Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry
ELSEVIER
Bioorganic & Medicinal Chemistry 12 (2004) 1781–1791

Design and synthesis of DNA-intercalating 9-fluoren-β-*O*-glycosides as potential IFN-inducers, and antiviral and cytostatic agents☆

S. Alcaro,[a] A. Arena,[b] S. Neri,[c] R. Ottanà,[c] F. Ortuso,[a] B. Pavone[b] and M.G. Vigorita[c,*]

[a]*Dipartimento di Scienze Farmacobiologiche, Università di Catanzaro 'Magna Græcia', Complesso Ninì Barbieri — 88021 Roccelletta di Borgia (CZ), Italy*
[b]*Dipartimento di Discipline Chirurgiche, Unità di Microbiologia, Facoltà di Medicina e Chirurgia, Azienda Ospedaliera Universitaria 'G. Martino' — 98125 Messina, Italy*
[c]*Dipartimento Farmaco-Chimico, Facoltà di Farmacia, Università di Messina, V.le SS. Annunziata, 98168 Messina, Italy*

Received 14 August 2003; accepted 16 December 2003

Abstract—Novel 9-fluoren-β-*O*-glycosides, designed as DNA-intercalating agents in structural correlation with antiviral tilorone and anticancer anthracyclines, have been prepared with yields in β-anomers ranging between 25 and 63%. They have been screened for antiproliferative, immunostimulating and antiviral properties against HSV-1 and HSV-2 viruses. Compounds displaying significant antiviral activity against HSV-2 are acetylated **1** and deprotected **6** 9-fluorenyl-*O*-D-arabinopyranoses, whereas 9-fluorenyl-*O*-D-glucopyranose **3** is the most effective on HSV-1 replication, followed by **1** and **6**. The conformational properties of these compounds have been evaluated by molecular modelling techniques.

1. Introduction

The trend of antitumoral and antiviral therapies of today relies on a double approach: the combined administration of chemotherapeutics and immunomodulators able to counteract immunological disorders, ground or consequence of the pathology.[1]

Titled 9-fluoren-β-*O*-glycosides, the synthesis and characterization of which are reported, have been designed as a sort of molecular hybrids of the intercalating antitumoral anthracyclines (daunomycin, doxorubicin etc.) and tilorone (2,7-bis2-(diethylamino)ethoxyfluoren-9-one), well-known interferon (IFN)-inducent and antiviral agent.[2]

In the novel compounds the fluorenic chromophore might be able to bind double-stranded DNA by intercalation reinforced by the sugar moiety in the same β-configuration as in daunomycin.[3]

Intercalating drugs are known to strongly bind to the DNA of chromatin in the cell nucleus by slipping between two base pairs of the double helix and forming charge–transfer complexes with the nucleotides. The four-membered ring system of daunomycin intercalates into DNA and distorts the double helix: the replication and transcription of genes are thus compromised, in particular those encoding for important enzymes such as topoisomerase.[4]

The mechanism of action of tilorone as well proves to be linked to DNA-intercalation: in fact, slipping between two adjacent base pairs of the minor groove, its planar molecule alters DNA chemo-physical properties, stimulating cytokines expression.[5] In particular, IFN-α and IFN-β play important roles in host resistance to viral infections.[6] They bind to IFN-α/β receptors that are expressed in all cell types.[7] Activation of such receptors modifies the transcriptional and translational environment in cells inducing an antiviral state.[8]

Tilorone was marketed in USA as a potent IFN-inducer and used for many years as an antiherpetic agent.[9] Some severe side effects were subsequently ascertained; in particular the onset of mucopolysaccharidosis which

☆A preliminary account of this work was presented at XIV Convegno Nazionale — Divisione di Chimica Farmaceutica – Società Chimica Italiana, Salsomaggiore Terme, PR, Italy, 21–25 September 1998.

* Corresponding author. Tel.: +39-090-6766469; fax: +39-090-355613; e-mail: vigorita@pharma.unime.it

0968-0896/$ - see front matter © 2004 Published by Elsevier Ltd.
doi:10.1016/j.bmc.2003.12.034

caused its withdrawal from trading. This effect was related to the presence of the symmetrical side chains in 2 and 7 positions.[10] In fact many non-symmetrically substituted analogues maintain the IFN inducent potential of the parent drug, lacking this adverse property.[11]

On the other hand, antitumoral anthracyclines are known to display severe cardiotoxicity mainly linked to quinonic B/C rings capable of generating noxious radical species.[12]

In view of these aspects, the non-beneficial moieties of parent drugs have been omitted in designing novel fluorenic DNA intercalating compounds. Moreover, as happens in several classes of xenobiotics, the insertion of sugar moieties would confer better pharmacokinetics and a more favourable toxicity profile.[13] In fact we are witnessing today a rapid growth of carbohydrate structures as new leads in pharmaceutical research, on the basis of the increased knowledge of many cellular processes in which carbohydrates are physiologically involved.[14]

In the present study we evaluated the novel 9-fluoren-β-*O*-glycosides as antiviral agents (against HSV-1 and HSV-2), IFN-α/β inducers and cytostatic agents.

