides and α-glucosides) of 9-fluorenylmethanol were investigated together with mono-β-galactoside and β-glucoside of this aglycone, produced by biocatalytic routes. By using marine glycoside hydrolases and inexpensive donors such as maltose or xylan, access to mono-α-glucoside or mono-β-xyloside of 9-fluorenylmethanol was obtained. Additionally, interesting polyglycoside derivatives were isolated. Biological testing indicated that in vitro treatment with these carbohydrate derivatives may influence the balance of cytokines in the environment of human peripheral blood mononuclear cells (PBMC), restricting the harmful effect of herpes simplex type 2 replication. In fact, these carbohydrate derivatives tested in WISH cells did not show any significant antiviral activity.

Introduction

Herpes simplex virus type 2 (HSV-2) contains its genetic information in a single, large double-stranded DNA. It is one of the most common and continuously increasing viral infections in humans, and is responsible for diseases ranging from gingivostomatitis to keratoconjunctivitis, genital disease, encephalitis, and infections of newborns and immune-compromised patients.[1] According to epidemiological surveys, the HSV infection rate has been continuously increasing in most countries.[2] After the primary infection, HSV tends to persist in the neuron of the ganglion.[3] Reactivation of latent HSV, which is very common during deficiency of immunity, causes recurrent herpetic infection. There are few drugs licensed for the treatment of HSV infections. The treatment of choice is acyclovir (ACV) and congeners. However its increasing use, especially in prophylaxis treatments among transplanted patients and in immune-compromised hosts (cancer, AIDS), has led to the emergence of resistant viral strains.[4,5] It is now generally agreed that cell mediated immunity to several infectious agents is regulated by two distinct T cell cytokine patterns. The production of Th1 cytokines such as interferon-γ (IFN-γ), interleukins-2 and -12 (IL-2, IL-12), is generally associated with resistance to infection.[6,7] Thus, efficient elimination of a viral infection requires a development of Th1-type immunity, that is characterized by the production of proinflammatory cytokines such as IFN, tumor necrosis factors (TNF), and ILs. These cytokines, may induce an antiviral state in the cells (via IFNs) or destroy virus-infected cells (via TNFα), may stimulate cytotoxicity and cytokine production (via IFN-γ and IL-12) by T cells and natural killer (NK) cells, and may initiate development of Th1 cells.[8] Moreover, it has been shown that Herpes viruses can indirectly counteract the phagocyte functions, namely by viral mechanisms of mimicry of cytokines and cytokine receptors.[9,10] The consequence of this argument is that there may be super networks of interacting host and viral molecules which determine the nature and effectiveness of host defenses.[11] This highlights the crucial need for the development of new antiviral drugs that seek to combine immunotherapeutic intervention, as an adjunct to antiviral activity, that may therefore confer added benefit by controlling viral replication.

Tilorone, 2,7-bis-[2-(diethylamino)ethoxy]-9*H*-fluoren-9-one, represents the lead compound of our continuing research and was the first low-molecular weight interferon (IFN) inducer orally effective against many DNA and RNA viruses, and against some neoplasias.[12–14] The mechanism of action of tilorone is linked to DNA intercalation; in fact, slipping between two adjacent base pairs of the minor groove, its planar molecule alters DNA physicochemical properties, stimulating cytokine production.[15] Several synthetic analogues possessing IFN-inducing activity have been reported[16] in the search for novel intercalat-

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ing agents which could induce IFN and other cytokine production.[17]

Various 9-fluorenone based compounds have been synthesized and assayed by us to produce relevant results.[18–21] In novel compounds, as in the well-known intercalators such as anthracyclines, the presence of carbohydrate residues might improve the intercalation mode thus stabilizing the DNA–ligand complexes.[22,23] In drug design, glycosylation was also judged as a direct element to improve pharmacokinetic and pharmacological properties.[24] Moreover, compounds not soluble in aqueous media, upon glycosylation generally become soluble and can be assayed. Indeed, this was the case of the title 9-fluorenylmethanol, the aglycon used in this research. In particular, its mono-β-glucoside, previously obtained by us using a thermophilic glycoside hydrolase,[18] has been reported to be an active anti-HSV-2 and interferon inducing agent, as are other monoglycosylated derivatives of 9-fluorenol, possessing a carbinol center and laboriously produced by chemical procedures.[25]

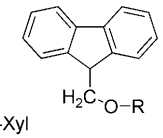
Enzymatic routes to obtain glycosyl derivatives including polyglycosides, difficult to obtain by chemical procedures, are based on glycoside hydrolases and a transglycosylation reaction using a suitable donor.[26] It was then considered of interest to evaluate the possibility of obtaining a variety of carbohydrate derivatives of 9-fluorenylmethanol by biocatalysis using different enzymes: 1) an endoxylanase activity present in the crude homogenate of the marine microorganism *Thermotoga neapolitana* (giving poly-β-xylosylated derivatives), 2) the α-glucosidase from marine mollusc *Aplysia fasciata* (giving poly-α-glucosyl derivatives), and 3) commercial glycoside hydrolases from almond (β-gluco) and *Aspergillus* (β-galacto). Enzymatic synthetic strategies based on endoglycoside hydrolases are extremely interesting allowing the one-pot transfer of a preformed oligosaccharide unit (di- or higher), thus giving easy access to complex molecules which are produced by very laborious chemical synthesis.[26] This is the case of the naturally occurring donor such as xylan for the production of oligoxylosides.[27,28] It is a useful donor for the synthesis of oligoxylosides of different acceptors using *T. neapolitana* xylanase activity.[29] The mollusc *A. fasciata* is one of the most promising marine organisms, rich in glycoside hydrolases of synthetic interest. In fact, the hepatopancreas and visceral mass of this sea hare contain a wide range of these enzymes which have already been successfully used for the hydrolysis and synthesis of glycosidic bonds.[30] Successful formation of α-D-oligoglucosides was recently reported using a purified enzyme.[31,32] This biocatalyst was also very recently used by us to synthesize structurally related *O*-α-oligoglucosides of different 9-fluorenones possessing spacers in different positions, as potential immunomodulators and anti-HSV-2 agents.[20] Commercial almond β-glucosidase and *Aspergillus oryzae* β-galactosidase are known enzymes usually adopted for the synthesis of β-gluco- and β-galactosides of a different nature,[26a,c] however, to the best of our knowledge they were never applied to tricyclic aromatic scaffolds such as 9-fluorenylmethanol.

