Available online at www.sciencedirect.com
ScienceDirect
European Journal of Medicinal Chemistry 43 (2008) 2656–2664
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Original article

2/4-Substituted-9-fluorenones and their *O*-glucosides as potential immunomodulators and anti-herpes simplex virus-2 agents. Part 5

Adriana Arena [a], Nicoletta Arena [b], Rosella Ciurleo [c], Ambra de Gregorio [d], Rosanna Maccari [c], Rosaria Ottana' [c], Bernadette Pavone [a], Annabella Tramice [e], Antonio Trincone [e], Maria Gabriella Vigorita [c,*]

[a] *Dipartimento di Discipline Chirurgiche, Sezione Microbiologia, Policlinico Universitario ''G. Martino'', 98123 Messina, Italy*
[b] *Dipartimento di Chimica Organica e Biologica, Università di Messina, Salita Sperone 31, 98166 Messina, Italy*
[c] *Dipartimento Farmaco-chimico, Università di Messina, Viale SS. Annunziata, 98168 Messina, Italy*
[d] *Dipartimento Farmaco-biologico, Università di Messina, Viale SS. Annunziata, 98168 Messina, Italy*
[e] *Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, 80078 Pozzuoli, Napoli, Italy*

Received 20 July 2007; received in revised form 14 November 2007; accepted 24 January 2008
Available online 14 February 2008

Abstract

In pursuing a research on the antiviral and immunomodulatory activity of tilorone congeners, two new series of compounds were prepared and pharmacologically explored: 9-fluorenone carboxyhydroxyesters, indicated as **AG**, and 9-fluorenone carboxyhydroxamides, indicated as **MG**. Two of them, **AG17** and **MG3**, were used as sugar acceptors in the transglycosylation reactions performed by α- and β-glucosidases extracted from the marine mollusc *Aplysia fasciata* providing different α- and β-, mono- and oligosaccharides. Then aglycons and saccharides were assayed for cytotoxicity, for anti-herpes virus-2 properties on peripheral blood mononuclear cells (PBMC) and for their capability to trigger human cells to produce antiviral cytokines such as IFNα and TNFα. Some promising compounds were individuated whereas the utility of the biocatalytic procedures in the preparation of pure anomeric material was further focused.

Keywords: 9-Fluorenone-4-carboxyhydroxyesters; 9-Fluorenone-2-carboxyhydroxamides; α and β-*O*-Glucosides; Enzymatic transglycosylation; Immunomodulatory; Antiviral activity

1. Introduction

For a long time, researchers and clinicians have been attracted by the possibility to control virus replication or tumoral growth by taking advantage of the immune system processes. Nowadays the immunopharmacology is going to be a central part of 21st century medicine.

An immunomodulator is any substance that helps to regulate the immune system. This ''regulation'' is a normalisation process, so that an immunomodulator helps to optimise immune response. In fact, immunomodulators do not tend to boost immunity, but to normalise it. Part of their benefit is due to their ability to naturally increase the production of messenger molecules such as cytokines and chemokines, which mediate and regulate the immune system [1].

The efficient elimination of a viral infection requires a proinflammatory host response and development of type 1 immunity. This type of response is characterised by activation of mononuclear cells and production of proinflammatory cytokines such as interferons (IFN), tumor necrosis factor (TNF) and interleukins (IL-12 and IL-18).

In fact, these cytokines, regulating the inflammatory and immune responses, may induce an antiviral state in the cells (i.e., via IFNs) or destroy virus-infected cells (i.e., via TNFα), may stimulate cytotoxicity and cytokine production by T-cells and NK cells, and may initiate development of Th1 cells (i.e., via IFNγ, IL-12 and IL-18) [2].

* Corresponding author. Tel.: +39 090 6766419/6408; fax: +39 090 355613.
E-mail address: vigorita@pharma.unime.it (M.G. Vigorita).

0223-5234/$ - see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2008.01.045

On the other hand, viruses have learned how to block cytokine production, activity and signal transduction, in order to evade the immune response of the host [3]. In fact many viruses possess several mechanisms to counteract the antiviral response raised by host immune system. Recently viral mechanisms of mimicry of cytokines and cytokine receptors have been identified [4].

Herpes simplex viruses establish lifelong, latent infections in the peripheral nervous system, thus escaping the immune response and persisting in the host [5]. Moreover, herpes viruses may reactivate from their latent state and cause severe recurrent and chronic diseases [6]. In addition, the ever-increasing number of immunocompromised (transplanted, cancer or AIDS) patients, has highlighted the involvement of herpes virus infections in these diseases [7]. On the other hand, the intensive use of antivirals (acyclovir and congeners) has led to the emergence of resistant viral strains, which are commonly found in immunocompromised patients [8].

Therefore novel drugs, possibly characterised by different mechanisms of action and improved pharmacokinetic features, are more and more required.

At cellular level, the IFN production can be caused by a series of interferences; among them, the DNA intercalation by which planar aromatic or heteroaromatic agents, arranging themselves among adjacent pairs of bases, alter the double helix of DNA and consequently disturb the physiological functions of many important DNA enzymes [9]. Several synthetic compounds possessing IFN-inducing activity have been reported in the search for novel agents that, due to intercalation, induce IFN production which, in turn, might stimulate antiviral and/or cytostatic effects in the cell [10]. Among them, tilorone, 2,7-bis[2(diethylamino)-ethoxy]-9*H*-fluoren-9-one, chosen as *lead compound* in our continuing research, was the first low-molecular weight IFN-inducer, orally effective in vivo against some DNA and RNA viruses as well as against some cancers [11]. Tilorone analogues, in which only a lateral basic chain was present, have been explored by some of us [12]. In such derivatives we designed to eliminate the symmetrical substitution pattern on the fluorenone skeleton, since it had been reported as responsible for the onset of mucopolisaccaridosis, the main ADR of tilorone [13] that, in turn, had been responsible for its withdrawing from the market [14].

In the novel compounds the presence of carbohydrate residues might have improved the intercalation mode, stabilising the DNA/compound complexes, as it happens in antitumoral anthracyclines, well-known intercalators [15].

Today, we can observe a renewed interest in carbohydrate chemistry on the basis of great progress of glycobiology [16]. In an ever-increasing number of drugs, the insertion of sugar moieties improves solubility, pharmacokinetic and sometimes pharmacodynamic properties [17], and many examples are reported of carbohydrate leads that are optimised in order to develop innovative drugs [18].