Worldwide, 60–95% of the population is infected by one or more viruses of the herpesviridae family. Herpes simplex viruses types 1 and 2 determine diseases appearing as dermatological, immunologic and neurological disorders.[15]

Moreover, the even wider number of immunocompromised, transplant, cancer or AIDS patients, has highlighted the involvement of herpesvirus infections in these patients. On the other hand the intensive use of antivirals (acyclovir and its congeners) has led to the emergence of resistant viruses, which are commonly found in severely immunocompromised patients.

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

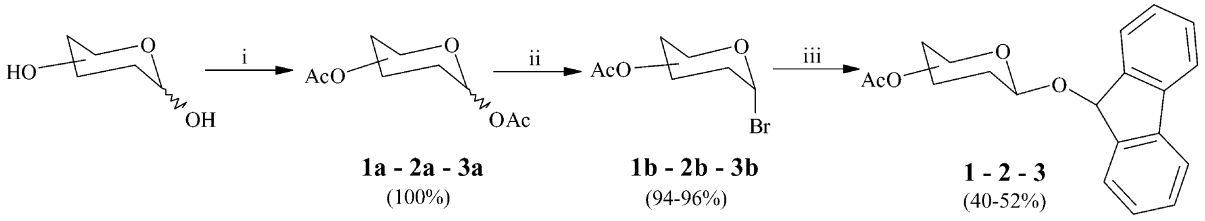
2. Chemistry

A large number of bioactive natural compounds possess β-glycosidic structures frequently containing one or more D-glucose, D and L-arabinose, D-xylose, and D-lyxose units.[16] Thus we selected these sugars to prepare our 9-fluoren-β-*O*-glycosides; classical glycosilation methods, sometimes adequately modified, were used for their preparation.

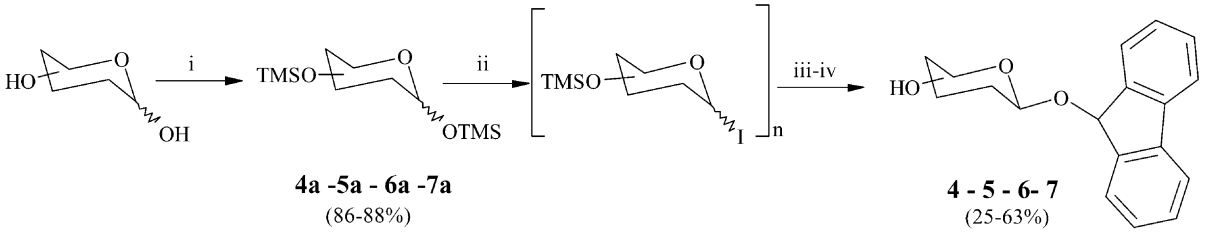
From a synthetic point of view, the crucial aspect of the carbohydrate chemistry has always been the achievement of a good level of stereocontrol on the formation of the glycosidic linkage. In addition, the multifunctional carbohydrate molecules require the use of protecting groups on the functionalities that must be manipulated at a later stage of the synthesis.[17]

We always used 9-fluorenol, obtained by commercial 9-fluorenone after reduction with $NaBH_4$, as aglicone. To prepare its glycosides, we employed at first the classical Koenigs–Knorr reaction, that has long been widely used.[18] The commercial sugars D-, L-arabinopyranoses, and D-glucopyranose yielded tetra-*O*-acetyl derivatives (as α/β anomer mixtures) (**1a**, **2a**, **3a**) from which glycosyl bromides as pure α anomers (**1b**, **3b**) or β anomer (**2b**) were obtained. In this procedure glycosyl bromide is the glycosyl donor and reacts with 9-fluorenol (alcoholic acceptor) in the presence of an excess of a heavy metal salt ($HgO/HgBr_2$) to give only the β-glycoconjugates **1**, **2**, **3** reported in Table 1 (Scheme 1 as *a* method). The yield ranged between 40 and 52%.

A valuable alternative to Koenigs–Knorr method was represented by the treatment of pyranose or furanose derivatives with bases; this generates anomeric alcoholates, which are known to react with suitable nitriles, providing stable *O*-alkyl imidates.[19] Therefore the reaction between 2,3,4,6-tetra-*O*-benzyl-glucopyranose and trichloroacetonitrile, in presence of a weak base such as potassium carbonate, allows the corresponding β-trichloroacetimidate to be isolated in good yield: the anomeric oxygen has been transformed into a good



Scheme 1. (i) Ac_2O, pyridine; (ii) HBr, CH_3COOH; (iii) HgO, $HgBr_2$, 9-fluorenol.



Scheme 2. (i) TMS-Cl, trietylammine, DMF; (ii) TMS-I, t.a.; (iii) 9-fluorenol, 2,6-di*ter*butylpyridine, CH_2Cl_2, 5 h, t.a.; (iv) CH_3OH.