The biological activities of an interesting series of mono- and oligosaccharides (β-xylo- and α-gluco-) of 9-fluorenylmethanol together with mono β-galactoside and β-glucoside of this aglycone, synthesized by the biocatalytic routes mentioned above, are reported herein. In the present paper, we studied the effects of carbohydrate derivatives on the replication of HSV-2 in human PBMC (peripheral blood mononuclear cell) and WISH cells. Furthermore, we investigate whether the anti-HSV-2 activity observed only in PBMC could be related to its modulatory activity on cytokine production by the same cells. The results indicate that these compounds may influence the balance of cytokines in the environment of human PBMC, restricting the harmful effect of HSV-2 replication.

Results and Discussion

Enzymatic syntheses

The thermophilic endoxylanase activity from *T. neapolitana* was used in the form of crude homogenate obtained as previously described (1.8 mg mL^{-1} of total protein).[29] The reaction for the formation of polyxylosides was conducted with 0.11 M 9-fluorenylmethanol as acceptor in the presence of 13% acetonitrile as co-solvent for 6.5 h at 70 °C. Enzyme activity was able to transfer xylan blocks up to tetra- and pentasaccharides to the aromatic acceptor. The resulting reaction mixture contained 9-fluorenylmethyl β-D-xylopyranoside (**1**) 1.5 mM, whereas disaccharide **2** was present at 0.35 mM, trisaccharide **3** at 0.5 mM, and the tetrasaccharide **4** at 0.15 mM (Figure 1). Additionally



1 R = β-Xyl
2 R = β-Xyl-(1,4)-β-Xyl
3 R = β-Xyl-(1,4)-β-Xyl-(1,4)-β-Xyl
4 R = β-Xyl-(1,4)-β-Xyl-(1,4)-β-Xyl-(1,4)-β-Xyl
5 R = α-Glc
6 R = α-Glc-(1,4)-α-Glc
7 R = α-Glc-(1,6)-α-Glc
8 R = α-Glc-(1,4)-α-Glc-(1,4)-α-Glc
9 R = trisaccharides
10 R = α-Glc-(1,4)-α-Glc-(1,4)-α-Glc-(1,4)-α-Glc
11 R = β-Glc
12 R = β-Gal

Figure 1. Carbohydrate derivatives of 9-fluorenylmethanol obtained by enzymatic transglycosylation reactions using *T. neapolitana* endoxylanase (compounds **1**–**4**), *A. fasciata* α-glucosidase (compounds **5**–**10**), almond (compound **11**), and *Aspergillus* (compound **12**) enzymes.

minor products, amounting to 13.2 mg g^{-1} xylan, were detected at a lower R_f in TLC and contain additional sugars different from xylose. Prior to acetylation, usually adopted to establish interglycosidic linkage by NMR spectroscopy, ESI-MS spectra of **1**–**4** indicated molecular weights of native materials thus ruling out the presence of residual acetyl groups found in the natural xylan: **1**, **2**, **3**, and **4** (*m/z* 351, 483, 615, and 747 $[M+Na]^+$, respectively). Furthermore ^{1}H NMR spectra and 2D COSY, TOCSY, and ^{1}H–^{13}C correlation experiments of acetylated derivatives, allowed the unambiguous structure determination of products.

Usually in the COSY spectra, starting from the anomeric proton signal of sugars and following the correlations through pyranosidic protons, it is easy to detect the glycosylation position for the upfield shift of the signal due to the absence of the acetyl group (see Experimental Section).

The enzymatic formation of α-glucosides of 9-fluorenylmethanol was based on a transglucosylation reaction performed by the α-glucosidase from *A. fasciata*[31] in 1 M maltose, adding the acceptor dissolved in DMSO. The reaction produced six different chromophoric α-glucosides (**5**–**10**, shown) purified by reverse phase column and preparative TLC. A total molar yield of approximately 53% based on the acceptor, was obtained in the enzymatic process: **5**: 31%; **6**: 13%; **7**: 2%; **8**: 5%; **9**: 1%; **10**: 1%. Acetylation was conducted for precise determination of interglycosidic linkages by 2D NMR spectroscopy.

Commercial almond β-glucosidase and *A. oryzae* β-galactosidase were used for the syntheses of β-gluco (**11**, shown) and β-galacto (**12**, shown) derivatives of 9-fluorenylmethanol in 20% acetonitrile but the yields obtained were very limited for both enzymes (1 and 4% for **11** and **12**, respectively) although a five molar excess of acceptor was used and total donor consumptions were observed.

In view of the complexity of the chemical procedures used to obtain the polyglycosides described above, the result is a remarkably easy access to these molecules.

Biological studies

As in the previously studied series of compounds[18,20,21,25] all derivatives **1**–**12** were used for a pharmacological screening to evaluate cytotoxicity, IFN-α, TNF-α, and IL-12 inducing properties, and antiviral activity against HSV-2 on human PBMC.

Table 1 shows the effects of different concentrations of compounds on cell viability. Data are represented as percentage of cytotoxicity ± standard deviation. The products were tested at the concentrations of 200, 150, 100, 60, 40, and 20 μg mL^{-1}. At concentrations of 200, 150, and 100 μg mL^{-1}, all the compounds were cytotoxic. At a concentration of 60 μg mL^{-1} all the molecules tested did not show any cytotoxicity. Concentrations of 40 and 20 μg mL^{-1} also did not show any cytotoxicity (data not shown). Similar results were obtained in WISH cells (data not shown). On these bases, we adopted the concentrations which were not cytotoxic (60, 40, and 20 μg mL^{-1}) in the experiments of antiviral activity and cytokine production.