In the stereocontrolled construction of glycosidic bonds, which is a great challenge for organic synthetists, biocatalysis offers many advantages. In particular, enzymatic strategies for stereospecific construction of glycosidic bonds are mainly based on the action of glycosyl hydrolases (*endo*- and *exo*-glycosidases) or on the use of glycosyltransferases [19]. The glycosyl hydrolases activities, present in several dozen enzymatic preparations, have been recently reviewed [20]. The marine environment has been shown to be very interesting for the isolation of new glycosyl hydrolases, not only for their hydrolytic potential but also in synthesis. The mollusc *Aplysia fasciata* is one of the most promising organisms among those cited. In fact the hepatopancreas and visceral mass of this sea hare contain a wide range of glycosyl hydrolases [21] (α-glucosidase, β-galactosidase, β-glucosidase, β-mannosidase and others) which have already been successfully used for the hydrolysis and synthesis of glycosidic bonds. The formation of α-D-oligoglucosides (up to tetra- and pentasaccharides) was recently reported [22,23].

The α-D-glucosidase activity of *Aplysia fasciata* was used here to synthesize α-glucosides of model fluorenones (**AG17** and **MG3**), and the β-glucosidase activity from the same organism was used to synthesize the corresponding β-glucosides. In pursuing our research, on the basis of the considerations above, we investigated the effects of new synthetic 9-fluorenone containing compounds, and their mono- and oligosaccharides on HSV2 replication in Peripheral Blood Mononuclear Cells (PBMC).

In addition, we investigated whether anti-HSV2 activity, observed in PBMC, could be correlated with the capacity to trigger PBMC in releasing cytokines involved in antiviral immune response such as TNFα and IFNα.

The extension of the structure/activity (SAR) study was another of our objectives.

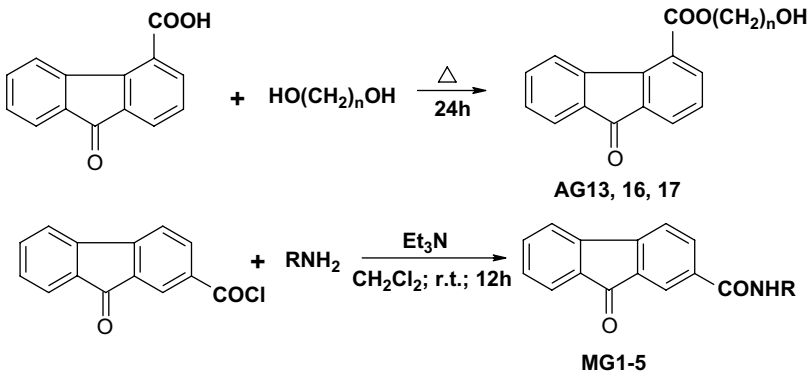
2. Chemistry

The synthesis of the 9-fluorenone-4-carboxyhydroxyesters (indicated as **AG**) and 9-fluorenone-2-carboxamides (indicated as **MG**) is depicted in Scheme 1.

In both series the lateral chains contain primary hydroxy groups suitable to be enzymatically glycosylated. In particular 9-oxo-9*H*-fluorene-4-carboxyhydroxyesters **AG13**, **AG16**, **AG17** were obtained in moderate to low yields, by esterification reaction between commercial 9-fluorenone-4-carboxylic acid and an excess of appropriate diol; the use of an acid catalyst was avoided because of unwanted rearrangements. The synthesis of 9-oxo-9*H*-fluorene-2-carboxyhydroxamides (**MG1–5**) was carried out by reaction of commercial 2-carboxy-9-fluorenone chloride with suitable aminoalcohols in the presence of triethylamine. When possible, the same lateral chains have been used in order to investigate the role exerted by the type and/or substitution position on the biological activity.

Table 1 collects analytical, physico-chemical and spectroscopic data relative to **AG** and **MG** fluorenones.

The enzymatic synthesis of the α- and β-*O*-glucosides of **AG17** and **MG3** fluorenones, used as sugar acceptors in transglycosylation reactions, was performed by the use of crude homogenate preparations of the α-glucosidase and β-glucosidase activities from *Aplysia fasciata* (Table 2).



Scheme 1. Synthesis of 9-oxo-9*H*-fluorene-4-carboxyhydroxyesters **AG 13**, **16**, **17** and 9-oxo-9*H*-fluorene-2-carboxyhydroxamides **MG1–5**.

Yields were satisfactory for the first enzyme showing an interesting polyglucosylation as in other cases [22,23], but not for the β-glucosidase; however both enzymatic reactions were not optimized to achieve more appreciable yields.

It was previously demonstrated that the α-glucosidase from *A. fasciata* was able to form mono- and diglucosides of pyridoxine in a reaction mixture where high concentration of maltose was present [23]. The activity and the good resistance of this enzyme in the presence of organic cosolvent DMSO up to 10–20% was previously established [21].

Here this enzyme was used for the α-glucosylation of **AG17** and **MG3** that were present in the reaction mixture at 8 mM concentration. For the former, up to trisaccharides were formed (Table 2), while for both acceptors, isomaltoside (**AG17B1** and **MG3A**) and maltoside (**AG17C1** and **MG3B**) derivatives were obtained, in accord to the action of this enzyme on maltose and pyridoxine [23]. The satisfactory overall yield of 30% obtained for **AG17** drops down to 10% for **MG3** probably due to the elongation of the spacer or different position on aromatic moiety.