Table 1. Yelds, melting points, elementary analysis and synthetic methods of glycosides

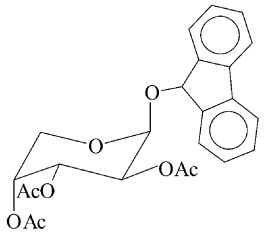
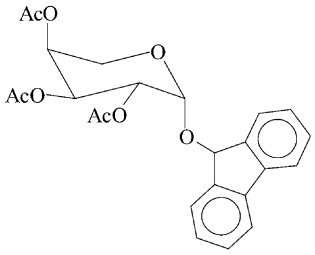
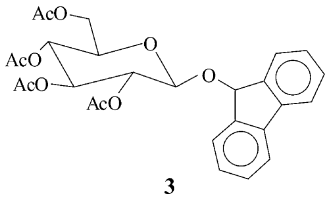
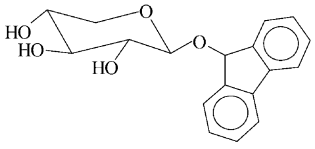
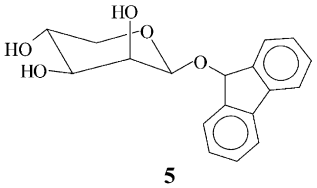
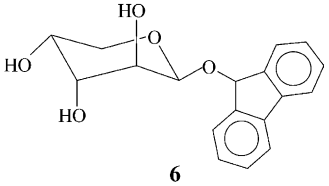
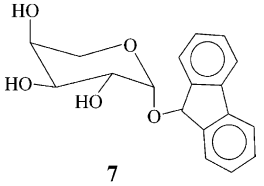
	Molecular formula Elemental analysis	mp (°C) Molecular weight	Synthetic method	Yield in β-anomer
 **1**	2,3,4-tri-*O*-acetyl-β-*O*-(9-fluorenyl)-D-arabinopyranose Anal. for $C_{24}H_{24}O_8$ (%): Calcd.: C = 65.45, H = 5.49 Found: C = 65.20, H = 5.50	146–149 MW 440.45	*a*	40%
 **2**	2,3,4-tri-*O*-acetyl-β-*O*-(9-fluorenyl)-L-arabinopyranose Anal. for $C_{24}H_{24}O_8$ (%): Calcd.: C = 65.45, H = 5.49 Found: C = 65.25, H = 5.51	134–136 MW 440.45	*a*	54%
 **3**	2,3,4,6-tetra-*O*-acetyl-β-*O*-(9-fluorenyl)–D-glucopyranose Anal. for $C_{27}H_{28}O_{10}$(%): Calcd.: C = 63.28, H = 5.51 Found: C = 63.05, H = 5.53	120–123 MW 511.37	*a*	52%
 **4**	β-*O*-(9-fluorenyl)–D-xylopyranose Anal. for $C_{18}H_{18}O_5$ (%): Calcd.: C = 68.78, H = 5.77 Found: C = 69.04, H = 5.74	115–117 MW 314.40	*b*	63%
 **5**	β-*O*-(9-fluorenyl)–D-lyxopyranose Anal. for $C_{18}H_{18}O_5$ (%): Calcd.: C = 68.78, H = 5.77 Found: C = 68.55, H = 5.79	117–120 MW 314.40	*b*	25%
 **6**	β-*O*-(9-fluorenyl)–D-arabinopyranose Anal. for $C_{18}H_{18}O_5$ (%): Calcd.: C = 68.78, H = 5.77 Found: C = 68.60, H = 5.78	102–105 MW 314.40	*b*	45%
 **7**	β-*O*-(9-fluorenyl)–L-arabinopyranose Anal. for $C_{18}H_{18}O_5$ (%): Calcd.:C = 68.78, H = 5.77 Found: C = 69.04, H = 5.75	124–127 MW 314.40	*b*	32%