Table 1. Cytotoxicity toward PBMC 48 h post-treatment.[a]

Compd	200 μg mL^{-1}	150 μg mL^{-1}	100 μg mL^{-1}	60 μg mL^{-1}
1	34 ± 4.1	22 ± 3.1	14 ± 1.1	0
2	23 ± 2.2	18 ± 1.6	6 ± 1.4	0
3	24 ± 1.9	9 ± 1.9	2 ± 0.8	0
4	29 ± 3.5	13 ± 1.6	7 ± 3.1	0
5	33 ± 7.2	24 ± 2.4	8 ± 2.2	0
6	27 ± 1.8	10 ± 1.3	5 ± 0.9	0
7	26 ± 2.7	13 ± 2.1	4 ± 0.7	0
8	33 ± 2.9	21 ± 3.2	7 ± 2.6	0
10	29 ± 4.4	19 ± 1.2	9 ± 3.8	0
11	33 ± 3.1	14 ± 2.1	10 ± 3.3	0
12	27 ± 2.7	12 ± 1.9	6 ± 2.1	0

[a] Values represent the mean of three experiments ± SD using the PBMC of the same donor.

In Table 2 results of the inhibition of HSV-2 replication are reported. At the concentration of 20 μg mL^{-1}, all compounds tested did not show any significant antiviral activity. Compounds **1** and **5** did not significantly influence the replication of HSV-2 at all concentrations tested. The addition of 60 μg mL^{-1} of compounds **2**, **3**, **4**, **6**, **7**, and **8** to PBMC resulted in a significant ($p < 0.05$) decrease in HSV-2 replication whereas at 40 μg mL^{-1} and 60 μg mL^{-1} compounds **10**, **11**, and **12** displayed a significant dose-dependent effect on viral replication. Conversely, all the compounds tested did not significantly influence the replication of HSV-2 on the WISH cell line (data not shown).

Table 2. Anti-HSV-2 activity toward human PBMC 48 h post-treatment.[a]

Compd	Mean no. PFU mL^{-1} [b]		
	60 μg mL^{-1} [c]	40 μg mL^{-1} [c]	20 μg mL^{-1}
1	$5.9\times10^4 \pm 3.7$	$7.1\times10^4 \pm 3.3$	$9.4\times10^4 \pm 1.9$
2	$3.6\times10^{4[d]} \pm 1.1$ (2.5)	$7.1\times10^4 \pm 2.8$	$9.3\times10^4 \pm 1.6$
3	$2.2\times10^{4[d]} \pm 2.9$ (4.2)	$6.1\times10^4 \pm 3.7$	$9.5\times10^4 \pm 1.3$
4	$1.1\times10^{4[d]} \pm 2.8$ (8.4)	$5.1\times10^4 \pm 3.6$	$9.1\times10^4 \pm 2.1$
5	$7.6\times10^4 \pm 3.1$	$8.1\times10^4 \pm 4.1$	$8.6\times10^4 \pm 2.8$
6	$2.8\times10^{4[d]} \pm 2.4$ (3.3)	$7.3\times10^4 \pm 2.7$	$8.1\times10^4 \pm 3.2$
7	$1.9\times10^{4[d]} \pm 3.3$ (4.8)	$6.1\times10^4 \pm 3.9$	$8.5\times10^4 \pm 4.8$
8	$2.2\times10^{4[d]} \pm 2.1$ (4.2)	$5.3\times10^4 \pm 3.9$	$8.3\times10^4 \pm 1.7$
10	$9.6\times10^{3[d]} \pm 3.1$ (9.6)	$4.1\times10^{4[d]} \pm 1.2$ (2.2)	$8.1\times10^4 \pm 2.1$
11	$1.8\times10^{4[d]} \pm 2.5$ (5.1)	$3.1\times10^{4[d]} \pm 1.4$ (3)	$8.1\times10^4 \pm 3.1$
12	$2.1\times10^{4[d]} \pm 3.4$ (4.4)	$3.5\times10^{4[d]} \pm 1.1$ (2.6)	$9.1\times10^4 \pm 3.1$
	Virus control: $9.3\times10^4 \pm 3.9$ PFU mL^{-1}		

[a] Values represent the mean of three experiments ± SD using the cells of the same donor. [b] PFU = plaque-forming unit. [c] Values in brackets indicate the fold decrease in each group, calculated as follows: (number of plaques in virus control)/(number of plaques in treatment). [d] $p < 0.05$ versus virus control ($p < 0.05$ considered significant).

With the aim to examine whether the observed antiviral activity on PBMC could be correlated to induction of cytokine synthesis by the compounds, we analyzed the release of IFN-α, TNF-α, and IL-12 (Table 3). Interestingly, in the experimental conditions in which the compounds showed a significant decrease in HSV-2 replication (Table 2), we found a clear induction of IFN-α and TNF-α. Treatment for 48 h with all compounds, except **5**, at a concentration of 60 μg mL^{-1} was able to trigger PBMC to release IFN-α and TNF-α although under these experimental conditions, compounds **4** and **10** were much more efficient in releasing IFN-α and TNF-α. Moreover, compounds **4**, **10**, **11**, and **12** at a concentration of 40 μg mL^{-1} were still able to release appreciable amounts of IFN-α and TNF-α. Concerning IL-12 production, at 48 h post treatment all compounds tested did not induce any detectable amount IL-12. A significant induction of IL-12 was observed at 24 h post treatment ($p < 0.05$) with 60 μg mL^{-1} of compounds **2**, **3**, **4**, **7**, **8**, **10**, **11**, and **12**. Furthermore, compounds **4**, **10**, **11,** and **12**

Table 3. Production of IFN-α, TNF-α, and IL-12 by PBMC.[a]