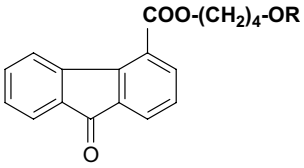
β-Glucosides of **AG17** and **MG3** were also formed exploiting a β-glucosidase activity present in the crude homogenate of *A. fasciata*. The harsh reaction conditions (13% organic solvent, overall organic acceptor and donor, about concentration 300 mM) in which the enzymes operate are an encouraging starting point toward optimization of these processes and can

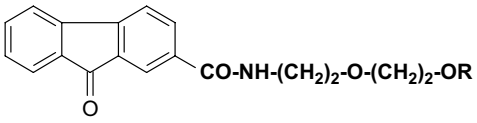
Table 1
Analytical, physico-chemical and ^{1}H NMR data of 9-fluorenone compounds **AG** and **MG**

Compound	R	M.p. (°C)	Yield (%)	Formula	Elementary analysis	^{1}H NMR[a] data
AG13	4-CO–O–$(CH_2)_2$OH	118–120	45	$C_{16}H_{12}O_4$	Calc. %: C 71.64; H 4.47; O 23.88. Found %: C 71.50; H 4.30; 23.88.	7.35–8.30 (m, 7H, ArH); 4.58 (m, 2H, COOCH_2); 4.03 (m, 2H, CH_2OH).
AG16	4-CO–O–$(CH_2)_3$OH	56–59	15	$C_{17}H_{14}O_4$	Calc. %: C 72.34; H 4.96; O 22.69. Found %: C 72.43; H 5.05; O 22.45.	7.34–8.29 (m, 7H, ArH); 4.60 (t, 2H, COOCH_2, $J = 6.3$); 3.84 (t, 2H, CH_2OH, $J = 6.3$); 2.08 (m, 2H, COOCH$_2CH_2$).
AG17	4-CO–O–$(CH_2)_4$OH	55–58	23	$C_{18}H_{16}O_4$	Calc.%: C 72.97; H 5.40; O 21.62. Found %: C 72.95; H 5.46; O 21.67.	7.32–8.29 (m, 7H, ArH); 4.46 (t, 2H, COOCH_2, $J = 6.6$); 3.75 (t, 2H, CH_2OH, $J = 6.3$); 1.93 (m, 2H, COOCH$_2CH_2$); 1.75 (m, 2H, HOCH$_2CH_2$).
MG1	2-CO–NH–$(CH_2)_2$OH	162–164	90	$C_{16}H_{13}NO_3$	Calc. %: C 71.91; H 4.86; N 5.24; O 17.97. Found %: C 72.10; H 4.97; N 5.13; O 18.11.	7.33–8.20 (m, 7H, ArH); 6,76 (br s, 1H, NH); 3.88 (t, 2H, CH_2OH, $J = 4.8$); 3.68 (m, 2H, CH_2N).
MG2	2-CO–NH–$(CH_2)_3$OH	175–177	90	$C_{17}H_{15}NO_3$	Calc. %: C 72.59; H 5.33; N 4.98; O 17.08. Found %: C 72.45; H 5.51; N 4.70; O 17.20.	7.37–8.10 (m, 7H, ArH); 3.77 (t, 2H, CH_2OH, $J = 3.0$); 3.67 (m, 2H, CH_2N); 1.85 (m, 2H, CH_2CH_2OH).
MG3	2-CO–NH–$(CH_2)_2O(CH_2)_2$OH	123–125	48	$C_{18}H_{17}NO_4$	Calc. %: C 69.45; H 5.46; N 4.50; O 20.57. Found %: C 69.60; H 5.55; N 4.66; O 20.33.	7.28–8.09 (m, 7H, ArH); 3.81 (m, 2H, CH_2OH); 3.71 (brs, 4H, CH_2OCH_2); 3.66 (m, 2H, CH_2N).
MG4	2-CO–NH–$(CH_2)_4$OH	140–143	44	$C_{18}H_{17}NO_3$	Calc. %: C 73. 22; H 5.76; N 4.74; O 16.27. Found %: C 73.41; H 5.80; N 4.70; O 16.32.	7.12–7.91 (m, 7H, ArH); 3.39 (t, 2H, CH_2OH, $J = 6.0$); 3.20 (m, 2H, CH_2N); 1.47 (m, 4H, CH_2).
MG5	2-CO–NH–$CH_2CHOHCH_3$	174–176	51	$C_{17}H_{15}NO_3$	Calc. %: C 72.59; H 5.33; N 4.98; O 17.08. Found %: C 72.40; H 5.40; N 5.20; O 16.90.	7.34–8.15 (m, 7H, ArH); 6.70 (brs, 1H, NH); 4.07[b] (m, 1H, *CH*); 3.73[c] (m, 1H, CH_2); 3.33[d] (m, 1H, CH_2); 1.29 (d, 3H, $J = 6.3$, CH_3).

[a] (δ) $CDCl_3$ solution, except for **MG4** DMSO-d_6 solution; *J* values are expressed in hertz.
[b] X part of an ABX system.
[c] B part of an ABX system.
[d] A part of an ABX system.

Table 2
Structures of α- and β-glucosides





Compound	R	Compound	R
AG17A1	−α-(Glc)$_3$		
AG17B1	−α-Glc−(1,6)-Glc	**MG3A**	−α-Glc−(1,6)-Glc
AG17C1	−α-Glc−(1,4)-Glc	**MG3B**	−α-Glc−(1,4)-Glc
AG17D1	−α-Glc	**MG3C**	−α-Glc
AG17β	−β-Glc	**MG3β**	−β-Glc

lead to interesting results although glucosides were obtained with very low yields.

After chromatographic purification and usual acetylation procedure, ^{1}H NMR spectra and 2D COSY experiments for the glucosidic derivatives allowed the unambigous structural assignment. 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 glycosylation position for the upfield shift of the signal due to the absence of the acetyl group (see Section 6).

3. Pharmacology

Like the previously studied series [12], these fluorenone derivatives were submitted to a pharmacological screening to evaluate cytotoxicity, IFNα and TNFα inducing properties and antiviral activity against Herpes simplex virus type 2 (HSV2) on human PBMC.

The stability of the glycosides in the test media over the assay timeframe was evaluated by TLC.

In Table 3 the effects of different concentrations of compounds on cell viability are reported.

Table 3
Cytotoxicity percentage on human PBMC[a]

Compound	60 μg/mL	40 μg/mL	20 μg/mL
MG1	2	0	0
MG2	15	0	0
MG3	4	0	0
MG4	45	28	20
MG5	5	0	0
MG3A	21	0	0
MG3B	18	0	0
MG3C	16	0	0
MG3β	6	0	0
AG13	14	6	0
AG16	41	22	0
AG17	38	21	0
AG17A1	42	19	0
AG17B1	52	29	0
AG17C1	69	37	26
AG17D1	57	38	22
AG17β	7	0	0

[a] Results represent the means of three experiments using the cells of the same donor. In all the experiments S.D. was less then 20%.