Table 2. ^{1}H NMR[a] data of glycosides

Compd	H-1	H-2	H-3	H-4	H-5	H-5′	H-6	H-6′	H-9 fluor.	OH	Oac	Arom.
1	4.75[d] (d, $J=6.9$ eq/ax)	5.28–5.23[d] (m)	5.04[d] (dd, $J_{3,4}=3.6$ ax/eq, $J_{3,2}=9.3$ ax/ax)	5.28–5.23[d] (m)	4.08[d(b)] (dd, $J_{AX}=3.3$, $J_{AB}=12.9$)	3.58[d(c)] (dd, $J_{BX}=1.5$, $J_{BA}=12.9$)	—	—	5.67 (s)		2.15, 2.02, 1.99 (3s)	7.65–7.27 (m)
2	4.75 (d, $J=6.9$ ax/eq)	5.25–5.22 (m)	5.02 (dd, $J_{3,2}=9.3$ ax/ax, $J_{3,4}=3.6$ ax/eq)	5.25–5.22 (m)	4.07[b] (dd, $J_{AX}=3.6$, $J_{AB}=12.9$)	3.58[c] (dd, $J_{BX}=1.8$, $J_{BA}=12.9$)	—	—	5.66 (s)		2.16, 2.15, 2.14 (3s)	7.62–7.26 (m)
3	5.40 (d, $J=6.9$ ax/ax)	4.26–4.19 (m)	5.09 (t, $J_{3,4}=J_{3,2}=9.6$ ax/ax)	5.26 (t, $J_{4,3}=J_{4\text{-}5}=9.6$ ax/ax)	3.79–3.71 (m)	—	4.33[b] (dd, $J_{AX}=3.9$, $J_{AB}=12.3$)	3.96[c] (dd, $J_{BX}=2.4$, $J_{BA}=12.3$)	5.71 (s)		2.13, 2.10, 2.05, 2.01 (4s)	7.73–7.30 (m)
4	4.67 (d, $J_{1,2}=7.5$ ax/ax)	3.57–3.42 (m)	5.18–5.02 (m)	5.18-5.02 (m)	4.32–4.10 (m)	4.32–4.10 (m)	—	—	5.68 (s)	5.18–5.02 (m), 4.32–4.10 (m)	—	7.68–7.26 (m)
5	5.23 (d, $J=4.2$ ax/eq)	4.25–3.80 (m)	4.25–3.80 (m)	4.25–3.80 (m)	4.25–3.80 (m)	4.25–3.80 (m)	—	—	5.65 (s)	2.20–1.40 (m)	—	7.66–7.28 (m)
6	5.49[d] (d, $J=5.4$ eq/ax) 5.33[e] (d, $J=5.1$ eq/ax)	5.71–5.63[d,e] (m)	4.22–3.64[d,e] (m)	4.22–3.64[d,e] (m)	4.22–3.64[d,e] (m)	4.22–3.64[d,e] (m)	—	—	5.71–5.63 (m)	2.20–1.40 [d+e] (m)	—	7.72–7.54 (m) 7.46–7.22 (m)
7	5.41[d] (d, $J=3.6$ eq/ax) 5.25[e] (d, $J=4.8$ ax/eq)	5.66–5.57[d,e] (m)	4.30–3.65[d,e] (m)	4.30–3.65[d,e] (m)	4.30–3.65 [d,e] (m)	4.30–3.65[d,e] (m)	—	—	5.47 (s)	3.47–3.01 (m)	—	7.68–7.55 (m) 7.40–7.26 (m)

[a] (δ) $CDCl_3$ solution; J values are expressed in hertz.
[b] A part of an ABX system.
[c] B part of an ABX system.
[d] conf. $^{1}C_4$.
[e] conf.

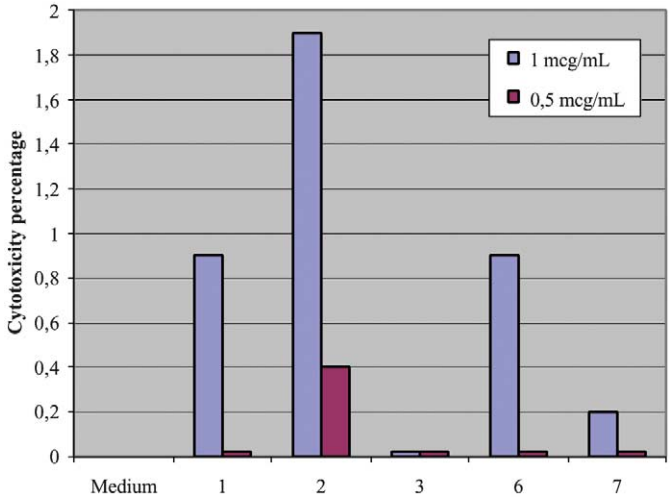


Figure 1. Cytotoxicity percentage on WISH cell lines.

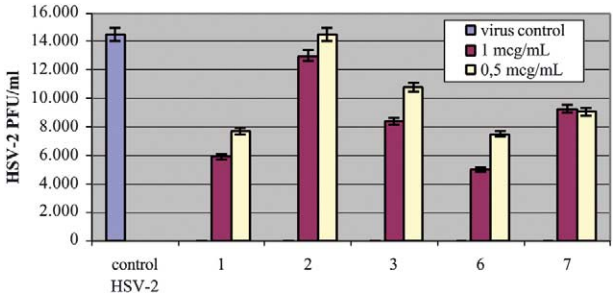


Figure 2. Anti-HSV-2 activity on WISH cell-lines.

leaving group ('activation step').[20] Unfortunately, the second reaction step, the synthesis of *O*-glycoside in presence of a catalytic amount of different acidic promoters ($BF_3.OEt_2$ in CH_2Cl_2 at −20 °C, TMSOTf in CH_2Cl_2 at −78 °C, $AlCl_3$ in CH_2Cl_2 at −10 °C),[21] yielded no product, probably because of the formation of adducts that prevent subsequent glycosidation reaction.

Deprotected glycosides **4**, **5**, **6**, **7** have been obtained only as β-anomers by using the reaction of Uchiyama–Hindsgaul, characterized by a very simple preparation of the glycosyl donor, through the use of iodotrimethylsilane (TMS-I)[22] and, on completion, by the addition of methanol to easily remove TMS-protecting groups without any glycosyl bond cleavage[23] (Scheme 2 as *b* method).

Table 1 carries analytical and physical data of glycosides **1**–**7**, whereas Tables 2 and 3 contain ^1H- and ^{13}C NMR data respectively.