Compd	c [μg mL^{-1}]	IFN-α [pg mL^{-1}] 24 h	IFN-α [pg mL^{-1}] 48 h	TNF-α [pg mL^{-1}] 24 h	TNF-α [pg mL^{-1}] 48 h	IL-12 [pg mL^{-1}] 24 h	IL-12 [pg mL^{-1}] 48 h
1	60	12±4	22±5	<0.13	7±1	12±6	<2.1
	40	<3.1	<3.1	<0.13	<0.13	<2.1	<2.1
2	60	26±6	43±11	<0.13	<0.13	<2.1	<2.1
	40	<3.1	18±5	<0.13	<0.13	<2.1	<2.1
3	60	37±5	55±13	<0.13	37±9	<2.1	<2.1
	40	<3.1	17±5	<0.13	<0.13	<2.1	<2.1
4	60	48±12	79±21	<0.13	46±12	69±15	<2.1
	40	19±9	28±8	<0.13	28±4	31±5	<2.1
5	60	<3.1	<3.1	<0.13	<0.13	<2.1	<2.1
	40	<3.1	<3.1	<0.13	<0.13	<2.1	<2.1
6	60	19±2	31±4	4±2	12±3	<2.1	<2.1
	40	<3.1	<3.1	<0.13	<0.13	<2.1	<2.1
7	60	26±4	43±7	14±2	34±7	21±2	<2.1
	40	<3.1	<3.1	<0.13	<0.13	<2.1	<2.1
8	60	21±3	37±6	10±2	31±5	17±2	<2.1
	40	<3.1	<3.1	<0.13	<0.13	<2.1	<2.1
10	60	44±9	68±12	19±4	59±7	49±5	<2.1
	40	27±5	33±6	11±2	31±5	16±3	<2.1
11	60	41±8	49±4	<0.13	44±5	56±10	<2.1
	40	28±4	50±12	<0.13	28±2	48±10	<2.1
12	60	39±5	53±9	<0.13	32±7	46±5	<2.1
	40	<3.1	31±5	<0.13	23±4	27±3	<2.1

[a] At 24 and 48 h after incubation in the presence of compound at 60 and 40 μg mL^{-1}; untreated PBMC and PBMC treated with 20 μg mL^{-1} of all the compounds tested did not release any cytokine; values represent the mean of three experiments ±SD using the cells of the same donor.

at 40 μg mL^{-1} were also able to trigger PBMC in producing increased amounts of IL-12 ($p < 0.05$) compared with the other compounds tested. These data are particularly interesting in light of the finding that these compounds also show a higher antiviral activity. In fact, the secretion of IL-12 from PBMC may provide a further signal that it synergizes with IFN-α and TNF-α, and renders cells far less permissive to HSV-2 replication. Thus, the 9.6-fold inhibition achieved by compound **10** could be the result of combined activities of IFN-α, TNF-α, and IL-12 in effectively hindering viral replication.

Although simple in nature, the tricyclic aromatic acceptor is not a good substrate for the commercial glycoside hydrolases tested (*Aspergillus* and almond) whereas reasonable yield was previously reported for the formation of anomerically pure monoglucoside **11** using a thermophilic enzyme.[18] Exploiting marine enzymes (*T. neapolitana* and *A. fasciata*) and inexpensive donors (xylan and maltose) useful production of monoxyloside **1** and mono-α-glucoside **5** is reported herein. Easy access to polyglycosides **2**–**4**, **6**–**8**, and **10**, has been also possible.

From the biological results reported herein, clear-cut relationships between structure and bioactive properties can be inferred. Conceivably in the intercalation process the steric disposition of these structures and consequently the capability of constructive H-bonds, could have a great role in stabilizing DNA–ligand complexes. Among the monoglycosides, β-isomers **1**, **11**, and **12** display better pharmacological features than mono-α-glucoside **5** which is deprived of any activity. Among the disaccharides, α-1,6 interglycosidic linkage such as in **7** confers better properties than the α-1,4 one present in **6**. This trend was also previously observed.[20] Interestingly in both the β-xyloside and α-glucoside series, polyglycosylation caused activity to increase in direct correlation with molecular weight: tetraxyloside **4** and tetraglucoside **10** prove to be the best derivatives, worthy of being analyzed for DNA binding properties.[19] In addition, subtle structural differences as in monoglycosides **1**, **11**, and **12**, influence pharmacological features. Hence we can anticipate that polyglycosides of **11** and **12** are compounds of interest and our research from a biocatalytic viewpoint is focused on enzymes (endoglycosidases) showing the ability to synthesize them. Conformational analysis along with molecular docking procedures, as in previously investigated 9-fluorenones[18,19] are also in progress with the aim of rationalizing the results obtained and designing new derivatives.

From a pharmaceutical point of view, various immunotherapeutic strategies including therapeutic immunization and immunomodulatory agents have been explored with limited success to date, highlighting the complexity of the interaction between host immunity, virus replication, and drug efficacy. In fact, it is well known that the first line of defense against viral infections involves production of IFN-α/β and activation of the innate immune response, which is regulated by cytokine and chemokines produced by leukocytes. For instance, IL-12 drives development of Th1 immune response which is generally believed to promote defense against viral infections.[33] It is a cytokine with multiple biological functions: it promotes cell-mediated immunity by activation of NK and Th1-type cells, up-regulates IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-2 receptor α (IL-2Rα) expression in T cells and down-regulates the Th2- type cytokines. Therefore the ability of immune cells to produce IL-12 appears to be a critical step in the activation of the first line of defense against viruses.[34,35] IFN-α also appears to play an important role in immune responses to viruses. In fact it directly inhibits viral replication in infected cells, enhances cytotoxic activity of macrophages and NK cells, facilitates the proliferation of memory $CD8^+$ T cells through the induction of IL-15, and enhances survival of T cells.[36,37] Mogensen et al.[38] have demonstrated that HSV-2 suppresses production of cytokines, including IFN-α TNF-α, and IL-12, which exert their effects at many different stages of the antiviral response. As a result the virus hampers the ability of the host to mount an antiviral response and in this way promotes the establishment of infection. Our

in vitro results indicate that treatment with carbohydrate derivatives play a protective role during HSV-2 infection by increasing inflammatory responses supported by Th1 cytokines such as IFN-α, TNF-α, and IL-12. Therefore the effects of these innovative compounds may contribute to improve immune surveillance of PBMC toward viral infection, by tipping the balance in favor of the Th1 immune response.