Data are represented as percentage of cytotoxicity of the tested molecules at the concentrations of 60, 40 and 20 μg/mL on PBMC. At the concentration of 60 μg/mL all compounds were cytotoxic, the most cytotoxic being **MG4**. At the concentration of 40 μg/mL, compounds **MG1−3**, **MG5** and glucosides **MG3A−C**, as well as **MG3β** and **AG17β** did not show cytotoxicity. At the concentration of 20 μg/mL, only **MG4**, α mono-Glc, **AG17C1** and α-Glc-(1−4)-Glc, **AG17D1** displayed cytotoxicity. On these bases, in the evaluation of antiviral activity and IFNα and TNFα production, we tested only the molecules and the concentrations which were not cytotoxic.

4. Results and discussion

The results concerning the inhibition of HSV2 replication are reported in Table 4.

The addition of **MG1−3**, **MG5**, **MG3A−C** and **MG3β** to PBMC resulted in a decrease in HSV2 replication. In particular, the most marked effect on viral replication was observed in the presence of 40 μg/mL of compounds **MG2**, **MG3** and

Table 4
Anti-HSV-2 activity (PFU/mL) on human PBMC[a]

Compound	40 μg/mL	20 μg/mL
MG1	3.7×10^4*	4.8×10^4*
MG2	4.1×10^3*	6.5×10^3*
MG3	3.9×10^3*	5.3×10^3*
MG5	3.5×10^3*	6×10^3*
MG3A	3.5×10^4*	4.2×10^4*
MG3B	3.2×10^4*	5.3×10^4
MG3C	3.4×10^4*	6×10^4
MG3β	3.6×10^4*	4.9×10^4*
AG13	nd[b]	5.8×10^4
AG16	nd[b]	6×10^4
AG17	nd[b]	7.1×10^4
AG17A1	nd[b]	7×10^4
AG17B1	nd[b]	6.8×10^4
AG17β	3.6×10^4*	7.8×10^4
Virus control	9.2×10^4	

In all the experiments S.D. was less then 20%.
*$p < 0.05$ vs virus control.
[a] Results represent the means of three experiments using the cells of the same donor.
[b] nd: not-determined because cytotoxic.

MG5 which produced 4.1×10^3, 3.9×10^3 and 3.5×10^3 PFU/mL, respectively, vs 9.2×10^4 PFU/mL of untreated cells ($p < 0.05$). At the concentration of 40 μg/mL, also glucosides from **MG3** significantly influenced the HSV2 replication with a reduction of viral replication from 62% (isomaltoside **MG3A**) to 65% (maltoside **MG3B**). **MG3A** and **MG3B** were also effective at 20 μg/mL. Monoglucosides **MG3C** and **MG3β** were almost equipotent at both doses.

On the contrary compounds **AG**, tested only at the concentration of 20 μg/mL, because cytotoxic at higher concentrations, were not effective in inhibiting viral replication, except β-mono-Glc **AG17β** that at 40 μg/mL provided the significant reduction of HSV2 replication (Table 4). It is interesting to note the beneficial effects exerted by the presence of a glucose unit in β-configuration on **AG17** structure. The same favourable trend was observed in the evaluation of cytotoxicity (Table 3) as well as in the production of cytokines by PBMC (Table 5).

Table 5
IFNα and TNFα production by PBMC at 24 and 48 h after incubation in presence of compounds **AG** and **MG**[a]

Compound	IFNα pg/mL 24 h	IFNα pg/mL 48 h	TNFα pg/mL 24 h	TNFα pg/mL 48 h
MG1				
20 μg/mL	<4.8	11	<5.8	13
40 μg/mL	9	29	14	36
MG2				
20 μg/mL	<4.8	7	<5.8	40
40 μg/mL	13	37	27	51
MG3				
20 μg/mL	<4.8	21	<5.8	17
40 μg/mL	6	38	24	48
MG5				
20 μg/mL	<4.8	18	<5.8	20
40 μg/mL	21	45	14	33
MG3A				
20 μg/mL	<4.8	14	<5.8	16
40 μg/mL	<4.8	25	<5.8	21
MG3B				
20 μg/mL	<4.8	10	<5.8	18
40 μg/mL	<4.8	30	<5.8	27
MG3C				
20 μg/mL	<4.8	17	<5.8	14
40 μg/mL	<4.8	31	<5.8	30
MG3β				
20 μg/mL	<4.8	17	9	31
40 μg/mL	8	31	13	39
AG13				
20 μg/mL	<4.8	16	<5.8	21
AG16				
20 μg/mL	<4.8	8	<5.8	11
AG17				
20 μg/mL	<4.8	12	<5.8	6
AG17A1				
20 μg/mL	<4.8	14	<5.8	9
AG17B1				
20 μg/mL	<4.8	9	<5.8	15
AG17β				
20 μg/mL	<4.8	10	<5.8	18
40 μg/mL	<4.8	29	<5.8	30

[a] Results represent the means of three experiments using the cells of the same donor. In all the experiments S.D. was less then 20%.

Similarly, in the previously investigated 9-fluorenone-2-carboxyhydroxyesters, the insertion of a β glucose unit had put at zero the cytotoxicity also at the concentration of 100 μg/mL, and improved other pharmacological properties [12a].

In order to assess whether the observed antiviral activity might be related to an immunomodulatory activity of the compounds, the production of cytokines IFNα and TNFα involved in the immune surveillance toward virus infection, was evaluated. The results are shown in Table 5.

Appreciable levels of such cytokines were detected in supernatants from PBMC at 48 h post treatment with active compounds **MG2**, **MG3**, **MG5** followed by **MG1**, glucosides **MG3A–C** and **MG3β**. Moreover, their effects were dose- and time-dependent.

By comparing aglycon **MG3** with its glucosides, the greater effectiveness of the aglycon was evident.

In contrast, derivatives **AG**, assayed only at the concentration of 20 μg/mL, induced the production of a lower amount of cytokines, except β-monoglucoside that, in both concentrations, produced relevant amounts of IFNα and, even more, TNFα. This datum was closely related with its significant antiviral activity displayed at the concentration of 40 μg/mL.

Infection of untreated PBMC with HSV2 did not induce any cytokine production.

In general the marked cytokine production observed in PBMC treated with all effective compounds might be closely related to the observed antiviral activity. Whereas, the low cytokine production by PBMC treated with ineffective compounds is correlated with marked amounts of viruses similar to those obtained in virus control.