3. Results and discussion

3.1. Antiviral and cytotoxicity assays

The cytotoxicity test was performed using trypan blue staining and WISH cell lines. The results were expressed as percentage of stained (dead) cells. Results presented in Figure 1 show that compounds **1**, **2**, **3**, **6**, **7**, tested at concentrations of 1 and 0.5 μg/mL, did not induce appreciable cytotoxicity (within 2%). Compounds **4** and **5** were not tested because of their low solubility in aqueous systems such as those required in cell cultures.

3.2. Plaque reduction and HSV-2 replication assay

HSV-2 replication was compared in WISH cells untreated or treated with compounds **1**, **2**, **3**, **6**, **7**. As shown in Figure 2, compounds **1** and **6** at a concentration of 1 μg/mL inhibited HSV-2 replication by 59% and 66% respectively ($p < 0.05$), whereas compounds **3** and **7** at the same concentration by 42% and 36% respectively ($p < 0.05$). Moreover, compounds **1**, **3** and **6** showed a dose-dependent inhibition of virus replication: in fact at a concentration of 0.5 μg/mL they displayed a lower antiviral activity than at a concentration of 1 μg/mL (but significative when compared to control virus $p < 0.05$). Compound **2** did not manifest any significant inhibition on HSV-2 replication.

3.3. Plaque reduction and HSV-1 replication assay

HSV-1 replication was compared in WISH cells untreated or treated with compounds **1**, **2**, **3**, **6**, **7** at two different concentrations. As shown in Figure 3, compound **3** at a concentration of 1 μg/mL inhibited HSV-1 replication by 80% ($p < 0.05$), whereas compounds **1** and **6** at the same concentration, compared with virus control, inhibited HSV-1 replication by 41% and 30% respectively ($p < 0.05$). Furthermore, the inhibition of HSV-1 replication induced by compounds **1**, **3** and **6** were dose-dependent: in fact, at a concentration of 0.5 μg/mL the compounds displayed a lower antiviral activity. Compounds **2** and **7** did not show any significant inhibition on HSV-1 replication.

3.4. IFN α/β production

Supernatants harvested from U937 cells, pre-treated with tested compounds **1**–**7** and then infected with HSV-1 or HSV-2, were analysed for the presence of IFN α/β. Our data demonstrated that none of the tested compounds induced appreciable amounts of IFN α/β.

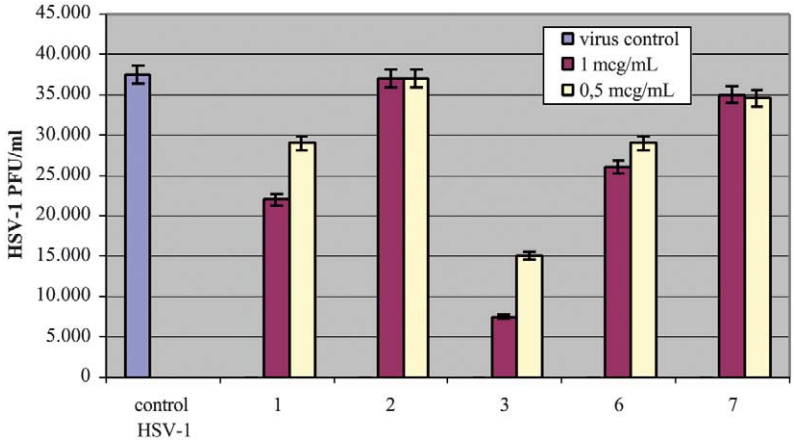


Figure 3. Anti HSV-1 activity on WISH cell-lines.

3.5. Anticancer in vitro pre-screen

The in vitro primary antitumor screening[24] consisted of the evaluation of the effects of each compound against 3 tumoral cell lines: MCF7 (breast), NCI-H460 (lung) and SF-268 (CNS). Such a 3-cell lines, one-dose assay, which has been in use by Developmental Therapeutics Program (DTP) of US-NCI for the evaluation of combinatorial libraries, has proven to be an effective antiproliferative pre-screen. Each cell line was inoculated and preincubated on a microliter plate. Test agents were then added at a single concentration (10^{-4} M) and the culture incubated for 48 h. End point determinations were made with sulforhodamine B, a protein-binding dye.

Results for compounds **1**, **2**, **3**, **4**, **5** are reported as percentage of growth of the treated cells when compared to the untreated control cells (Fig. 4). None of them reached the level of 32% growth reduction, assigned by US-NCI to pass examined compounds for evaluation in the full panel of 60 cell lines.

Compound **2** however reached 60% for two over three lines, followed by compound **3**, relatively to MCF7 breast cell line.

3.6. Conformational search study

The conformational search was carried out by molecular mechanics techniques coupled to the analysis of the averaged solvent accessible surface area (ASASA) computed onto the fluorene moiety. This descriptor was considered as able to take into account the putative mechanism of action of these compounds that could intercalate the duplex DNA by the fluorene moiety.

On the other hand, ASASA allowed the estimation of the degree of exposition of the planar tricyclic ring to the aqueous solvent, providing an indirect measure of pharmacokinetic properties of our compounds in which the fluorene lipophilic moiety is linked to hydrophilic sugar rings.