Conclusions

In summary, facile access to polyglycosides of 9-fluorenylmethanol using biocatalytic procedures is described herein. Biological tests indicated that most of them influenced the balance of cytokines in the environment of human PBMC.

Initial screening, such as this, will serve to identify novel compounds which might be worth to be developed further as a potential therapeutic compound. Although reaction yield of biocatalytic routes for the synthesis of these compounds are still not satisfactory, complex anomerically-pure molecules can be obtained by these easy, inexpensive, and environmentally friendly procedures.

Experimental Section

General

Nitrophenyl glycosides, maltose, commercial enzymes, and reverse-phase silica gel and TLC silica gel plates were obtained from Sigma (St. Louis, MO, USA) and from E. Merck (Darmstadt, Germany) respectively. Protein concentration was determined using the Bradford assay system (BioRad). Compounds on TLC plates were visualized under UV light or charring with α-naphthol reagent. Acetylation of compounds was performed with pyridine/Ac_2O at room temperature; the solvents were removed by N_2 stream, and the reaction mixture was purified by silica gel chromatography or preparative TLC. Chromatographic purifications were performed using methanol/water or EtOAc/MeOH gradients for Lobar reverse phase and silica-gel chromatography respectively. NMR spectra were recorded on Bruker instruments at 600, 400, or 300 MHz. Samples for NMR analysis were dissolved in suitable solvents and the signal of the solvent was used as internal standard. ESI-MS and spectra were obtained on a Q-Tof mass spectrometer, Micro (Micromass).

Biocatalysts

Crude homogenate of *T. neapolitana* (1.8 mg mL^{-1} of total protein) used for the synthesis of β-xylosides was obtained as previously described.[29] A partial purified extract of *A. fasciata* containing α-glucosidase activity (4.2 mg mL^{-1}, 3.3 U mg^{-1}) obtained as previously reported[30,31] was used for the synthesis of α-glucosides. Almond β-glucosidase and *A. oryzae* β-galactosidase were acquired from Sigma.

Oligosaccharide syntheses and structural determination

The reaction for the formation of xylosides using 9-fluorenylmethanol as acceptor was conducted in CH_3CN/Na-phosphate 100 mM pH 7.5 (13:87 *v/v*), containing 257 μg mL^{-1} of crude protein, 20 g L^{-1} xylan from birch wood as donor, and 0.11 M 9-fluorenylmethanol. After 6.5 h at 70 °C under agitation, the reaction mixture was subjected to reverse-phase column chromatography (Lobar RP-18) eluting with water, thus efficiently separating chromophoric xylosylated fraction (150 mg g^{-1} xylan used) from anomerically free saccharides (xylose and xylo-oligosaccharides). Single chromophoric xylosides were further purified by preparative TLC (solvent EtOAc/MeOH/H_2O 70:20:10 by volume). ^{1}H NMR spectra and 2D COSY, TOCSY, and ^{1}H–^{13}C correlation experiments, allowed the unambiguous structure determination of products.

1. Peracetyl derivative ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.76$, 7.60, 7.38, 7.29 (8H, aromatic ring protons), 5.17 (t, 1H, H-3 xylose), 5.06 (dd, 1H, H-2 xylose), 4.98 (m, 1H, H-4 xylose), 4.55 (d, $J = 7.8$ Hz, 1H, H-1 xylose), 4.24 (dd, 1H, Ar-CH_{2a}-*O*-β-Xyl), 4.18 (t, 1H, Ar-C*H*CH_2-*O*-β-Xyl), 4.16 (dd, 1H, H-5a xylose), 3.58 (t, 1H, Ar-CH_{2b}-*O*-β-Xyl), 3.39 (dd, 1H, H-5b xylose), 2.03 ppm (acetyl group protons). ^{13}C NMR ($CDCl_3$, 400 MHz): xylose moiety $\delta = 100.5$ (C1), 71.3 (C3), 70.6 (C2), 68.7 (C4), 61.9 ppm (C5). Native material ESI-MS *m/z* 351 [*M*+Na]$^+$. ^{1}H NMR (CD_3OD, 400 MHz): $\delta = 7.85$–7.68, 7.49–7.25 (8H, aromatic ring protons), 4.40 (d, 1H), 4.25 (t, 1H), 4.10 (dd, 1H), 3.90 (dd, 1H), 3.78 (dd, 1H), 3.55 (m, 1H), 3.40–3.10 ppm (m). ^{13}C NMR (CD_3OD, 300 MHz): $\delta = 146.1$, 145.9, 142.4, 128.5, 127.9, 126.6, 126.5, 120.7, 105.3, 77.8, 75.0, 73.3, 71.2, 66.9, 50.0 ppm, (some signal is attributable to more than one carbon atom).

2. Peracetyl derivative ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.76$, 7.58, 7.39, 7.30 (8H, aromatic ring protons), 5.12 (t, 1H, H-3 internal xylose), 5.09 (t, 1H, H-3 external xylose), 4.99 (dd, 1H, H-2 internal xylose), 4.87 (m, 1H, H-4 external xylose), 4.79 (dd, 1H, H-2 external xylose), 4.56 (d, $J = 7.8$ Hz, 1H, H-1 external xylose), 4.49 (d, $J = 7.8$ Hz, 1H, H-1 internal xylose), 4.26 (dd, 1H, Ar-CH_{2a}-*O*-β-Xyl), 4.15 (t, 1H, Ar-C*H*CH_2-*O*-β-Xyl), 4.10 (dd, 1H, H-5a external xylose), 3.98 (dd, 1H, H-5a internal xylose), 3.86 (m, 1H, H-4 internal xylose), 3.54 (dd, 1H, Ar-CH_{2b}-*O*-β-Xyl), 3.39 (dd, 1H, H-5b external xylose), 3.29 (dd, 1H, H-5b internal xylose), 2.03 ppm (acetyl group protons). Native material ESI-MS *m/z* 483 [*M*+Na]$^+$. ^{1}H NMR (CD_3OD, 300 MHz): $\delta = 7.85$–7.68, 7.49–7.25 (8H, aromatic ring protons), 4.50 (d, 1H), 4.45 (d, 1H), 4.35 (t, 1H), 4.28–3.40 ppm (signals of remaining sugar protons and nonaromatic protons of aglycone). ^{13}C NMR (CD_3OD, 300 MHz): 146.1, 145.9, 142.4, 128.5, 127.9, 126.6, 126.5, 120.7, 105.0, 104.0, 78.2, 77.6, 75.9, 74.8, 74.3, 73.3, 71.1, 67.1, 64.4, 49.8 ppm (some signal is attributable to more than one carbon atom).