Taken together the results discussed so far demonstrate that 9-fluorenone-2-carboxamides **MG**, except **MG4**, possess better pharmacological profiles than those of carboxyesters **AG**.

In order to explore the role exerted by the nature of substituents on fluorenone moiety, we have compared 2-carboxamides **MG** with the previously explored 2-carboxyesters [12a]. Compounds **MG** proved to be slightly more cytotoxic than the corresponding 2-carboxyesters. In fact, at 60 μg/mL, they displayed percentages from 45% (**MG4**) to 2% (**MG1**), whereas corresponding esters, assayed at a concentration of 100 μg/mL, had displayed percentages from 24% to 8% [12a].

Moreover, 9-fluorenone-4-carboxamides explored in a precedent note had shown a general greater cytotoxicity than **MG** compounds, following the same trend that was observed also in the series of 2- and 4-substituted carboxyesters. This suggests that in general the substitution in position 4 of the aromatic moiety, produces an increase in cytotoxicity, while anti-HSV2 activity and cytokines production decrease [12b].

The main SAR that can be drawn from the evaluation of glucosides and oligosaccharides, is that the β-configuration is always able to confer the most interesting features, along with scarce or no cytotoxicity. This finding proved to be independent of the type of lateral chain and the substitution in position 2 or 4 of fluorenone skeleton. In fact, similar levels of cytotoxicity were displayed by **MG3β** and **AG17β**. As above said, the same trend was evident in anti-HSV2 and in cytokines production evaluation (Tables 3–5), whereas β-glucosides of

previously studied 9-fluorenone-2-carboxyhydroxyesters had also displayed good profiles [12a].

In contrast, α-glucoconjugates obtained by **MG3** and **AG17** aglycons, exhibited worse pharmacological profiles than β-isomers. However, derivatives **MG** are still better than **AG** ones (Tables 3–5). Among these latter, the mono-Glc **AG17D1** and the 1,4-disaccharide **AG17C1** were found to be the most cytotoxic, thus not further evaluated.

A conformational analysis, as in previously explored fluorenones [12a,b] along with molecular docking procedures are in progress to try to rationalise at molecular level, the results so far obtained.

5. Conclusions

Since the immune system plays a fundamental role in host defense against pathogens as well as against tumors, a safe compound with immunomodulatory property for clinical use has become a major goal of many research groups. It is well-known that efficient elimination of viruses relies on the ability of the infected host to mount a proinflammatory immune response and develop a Th1-type immunity that is able to restrict viral replication [25]. This response is strictly characterized by the activation of monocytes/macrophages, natural killer cells, cytotoxic T-lymphocytes and production of proinflammatory cytokines and chemokines, including IFNs, TNFα and various interleukins.

In this context 9-fluorenone-2-carboxamides **MG1–3**, **MG5** and **MG3A–C** and **MG3β** were found to be able to inhibit virus replication in PBMC probably by up-regulating expression of peculiar cytokines such as IFNα and TNFα: thus they may contribute to improve immune surveillance of PBMC toward HSV2 infection.

Better activity profiles were observed in 2-carboxamides **MG** than in 4-carboxyesters **AG**. By comparing these results with our previous results relative to 2-carboxyesters [12a] and 4-carboxamides [12b], we have deduced that the 2 substitution on 9-fluorenone skeleton is more beneficial than that in 4 position. Furthermore the amidic nature of lateral chain also have a positive role that is conserved in the previously studied 4-carboxamides, whose interesting DNA binding properties have been recently reported [26].

On the whole the data here presented point out the possible use of some 9-fluorenone-2-carboxamides in the elicitation of therapeutic responses in clinical settings of viral diseases as well as in immunocompromised host.

6. Experimental protocols

6.1. General chemistry

Melting points were determined with a Kofler–Reichert hot-stage apparatus and are uncorrected. Precoated silica gel plates F 254 for analytical controls and chromatographic columns 70–230 mesh SiO_2 were used for separations. Chromatographic purification of crude glucosides was performed by reverse-phase column chromatography (Lobar RP-18). Elemental analyses (C, H, N), determined by means of a C. Erba mod. 1106 elem. analyzer, were within ±0.4% of theory.

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker instruments at 600, 400, and/or 300 MHz. Samples for NMR analysis were dissolved in the appropriate solvent and the downfield shift of the signal of the solvent was used as internal standard.

Electrospray ionization mass spectrometry (ESIMS) spectra were obtained on a Q-Tof mass spectrometer, Micro (Micromass).

The enzymes were obtained as previously reported [21] from the marine mollusc *Aplysia fasciata*. Partially purified or simply concentrated crude homogenates were used. Analytical scale reactions to assess aptness of biocatalysts were conducted by using enzymes in presence of organic solvent and acceptors.

6.2. General synthesis of 9-oxo-9H-fluorene-4-carboxyhydroxyesters (*AG13*, *16*, *17*)

An excess of appropriate diol (30 ml) was added to 9-fluorenone-4-carboxylic acid (0.5 g, 2,23 mmol). The mixture was stirred at room temperature for 12 h, then the solution was evaporated under vacuum. The residue was dissolved in CH_2Cl_2 and washed with a saturated solution of $NaHCO_3$ and then with distilled water. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under vacuum. The product was purified by silica gel chromatography (CH_2Cl_2/CH_3OH 9:1).

The analytical, physico-chemical and spectroscopic data of compounds **AG** are collected in Table 1.

6.3. General synthesis of 9-oxo-9H-fluorene-2-carboxyhydroxamides (*MG1–5*)

A solution of appropriate aminoalcohol (6 mmol) and triethylamine (0.42 ml, 3 mmol) in anhydrous CH_2Cl_2 was added to a solution of 9-oxo-9*H*-fluorene-2-carbonyl chloride (0.5 g, 2 mmol) in anhydrous CH_2Cl_2 (10 ml). The mixture was stirred at room temperature for 24 h and then washed with distilled water. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated under vacuum. The residue was purified by silica gel chromatography (CH_2Cl_2/CH_3OH 9:1) or by crystallization from CH_3OH.

Their analytical, physico-chemical and spectroscopic data are collected in Table 1.

6.4. Enzymatic reactions

α and β-Glucosidations of **AG17** and **MG3** were performed by the use of crude homogenate of *Aplysia fasciata* in presence of organic cosolvent. After purification, the glycosides were TLC pure and presented homogeneous proton NMR spectra; ionization mass spectra confirm their molecular weights. After usual acetylation procedure, ^1H NMR and 2D COSY spectra allowed the unambigous structure assignment.