In detail, after building the 3-D structures of each compound, we proceeded with the exploration of the internal degrees of freedom by Monte Carlo (MC) randomization of any rotatable bond, including those pertinent to the sugar ring. 5000 conformations were generated and energy minimized with the AMBER* force field using the notation of all the atoms and the implicit model of solvation GB/SA water[25] as implemented in Macro Model ver. 7.2.[26] The convergence in the conformational search was evaluated for each molecule using the averaged number of duplicate conformers (AND). Usually an AND value higher than 2 indicates a good conformational space exploration.

In the most flexible case, compound **3**, AND was 1.9, so we repeated the MC search of this molecule running

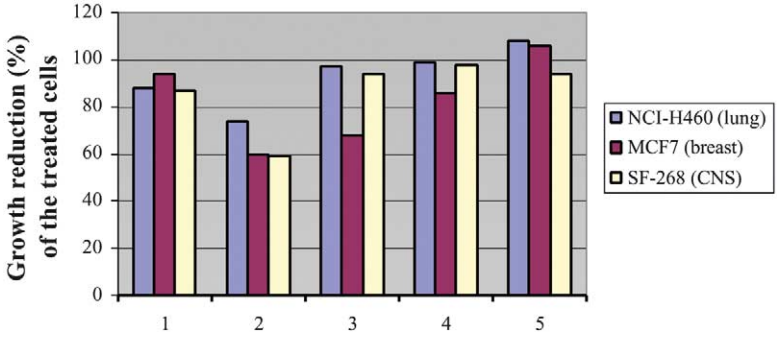


Figure 4. Anticancer in vitro pre-screen (10^{-4} M).

Table 3. ^{13}C NMR[a] data of glycosides

Compd	CH-1	CH-2	CH-3	CH-4	CH-5	CH_2-5	CH_2-6	C quat.	COO	$\underline{C}H_3COO$	CH-9 fluor.	CH-arom.
1	81.71	70.23	81.63	69.37		67.58		142.49, 141.25, 140.57, 139.62	170.53, 168.84, 168.21	22.39, 21.54, 19.47	100.56	129.23, 127.65, 127.31, 126.18, 125.65, 119.95, 119.92, 119.78
2	81.70	70.24	81.63	67.59		63.00		142.59, 142.51, 140.51, 139.63	170.42, 169.96, 169.32	21.02, 20.88, 19.93	100.56	129.25, 129.23, 127.66, 127.33, 126.20, 125.66, 119.93, 119.79
3	81.59	71.02	73.25	69.80	67.86		61.80	142.06, 141.45, 140.19, 139.63	174.20, 172.34, 170.83, 168.56	24.89, 23.85, 20.76, 19.84	98.50	129.66, 128.38, 127.84, 125.96, 125.16, 125.07, 123.80, 120.28
4	82.30	79.50	80.65	70.12		62.74		143.24, 142.90, 142.40, 140.55			89.27	129.27, 129.25, 127.69, 127.43, 126.35, 125.76, 120.03, 119.89
5	81.76	78.92	80.97	70.36		63.12		143.39, 142.64, 142.05, 140.15			88.58	129.35, 129.22, 127.62, 127.38, 126.30, 125.69, 119.98, 119.82
6	82.49	79.24	81.13	70.50		62.45		143.09, 142.80, 142.10, 140.20			88.62	129.58, 128.72, 127.82, 126.02, 125.81, 125.64, 125.21, 120.12
7	82.45	79.40	81.20	70.60		62.39		143.18, 142.61, 142.10, 140.73			88.66	129.58, 127.82, 126.03, 125.81, 125.63, 125.21, 124.63, 120.12

[a] (δ) $CDCl_3$ solution, 75.46 MHz.

Table 4. Summary of the Monte Carlo conformational search conditions and results

Compd	RB[a]	MC ite[b]	NCONF[c]	AND[d]
1	7	5000	380	8.06
2	7	5000	370	8.19
3	9	15,000	3697	3.48
4	7	5000	452	9.15
5	7	5000	496	7.84
6	7	5000	404	10.08
7	7	5000	408	9.98

[a] Number of rotatable bonds.
[b] Monte Carlo iterations.
[c] Number of conformers within 12.5 kcal/mol.
[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.

15,000 iterations and increasing its AND to 3.48. In all other cases AND was higher than 7.

In Table 4 the MC simulation conditions and results are summarized.

For all conformers, the solvent accessible surface area, according to the water property suggested by the Macro Model program, was performed, considering the interaction with a 1.4 Å radius probe. The fluorene moiety atoms were selected and the averaged value (ASASA) was computed for each compound (Fig. 5).

As indicated in Figure 5, we found three different kinds of fluorene moiety solvent accessibility. Compounds **4**–**7** showed the higher ASASA values associated to low variability. *Vice versa*, **3** demonstrated the lowest averaged solvent accessible surface area and the highest variability. Finally, compounds **1** and **2** proved to have an intermediate behaviour.