3. Peracetyl derivative ^{1}H NMR ($CDCl_3$, 600 MHz): $\delta = 7.76$, 7.56, 7.37, 7.28 (8H, aromatic ring protons), 5.11, 5.09, 5.06 (m, 3H, H-3 xyloses external, middle, and internal respectively), 4.98 (dd, 1H, H-2 external xylose), 4.87 (m, 1H, H-4 external xylose), 4.79 (dd, 1H, H-2 middle xylose), 4.75 (dd, 1H, H-2 internal xylose), 4.55 (d, 1H, H-1 $J = 7.8$ Hz middle xylose), 4.49 (d, 1H, H-1 $J = 7.8$ Hz internal xylose), 4.47 (d, 1H, H-1 $J = 7.8$ Hz external xylose), 4.25 (dd, 1H, Ar-CH_{2a}-*O*-β-Xyl), 4.17 (t, 1H, Ar-C*H*CH_2-*O*-β-Xyl), 4.09 (dd, 1H, H-5a external xylose), 4.05 (m, 2H, H-5 internal and middle xyloses) 3.84 (m, 1H, H-4 middle xylose), 3.81 (m, 1H, H-4 internal xylose), 3.50 (t, 1H, Ar-CH_{2b}-*O*-β-Xyl), 3.45 (dd, 1H, H-5b external xylose), 3.25 ppm (m, 2H, H-5b internal and middle xyloses). ^{13}C NMR ($CDCl_3$, 400 MHz): xylose moieties $\delta = 100.8$, 100.5, 99.4, 75.3, 74.1, 72.2, 71.8, 71.0, 70.9, 70.4, 70.2, 68.2, 62.6, 62.4, 61.5 ppm. Native material ESI-MS *m/z* 615 [*M*+Na]$^+$. ^{1}H NMR (D_2O, 600 MHz, 3 mM] 7.92, 7.76, 7.49, 7.43 (8H, aromatic ring protons), 4.46, 4.44, 4.38, 4.34, 4.28, 4.15, 4.10, 3.98, 3.79, 3.71, 3.64, 3.56, 3.50, 3.44, 3.36, 3.31, 3.29, 3.28, 3.26, 3.21–3.20 ppm. ^{13}C NMR (CD_3OD, 300 MHz): 146.0, 145.9, 142.4, 128.4, 127.9, 126.6, 126.5, 120.6, 104.9, 103.9, 103.6, 78.1, 78.0, 77.5, 75.8, 75.6, 74.7, 74.2, 74.0, 73.3, 71.0, 67.0, 64.6, 64.3, 50.6 ppm (some signal is attributable to more than one carbon atom).

4. Peracetyl derivative ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.76$, 7.58, 7.39, 7.30 (8H, aromatic ring protons), 5.15–5.02 (m 4H xyloses H-3 signals), 4.96 (dd, 1H xylose H-2 signal), 4.87 (m 1H, H-4 external xylose), 4.80–4.70 (m, 3-H xyloses H-2 signals), 4.56 (d, 1H, anomeric xylose signal, $J = 7.8$ Hz), 4.50–4.46 (m 3H, xyloses anomeric signals), 4.26 (dd, 1H, Ar-CH_{2a}-*O*-β-Xyl), 4.17 (t, 1H, Ar-*CH*CH_2-*O*-β-Xyl), 4.05 (m, 2H xyloses H-5), 3.95 (m, 3H xyloses H-5), 3.80 (m, 3H, xyloses H-4), 3.56 (t, 1H, Ar-CH_{2b}-*O*-β-Xyl), 3.38–3.25 ppm (m, 3H xyloses H-5). Native material ESI-MS *m/z* 747 $[M+Na]^+$. ^{1}H NMR (CD_3OD, 600 MHz): $\delta = 7.85$–7.68, 7.49–7.30 (8H, aromatic ring protons), 4.43, 4.39–4.33, 4.25, 4.17–4.10, 3.92, 3.81, 3.70, 3.53, 3.47, 3.41–3.21 ppm.

The reaction for the formation of α-glucosides was conducted at 34 °C using 9-fluorenylmethanol, (50 mg 0.255 mmol), in 200 μL of DMSO added to a 10 mL of 1 M maltose in phosphate buffer (50 mM, pH 5.8). Enzyme solution (500 μL of partially purified α-glucosidase 4.2 mg mL^{-1}, 3.3 U mg^{-1})[31] was added and the mixture stirred at 34 °C for 24 h. TLC monitoring was performed by using EtOAc/MeOH/H_2O, 70:20:10 by volume. Chromatographic purification of the reaction mixture was based on reverse-phase column chromatography (Lobar RP-18), which efficiently separated the chromophoric products from maltose, glucose, and oligosaccharides produced by maltose bioconversion. This anomerically free-carbohydrate part eluted first in water whereas increasing gradients of methanol, allowed the separation of six different chromophoric α-glucosides (**5**–**10**). Preparative TLC was performed to obtain pure material for biological tests, and acetylation was conducted for precise determination of interglycosidic linkages by NMR spectroscopy.