6.4.1. Synthesis of α-glucosides of *AG17*

AG17 (50 mg, 0.169 mmol) dissolved in 100 μl of DMSO was added to 20 ml of 500 mM maltose solution in phosphate

buffer (50 mM, pH 5.8). Partially purified crude homogenate (150 μl) of *Aplysia fasciata* containing α-glucosidase (31 U/μl) was added to the resulting suspension in three portions during reaction time. The mixture was stirred at 34 °C for 24 h. TLC monitoring was performed by using EtOAc/MeOH/H_2O 70:20:10 by vol. 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. The latter eluted first in water while increasing gradients of methanol allowed the separation of different α-glucosides of the acceptor used. Acetylation was conducted by standard procedures using Ac_2O/pyridine for 24 h.

The products were formed in a total yield of about 30% with respect to aglycone added and were composed by **AG17D1** (13%), two different disaccharides **AG17B1** and **AG17C1** (14%, 75:25 ratio, respectively) and a trisaccharide **AG17A1** (3%) as indicated by mass spectra of acetylated derivatives (**AG17D1** 649.27 $M^+ + Na^+$, **AG17B1** 937.64 $M^+ + Na^+$, **AG17C1** 937.64, **AG17A1** 1225.92 $M^+ + Na^+$). The interglycosidic linkages were established by 2D NMR spectroscopy. Coupling constants of the newly formed anomeric linkages secured for α-configuration and aromatic rings show signals in accord with the proposed structures.

AG17D1. *Saccharidic moiety* δ ^{1}H (^{13}C): H1 5.09 (95.80), H2 4.87 (70.91), H3 5.49 (70.17), H4 5.05 (68.63), H5 4.02 (67.31), H6 4.08–4.25 (61.94). *Spacer moiety*: δ ^{1}H (^{13}C): CH_2–O–α-Glc 3.77–3.47 (68.02), CH_2–CH_2–O–α-Glc 1.80 (25.51), CH_2–CH_2–O–aromatic 1.92 26.00, CH_2–O–aromatic 4.45 (65.15).

AG17C1. *Saccharidic moiety* δ ^{1}H: O–α-Glc–(1,4)-*Glc*, H1 5.43, H2 4.86, H3 5.38, H4 5.08, H5 4.05, H6 4.27–4.47; O–α-*Glc*–(1,4)-Glc, H1 4.98, H2 4.75, H3 5.54, H4 3.98, H5 4.02, H6 4.30–4.16. ^{13}C: 95.65 × 2, 72.88, 72.63, 71.44, 70.01, 69.36, 68.51, 68.05, 67.69, 62.81, 61.45. *Spacer moiety* δ ^{1}H (^{13}C): CH_2–O–α-Glc 3.80–3.50 (68.14), CH_2–CH_2–O–α-Glc 1.81 (25.49), CH_2–CH_2–O–aromatic 1.90 (26.00), CH_2–O–aromatic 4.45 (65.22).

AG17B1. *Saccharidic moiety* δ ^{1}H: O–α-Glc–(1,6)-*Glc*, H1 5.12, H2 4.85, H3 5.43, H4 5.06, H5 4.10, H6 4.20–4.30; O–α-*Glc*–(1,6)-Glc, H1 5.06, H2 4.80, H3 5.50, H4 5.02, H5 4.02, H6 3.73–3.53. ^{13}C: 95.5, 95.4, 71.0, 70.7, 70.2, 69.3, 68.2, 67.9, 67.4, 66.1, 65.3 60.9. *Spacer moiety* δ ^{1}H (^{13}C): CH_2–O–α-Glc 3.49–3.83 (68.02), CH_2–CH_2–O–α-Glc 1.82 (25.53), CH_2–CH_2–O–aromatic 1.92 (25.99), CH_2–O–aromatic 4.45 (66.1).

AG17A1. *Saccharidic and aglycon moieties* δ ^{1}H: anomeric signals 5.07 × 2, 5.14, H2 4.75–4.89 (three protons), H3 5.42–5.53 (three protons), H4 5.01–5.10 (three protons), H5 4.01–4.10 and H6 external (seven protons) 4.11–4.33, H6 internal and CH_2–O–α-Glc 3.47–3.90 (seven protons), CH_2–O–aromatic 4.47. Selective ^{13}C NMR signals: 95.46 × 2 and 95.65 anomeric signals, 61.86 (C6 external glucose moiety), 66.15, 65.99 (C6 of the two internal glucose moiety), CH_2–O–α-Glc 3.80–3.50 (67.88), CH_2–O–aromatic 4.45 (65.32), CH_2–CH_2–O–α-Glc (25.55), CH_2–CH_2–O–aromatic (26.00).

*6.4.2. Synthesis of α-glucosides of **MG3***

MG3 (50 mg, 0.161 mmol) dissolved in 200 μl of DMSO was added to 20 ml of maltose solution 1 M in phosphate buffer (50 mM, pH 5.8). Partially purified crude homogenate (150 μl) of *Aplysia fasciata* containing α-glucosidase (31 U/μl) was added, to the resulting suspension, in three portions during reaction time. The mixture was stirred at 34 °C for 48 h. TLC monitoring was performed by using EtOAc/MeOH/H_2O 70:20:10 by vol. 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. The latter eluted first in water, while increasing gradients of methanol allowed the separation of different α-glucosides of the acceptor used. Acetylation was conducted by standard procedures using Ac_2O/pyridine for 24 h.

The products were formed in a total yield of about 10% with respect to aglycon added and were composed by the monoglucoside **MG3C** (4%), two different disaccharides **MG3B** and **MG3A** (6.5%, 30:70 ratio, respectively) and traces of different trisaccharides as judged by R_f in TLC.

The mass spectra of acetylated derivatives of **MG3C** (664.56 $M^+ + Na^+$), **MG3B** and **MG3A** (952.84 $M^+ + Na^+$) secured on monosaccharidic and disaccharidic nature of the products. The interglycosidic linkages were established by 2D NMR spectroscopy. Coupling constants of the newly formed anomeric linkages secured for α-configuration and aromatic rings show signals in accord with the proposed structures.