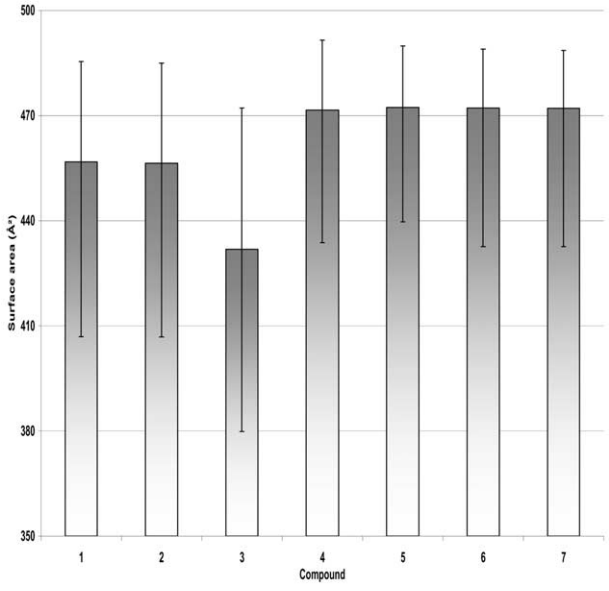


Figure 5. Fluorene ASASA results for compounds **1**–**7**. Bar plots refer to the averaged solvent accessible surface area in Å^2 for the fluorene moiety. Error bars regard the maximum and minimum ASASA values in the conformational ensemble generated in the Monte Carlo search.

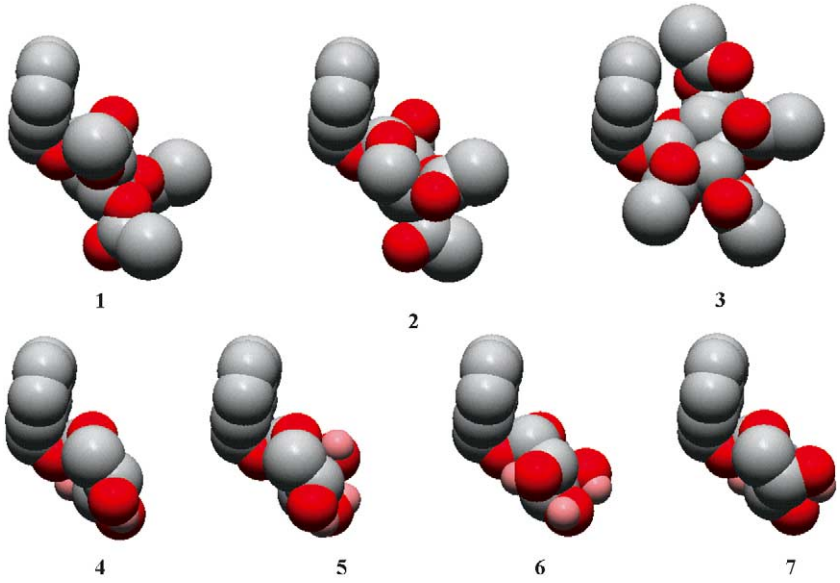


Figure 6. Energy global minimum conformers of compounds **1**–**7** (CPK models) obtained in the Monte Carlo conformational search.

In Figure 6 the comparison of the energy global minimum conformers of all compounds is depicted.

Both Figures 5 and 6 clearly show the high capability of compound **3** to hinder the fluorene moiety. Such an observation suggests a possible better diffusion through biomembranes that will be evaluated with appropriate methods in a future communication.

4. Conclusion

Taken together, our results allow some considerations to be preliminarily pointed out.

Compounds displaying significant antiviral activity against HSV-2 are acetylated **1** and deprotected **6** 9-fluorenyl-*O*-D-arabinopyranoses, whereas 9-fluorenyl-*O*-D-glucopyranose **3** is the most effective on HSV-1 replication, followed by **1** and **6**.

At a concentration of 1 mcg/mL, deprotected compounds **6** and **7** are less cytotoxic than equivalent acetylated compounds **1** and **2** and show a better inhibition on HSV-2 virus replication.

Apparently the presence of L-arabinose moiety is not beneficial, in fact compounds **1** and **6** appear to have a greater inhibitor effect than L-arabinopyranosides **2** and **7** on HSV-1 and HSV-2 replication.

As regards antitumor screening, only the most cytotoxic compound **2** (2,3,4-tri-*O*-acetyl-β-*O*-(9-fluorenyl)-L-arabinopyranose), reduces the growth of the treated cells by 59% (SF-268 cell line) and 60% (breast cell line) without reaching, however, the stated criterion of 32%.[24]

On the contrary 2,3,4,6-tetra-*O*-acetyl-β-*O*-(9-fluorenyl)-D-glucopyranose **3**, deprived of cytotoxicity (Fig. 1), inhibits only MCF-7 (breast) cell line.

The presence of 5-CH_2OH in the sugar ring of **3** could be invoked as responsible for its better profile. Other congener fluoren-*O*-β-glucosydes with D-glucose moiety linked at the end of a lateral chain in position 2 of fluorene ring, present similar biological features.[27]

In apparent accordance, the conformational study of **3** reveals that the fluorene moiety is partially hindered by the acetylated sugar, thus contributing to improve pharmacokinetics. The larger conformational variability of **3** with respect to other compounds shouldn't exclude the possibility of a productive duplex DNA intercalation, rationalizing its pharmacological profile.