5. Peracetyl derivative ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.80$–7.29 (8H, aromatic ring protons), 5.63 (t, 1H, H-3 glucose), 5.12 (d, 1H, $J =$ 3.1 Hz H-1 glucose), 5.10 (t, 1H, H-4 glucose), 4.94 (dd, 1H, H-2 glucose), 4.22 (m 2H, H-6a glucose, Ar-CH_{2a}-*O*-α-Glc), 4.07–4.05 (m 3H, H-6b glucose, Ar-*CH*CH_2-*O*-α-Glc, H-5 glucose), 3.54 ppm (t, Ar-CH_{2b}-*O*-α-Glc). ^{13}C NMR ($CDCl_3$, 300 MHz): $\delta = 144.5$, 143.6, 141.3, 141.2, 127.8, 127.7, 127.1, 127.0, 125.5, 124.9, 120.1, 120.0, 95.9, 70.8×2, 70.3, 68.6, 67.5, 61.9, 47.4 ppm. Carbonyl and methyl signals sets of acetyl groups are also present in the spectrum. DEPT experiments are in accord to the structure proposed. Native material ESI-MS *m/z* 381 $[M+Na]^+$.

6. Peracetyl derivative ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.80$–7.29 (8H, aromatic ring protons), 5.74 (dd, 1H, H-3 internal glucose), 5.45 (d, 1H $J = 3.1$ Hz H-1 external glucose), 5.42 (t, 1H, H-3 external glucose), 5.08 (t, 1H, H-4 external glucose), 5.01 (d, 1H $J = 3.1$ Hz H-1 internal glucose), 4.89 (dd, 1H, H-2 external glucose), 4.83 (dd, 1H, H-2 internal glucose), 4.40–3.92 (9H, H-5 and H-6 of both glucose units, Ar-CH_{2a}-*O*-α-Glc, Ar-*CH*CH_2-*O*-α-Glc, H-4 internal glucose), 3.54 ppm (t, 1H Ar-CH_{2b}-*O*-α-Glc).). ^{13}C NMR ($CDCl_3$, 300 MHz): $\delta = 144.5$, 143.7, 141.3×2, 127.8, 127.7, 127.2, 126.9, 125.6, 125.2, 120.1, 120.0, 95.8, 95.6, 72.9, 72.7, 71.3, 70.7, 70.1, 69.4, 68.5, 68.1, 68.0, 62.8, 61.4, 47.4 ppm. DEPT experiments are in accord to the structure proposed. Native material ESI-MS *m/z* 543 $[M+Na]^+$.

7. Peracetyl derivative ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.80$–7.29 (8H, aromatic ring protons), 5.64 (t, 1H, H-3 internal glucose), 5.50 (t, 1H, H-3 external glucose), 5.08 (d, 1H $J = 3.1$ Hz external glucose), 5.06 (d, 1H $J = 3.1$ Hz internal glucose), 5.02 (m, 2H, H-4 both glucoses), 4.87 (m 2H, H-2 both glucoses), 4.18 (m, 2H Ar-*CH*CH_2-*O*-α-Glc, Ar-CH_{2a}-*O*-α-Glc), 4.18–4.10, (m 2H, H-6 external glucose), 4.07 (m 2H, H-5 both glucoses), 3.73–3.56, (m 2H, H-6 internal glucose), 3.47 ppm (t, 1H Ar-CH_{2b}-*O*-α-Glc). Native material ESI-MS *m/z* 543 $[M+Na]^+$.

8. Peracetyl derivative ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.80$–7.29 (8H, aromatic ring protons), 5.73 (dd, 1H, H-3 internal glucose), 5.45 (dd, 1H, H-3 middle glucose), 5.43 (d, 1H $J = 3.1$ Hz external glucose), 5.37 (t, 1H, H-3 external glucose), 5.31 (d, 1H $J = 3.1$ Hz middle glucose), 5.08 (t, 1H, H-4 external glucose), 4.99 (d, 1H $J =$ 3.1 Hz internal glucose), 4.90 (dd, 1H, H-2 external glucose), 4.82 (dd, 1H, H-2 internal glucose), 4.77 (dd, 1H, H-2 middle glucose), 4.49–3.90 (m, 13H, H-5 and H-6 of the three glucose units, Ar-CH_{2a}-*O*-α-Glc 4.05, Ar-*CH*CH_2-*O*-α-Glc 4.30, H-4 of internal (3.99) and middle (4.00) glucoses), 3.51 ppm (t, 1H Ar-CH_{2b}-*O*-α-Glc). Native material ESI-MS *m/z* 705 $[M+Na]^+$. An additional TLC band (**9**,) contains a mixture of at least other two minor trisaccharides as indicated by NMR spectra.

10. Peracetyl derivative ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.80$–7.29 (8H, aromatic ring protons), 5.74 (dd, 1H, H-3 glucose), 5.49 (t, 1H, H-3 glucose), 5.43 (d, 1H, H-1 $J = 3.1$ Hz glucose), 5.42 (m, 1H, H-3 glucose), 5.37 (t, 1H, H-3 glucose), 5.33 (d, 1H $J = 3.0$ Hz external glucose), 5.30 (d, 1H, H-1 $J = 3.1$ Hz glucose), 5.08 (t, 1H, H-4 glucose), 5.00 (d, 1H, H-1 $J = 3.1$ Hz glucose), 4.86 (dd, 1H, H-2 glucose), 4.82 (dd, 1H, H-2 glucose), 4.76 (m, 2H, H-2 glucose), 4.49–3.90 (m, 17H, H-5, and H-6 of the four glucose units, Ar-CH_{2a}-*O*-α-Glc 4.08, Ar-*CH*CH_2-*O*-α-Glc 4.30, H-4 of three internal glucoses), 3.51 ppm (t, 1H Ar-CH_{2b}-*O*-α-Glc). ^{13}C NMR (CD_3OD, 600 MHz): $\delta =$ 145.4, 144.6, 143.7, 141.3, 141.2, 141.1, 127.8, 127.6, 127.2, 126.9, 126.8, 125.7, 125.2, 120.1, 120.0, 95.8, 95.7, 95.6×2, 73.8, 73.4, 72.7, 72.3, 71.8, 71.6, 71.3, 70.7, 70.5, 70.0, 69.4, 68.8, 68.4, 67.9, 63.0, 62.4, 62.2, 61.4, 47.3 ppm. Native material ESI-MS *m/z* 867, $[M+Na]^+$. Interglycosidic linkages are indicated by the absence of proton signals in the range 3.7–3.5 (glycosylated methylene position) as found in the isomaltoside **7**, by DEPT experiments and by the presence of a set of three signals at 82.4, 81.9, 81.7 ppm (three α-1,4 glycosylated positions) in the ^{13}C NMR spectra of native material.