MG3C. *Saccharidic moiety* δ ^{1}H (^{13}C): H1 5.25 (95.5), H2 4.85 (71.0), H3 5.51 (69.8), H4 5.05 (68.7), H5 4.11 (67.2), H6 4.13–4.22 (62.0). *Spacer moiety* δ ^{1}H (^{13}C): –NH–CH_2–CH_2–O–CH_2–CH_2–α-Glc 3.62 (40.0), –NH–CH_2–CH_2–O–CH_2–CH_2–α-Glc 3.67–3.75 (69.9, 70.6), –NH–CH_2–CH_2–O–CH_2–CH_2–α-Glc 3.71–3.79 (67.0).

MG3B. *Saccharidic moiety* δ ^{1}H: O–α-Glc–(1,4)-*Glc*, H1 5.42, H2 4.88, H3 5.38, H4 5.08, H5 4.09, H6 4.09–4.26; O–α-*Glc*–(1,4)-Glc: H1 5.18, H2 4.76, H3 5.57, H4 4.09, H5 4.26, H6 4.26–4.48. *Spacer moiety* δ ^{1}H: –NH–CH_2–CH_2–O–CH_2–CH_2–α-Glc 3.61, –NH–CH_2–CH_2–O–CH_2–CH_2–α-Glc 3.65–3.74, –NH–CH_2–CH_2–O–CH_2–CH_2–α-Glc 3.70–3.80.

MG3A. *Saccharidic moiety* δ ^{1}H: O–α-Glc–(1,6)-*Glc*, H1 5.22, H2 4.78, H3 5.53, H4 4.95, H5 4.13, H6 3.75–3.56; O–α-*Glc*–(1,6)-Glc: H1 5.13, H2 4.88, H3 5.49, H4 5.04, H5 4.13, H6 4.13. *Spacer moiety* δ ^{1}H: –NH–CH_2–CH_2–O–CH_2–CH_2–α-Glc 3.75, –NH–CH_2–CH_2–O–CH_2–CH_2–α-Glc 3.70–3.74, –NH–CH_2–CH_2–O–CH_2–CH_2–α-Glc 3.70–3.85.

*6.4.3. Synthesis of β-glucosides of **AG17** and **MG3***

AG17 (90 mg, 0.304 mmol) or **MG3** (90 mg, 0.29 mmol) and *p*-nitrophenyl-β-D-glucopyranoside (30.5 mg, 0.101 mmol) dissolved in 300 μl of DMSO were added to 2 ml of phosphate buffer (50 mM, pH 6); 1 ml of concentrated crude homogenate of visceral mass of *A. fasciata* (6 mg/ml total proteins) containing

β-glucosidic activity was added to this suspension under stirring. The formation of products was monitored by TLC in $CHCl_3$/MeOH/H_2O (65:25:4 by vol). When the donor, *p*-nitrophenyl β-D-glucopyranoside, was exhausted (2.5 h), chromatographic purification of the reaction mixture was performed based on reverse-phase column chromatography (Lobar RP-18); it efficiently separated both the product and remaining aglycon from glucose. The products **AG17-β-Glc** (1.4 mg, 3% yield with respect to donor) and **MG3-β-Glc** (1.1 mg, 2.3% yield with respect to donor), were then obtained pure by preparative TLC.

AG17-β-Glc. δ ^{1}H: 7.4–8.4 (aromatic signals), 4.5 CH_2–O–aromatic, 4.3 ($J = 7.8$ Hz, H1 glucose), 4.1–3.1 CH_2–O–α-Glc and H2–H6 glucose, 1.8–1.9 CH_2–CH_2–O–α-Glc and CH_2–CH_2–O–aromatic.

MG3-β-Glc. δ ^{1}H: 7.4–8.2 (aromatic signals), 4.4 ($J = 7.8$ Hz, H1 glucose), 4.2–3.1 spacer arm protons and H2–H6 of glucose. Selected ^{13}C NMR signals: 104.5 (C1 glucose), 62.80 (C6 glucose), 40.9 (–NH–CH_2–spacer arm).

6.4.4. Isolation of human peripheral blood mononuclear cells (PBMC) and compound treatment

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 washed three times in RPMI 1640 medium and cultured in 24-well plates at a concentration of 2×10^6 cells/ml per well in RPMI 1640 medium. PBMC were cultured at 37 °C in 5% CO_2 atmosphere, in RPMI 1640 supplemented with 50 mcg/ml gentamicin and 5% fetal calf serum (FCS). All tested compounds were diluted in DMSO at a concentration of 0.1 mg/mL, and then diluted in RPMI 1640 medium to obtain the final concentrations employed. PBMC were then treated with the compounds at different concentrations.

Lipopolysaccharide (LPS) from *E. coli* strain 055:B5 was used as positive control.

The supernatants were harvested 24 and 48 h post treatment, aliquoted and stored at −80 °C until cytokine analysis.

6.5. Cytotoxicity test

To determine the effect of different concentrations of title compounds on cells viability, a colorimetric assay was used as described by Mosmann [24]. The assay is based on the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), a pale yellow substrate that is cleaved by active mitochondria to produce a dark blue formazan product. PBMC 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 compounds were added into each well. The plate was incubated at 37 °C with atmosphere of 5% of CO_2 for 48 h. Then 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:

$$1 - [(\text{experimentalOD} - \text{lysiscontrolOD})/(\text{cellcontrolOD} - \text{lysiscontrolOD})] \times 100.$$

6.6. Virus

HSV-type 2 was used throughout the study. HSV2 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 plaque forming unit (PFU) per mL.

6.7. Limulus test

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 per mL.

6.8. Treatment and infection

In order to evaluate antiviral activity, PBMC were seeded onto 24-well culture plates at a density of 2×10^6 cells per well. Then, PBMC were added with compounds (40 and 20 μg/mL) and incubated for 24 h at 37 °C in 5% CO_2. After this period, cells were infected with HSV2 at a multiplicity of infection (MOI) 0.1 and incubated for 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 °C until virus titration. The virus titre was expressed as plaque forming unit (PFU) per mL.

6.9. Cytokine evaluation

In a second series of experiments, we evaluated the production of IFNα and TNFα by PBMC added with compounds at the concentrations of 40 and 20 μg/mL. 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 °C. The titration of IFNα and TNFα was performed by an immunoenzymatic method (ELISA) from Bender MedSystems (Milan, Italy). The limit of detection of the assay was <4.8 pg/mL for IFNα and <5.8 pg/mL for TNFα.