The reaction for the formation of 9-fluorenylmethyl β-D-glucopyranoside was conducted in 10 mL of 100 mM sodium acetate buffer (20% acetonitrile), pH 5 using five molar excess of 9-fluorenylmethanol in 33 mM *p*-nitrophenyl β-glucopyranoside and 1.4 U of almond β-glucosidase for 24 h at 30 °C. The reaction for the formation of 9-fluorenylmethyl β-D-galactopyranoside was conducted in 12.5 mL of 100 mM sodium acetate buffer (20% acetonitrile), pH 5 using five molar excess of 9-fluorenylmethanol in 52 mM *p*-nitrophenyl β-galactopyranoside and 10 U of *A. oryzae* β-galactosidase for 4 h at 40 °C. At total consumption of donors both reaction mixtures were placed 3 min at 100 °C. Then **11** was purified and characterized as previously described[18] and **12** was acetylated for structural determination by NMR spectroscopy.

12. Peracetyl derivative ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.80$–7.29 (8H, aromatic ring protons), 5.42 (d, 1H, H-4 galactose), 5.38 (dd, 1H, H-2 galactose), 5.00 (dd, 1H, H-3 galactose), 4.52 (d, 1H, $J =$ 8.0 Hz H-1 galactose), 4.42 (dd, 1H Ar-CH_{2a}-*O*-β-Gal), 4.12 (m, 3H, H-6 Gal, Ar-CH_{2b}-*O*-β-Gal), 3.87 (t, 1H, H-5 galactose), 3.48 ppm (t, 1H Ar-$CHCH_{2b}$-*O*-β-Gal). Native material ESI-MS *m/z* 381 $[M+Na]^+$.

Isolation and treatment of human PBMC

PBMC were isolated from freshly collected buffy coats of healthy blood donors (Centro Trasfusionale, Policlinico Universitario "G. Martino", Messina, Italy), after centrifugation over Ficoll–Hypaque gradient. PBMC were then washed three times in RPMI-1640 medium and cultured in 24-well plates at a concentration of 2×10^6 cells mL^{-1} per well in RPMI 1640 medium. PBMC were cultured at 37 °C in 5% CO_2 atmosphere, in RPMI-1640 supplemented with

50 μg mL^{-1} gentamicin and 5% fetal calf serum (FCS). All culture media, reagents, and water were tested for the presence of endotoxin by E-Toxate kit (Sigma), and found to contain <10 pg endotoxin mL^{-1}. All tested compounds were diluted in DMSO at a concentration of 0.1 mg mL^{-1}, and then diluted in RPMI-1640 medium to obtain the final concentrations employed. PBMC were then treated with the compounds at different concentrations. After 24 and 48 h post treatment, the supernatants were harvested, separated into aliquots, and stored at $-80\,^{\circ}$C until cytokine analysis. In a second series of experiments, we evaluated the production of IFN-α and TNF-α by PBMC added with compounds at the concentrations of 60, 40, and 20 μg mL^{-1}. After 24 and 48 h of incubation at 37 °C in 5% CO_2, supernatants from untreated and treated PBMC were harvested, centrifuged, and kept at $-80\,^{\circ}$C.

Cytotoxicity tests

To determine the effect of different concentrations of the named compounds on cell viability, a colorimetric assay was used as described by Mosmann.[39] The assay is based on the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a pale yellow substrate that is cleaved by active mitochondria to produce a dark blue formazan product. Briefly, PBMC and WISH cells were seeded onto 96-well culture plates at a number of 10^4 per well. After 4 h of incubation to allow seeding of the cells, various concentrations of the compounds were added into each well. The plate was then incubated at 37 °C with an atmosphere of 5% of CO_2 for 48 h. Later, the medium was discarded and the MTT reagent was added. The plate was re-incubated at 37 °C for an additional 3 h to allow the development of formazan. The microplates were read with a microELISA reader using a wavelength of 570 nm. Cytotoxicity percentage was calculated as follows: 1−[(experimental OD−lysis control OD)/(cell control OD−lysis control OD)] 100.

Cytokine evaluation

The titration for the presence of IFN-α, TNF-α, and IL-12 was performed by an immunoenzymatic method (ELISA) from Bender MedSystems (Milan, Italy). The limit of detection of the assay was <3.1 pg mL^{-1} for IFN-α, <0.13 pg mL^{-1} for TNF-α, and <2.1 pg mL^{-1} for IL-12.

Virus

HSV-2 strain G was used throughout the study. HSV-2 infection was propagated on WISH cell lines. Viral stocks were prepared by pelleting infected cells exhibiting cytopathic effect, and freezing aliquots at $-80\,^{\circ}$C. The virus titer was assessed on WISH cells and expressed as plaque-forming units (PFU) per mL.

Treatment and infection

To evaluate antiviral activity, PBMC or WISH cells were seeded onto 24-well culture plates at a density of 2×10^6 cells per well. Then, PBMC was added with compounds at the concentration indicated and incubated for 24 h at 37 °C in 5% CO_2. After this period, cells were infected with HSV-2 at a multiplicity of infection (MOI) 0.1 and incubated for a further 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 $-80\,^{\circ}$C until virus titration. The virus titer was expressed as plaque-forming units (PFU) per mL.

Statistical evaluation

Results are expressed as the mean ± standard deviation (SD) of four experiments. Data were analyzed by one-way analysis of variance (ANOVA) and the Student–Newman–Keults test. Differences were considered statistically significant for a p value of <0.05.

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