6.10. Statistical evaluation

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

Acknowledgements

We wish to thank E. Pagnotta for technical assistance, and S. Zambardino, D. Melck and V. Mirra (NMR service of

Istituto di Chimica Biomolecolare, CNR, Pozzuoli, Italy) and M. Zampa for mass spectra. Dr. A. Tramice wishes to thank Centro Regionale di Competenza in Applicazioni Tecnologico-Industriali di Biomolecole e Biosistemi for a fellowship.

References

[1] A. Arena, T.L. Maugeri, B. Pavone, D. Iannello, C. Gugliandolo, G. Bisignano, Int. Immunopharmacol. 6 (2006) 8–13.
[2] J.A. Murphy, R.J. Duerst, T.J. Smith, L.A. Morrison, J. Virol. 77 (2003) 9337–9345.
[3] (a) A. Alcami, U.K. Koszinowski, Trends Microbiol. 8 (2000) 447–455;
(b) T.H. Mogensen, J. Melchjorsen, L. Malmgaard, A. Casola, S.R. Paludan, J. Virol. 78 (2004) 5883–5890.
[4] (a) A. Alcami, Nat. Rev. Immunol. 3 (2003) 36–50;
(b) M.J. Boulanger, D.C. Chow, E. Breynova, M. Martick, D. Sanford, J. Nicholas, K.C. Garcia, J. Mol. Biol. 335 (2004) 641–654.
[5] C.L. Celum, R. Levine, M. Weaver, A. Wald, Bull. World Health Organ. 82 (2004) 447–453.
[6] A.J. Simmons, Infect. Dis. 186 (2002) 71–77.
[7] (a) V. Panasiti, V. Devirgiliis, R.G. Borroni, A. Spataro, L. Melis, M.C. Petrella, S. Pala, J. Infect. 54 (2007) 55–57;
(b) W.A. Duffus, J. Mermin, R. Bunnell, R.H. Byers, G. Odongo, P. Ekwaru, R. Downing, Int. J. STD AIDS 16 (2005) 733–735.
[8] D. Malvy, M. Treilhaud, S. Bouee, A. Crochard, D. Vallee, A. El Hasnaoui, M. Aymard, RESSAC Study Group, Clin. Infect. Dis. 41 (2005) 320–326.
[9] T. Nogrady, Medicinal Chemistry. A Biomedical Approach, second ed. Oxford University Press, 1998, pp. 408–412.
[10] K. Hirota, K. Kazaoka, I. Niimoto, H. Kumihara, H. Sajiki, Y. Isobe, H. Takaku, M. Tobe, H. Ogita, T. Ogino, S. Ichii, A. Kurimoto, H. Kawakami, J. Med. Chem. 45 (2002) 5419–5422.
[11] (a) E.R. Andrews, R.W. Fleming, J.M. Grisar, J.C. Kihm, D.L. Wenstrup, G.D. Mayer, J. Med. Chem. 17 (1974) 882–886;
(b) W.L. Albrecht, R.W. Fleming, S.W. Horgan, J.C. Kihm, G.D. Mayer, J. Med. Chem. 17 (1974) 886–889;
(c) J. Fischer, Biochem. J. 312 (1995) 215–222.
[12] (a) S. Alcaro, A. Arena, R. Di Bella, S. Neri, R. Ottanà, F. Ortuso, B. Pavone, A. Trincone, M.G. Vigorita, Bioorg. Med. Chem. 13 (2005) 3371–3378;
(b) S. Alcaro, A. Arena, R. Di Bella, S. Neri, R. Ottanà, F. Ortuso, B. Pavone, M.G. Vigorita, Arkivoc 5 (2004) 334–348.
[13] (a) J. Fischer, H. Lullmann, R. Lullmann-Ranch, Gen. Pharmacol. 27 (1996) 1317–1324;
(b) M. Prokopek, Biochem. Pharmacol. 42 (1991) 2187–2191.
[14] (a) J. Fisher, L. Hein, R. Lullmann-Rauch, B. von Witzendorff, Biochem. J. 315 (1996) 369–375;
(b) R. Lullmann-Rauch, R. Pods, B. von Witzendorff, Biochem. Pharmacol. 49 (1995) 1223–1233.
[15] F. Arcamone, Doxorubicin-Anticancer Antibiotics, Academic, New York, 1981.
[16] (a) E. Sidransky, Mol. Genet. Metab. 83 (2004) 6–15;
(b) C.T. Campbell, K.J. Yarema, Genome Biol. 6 (2005) 236–244;
(c) D. Zopf, S. Roth, Lancet 347 (1996) 1017–1021.
[17] (a) A.A. Vjas, H.V. Patel, S.E. Fromholt, M. Heffer-Lauc, K.A. Vyas, J. Dang, M. Schachner, R.L. Schnaar, PNAS 99 (2002) 8421–8427;
(b) V. Kren, L. Martinkova, Curr. Med. Chem. 8 (2001) 1313–1338.
[18] A. Dondoni, A. Massi, E. Minghini, V. Bertolasi, Helv. Chim. Acta 85 (2002) 3331–3348.
[19] K.M. Koeller, C.H. Wong, Chem. Rev. 100 (2000) 4465–4493.
[20] M. Scigelova, S. Singh, D.H.G. Crout, J. Mol. Catal., B Enzym. 6 (1999) 483–494.
[21] A. Giordano, G. Andreotti, E. Mollo, A. Trincone, J. Mol. Cat., B Enzym. 30 (2004) 51–59.
[22] G. Andreotti, A. Giordano, A. Tramice, E. Mollo, A. Trincone, J. Biotechnol. 122 (2006) 274–284.
[23] (a) A. Tramice, A. Giordano, G. Andreotti, E. Mollo, A. Trincone, Mar. Biotechnol. 8 (2006) 448–452;
(b) A. Trincone, A. Giordano, Curr. Org. Chem. 10 (2006) 1163–1193.
[24] T. Mossman, J. Immunol. Methods 65 (1989) 55–63.
[25] R. Romagnani, Immunol. Today 18 (1997) 263–266.
[26] S. Alcaro, A. Artese, J.N. Iley, R. Maccari, S. Missailidis, F. Ortuso, R. Ottanà, P. Ragazon, M.G. Vigorita, Bioorg. Med. Chem. Lett. 17 (2007) 2509–2514.