Compounds **4**–**7** should be able to achieve a faster DNA recognition and intercalation but the exposition of the fluorene moiety to the solvent could cause a reduced diffusion through biomembranes. Finally, compounds **1** and **2** show ASASA values higher than **3** but lower than **4**–**7** with intermediate conformational variability.

The on-going in vitro intercalation tests and docking studies with duplex DNA sequences will be useful to clarify the mechanism of action to establish a rational design of new analogues.

Likewise these preliminary biological screening results will help the optimisation work in this new series of compounds.

5. Experimental

5.1. General chemistry

^1H 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 ^1H 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).

$CHCl_3$, CH_2Cl_2, Ethyl acetate, THF, pyridine, acetic anhydride, *N*,*N*-dimethylformamide (DMF) and triethylamine were dried following standard procedures. All glycosylation reactions were carried out under N_2 atmosphere in oven-dried glassware.

5.2. Synthesis of 1,2,3,4-tetra-*O*-acetyl-L-arabinose (2a)

Acetic anhydride (2.5 mL, 6.66 mmol), pyridine (1.05 mL, 13.32 mmol) and 4-dimethylaminopyridine (catalytic amount) were added to sugar (1 g, 6.66 mmol) in dry CH_2Cl_2 (3 mL) and maintained at room temperature for 24 h. The mixture was then neutralized with diluted HCl and the resulting crude was concentrated and put through a flash silica gel column (CH_2Cl_2–CH_3OH 25:1). The final product was isolated, concentrated as a mixture of α and β anomers (2.07 g, 6.50 mmol, 100%) and characterized by spectroscopic (^1H NMR) method.

Compounds **1a** and **3a** were prepared according to the same procedure.

5.3. Synthesis of 1-Br-2,3,4-tri-*O*-acetyl-β-L-arabinose (2b)

A solution of HBr/acetic acid 30% (1.27 g, 90 μL, 15.7 mmol) was added to **2a** (500 mg, 1.57 mmol) at 0 °C. The mixture was stirred at room temperature for 2–3 h, then dissolved in CH_2Cl_2, washed with ice-water and sat. $NaHCO_3$, dried over anhydrous Na_2SO_4. Evaporation gave product **2b** (501 mg, 1.47 mmol, 94%, β anomer only) which requires no further purification.

Compounds **1b** and **3b** were prepared according to the same procedure.

5.4. Synthesis of 2,3,4-tri-*O*-acetyl-β-*O*-(9-fluorenyl)-L-arabinose (2)

HgO (27,3 mg, 0.13 mmol), $HgBr_2$ (catalytic amount) and 9-fluorenol (23 mg, 0.13 mmol) were added to a solution of 1-Br-2,3,4-tri-*O*-acetyl-β-L-arabinose **2b** (45 mg, 0.13 mmol) in anhydrous CH_2Cl_2 (5 mL) under nitrogen with stirring at room temperature for 72 h. After 10 days the mixture was filtered and evaporated under vacuum. The solid residue was dissolved in CH_3OH, purified by flash chromatography on silica gel (petroleum ether-ethyl acetate 2:1) to afford **2** as a solid (18 mg, 0.04 mmol, 54%).

Compounds **1** and **3** were prepared according to the same procedure.

5.5. Synthesis of 1,2,3,4-tetra-*O*-trimethylsilyl-D-xylopyranose (4a)

Chlorotrimethylsilane (2.11 mL, 16.65 mmol) was added at 0 °C to a stirred solution of sugar (500 mg, 3.33 mmol) and triethylamine (2.32 mL, 16.65 mmol) in dry DMF (3 mL), and the reaction mixture was stirred for 4 h at room temperature. *N*-hexane (50 mL) and crushed ice (20 mL) were added to the reaction mixture successively, and the organic layer was extracted with ice-water (6×15 mL), dried over Na_2SO_4 and evaporated in vacuum to afford **4a** (1.27 g, 2.91 mmol, 88%) as an oil.

Compounds **5a**, **6a** and **7a** were prepared according to the same procedure.

5.6. Synthesis of β-*O*-(9-fluorenyl)-D-xylopyranose (4)

Iodotrimethylsilane (580 mg, 417 μL, 2.91 mmol) was added at room temperature to a stirred solution of 1,2,3,4-tetra-*O*-trimethylsilyl-D-xilose **4a** (1.27 g, 2.91 mmol) in dry CH_2Cl_2 (5 mL). After 20 min the reaction mixture was added to the solution of 9-fluorenol (1.060 g, 5.82 mmol) and 2,6-di-*ter*-butylpyridine (560 mg, 650 μL, 2.91 mmol) in dry CH_2Cl_2 (7 mL) and stirred for 24 h at room temperature. After removing TMS groups with CH_3OH (4 mL), the reaction mixture was neutralized with pyridine, concentrated and purified by silica gel chromatography (petroleum ether–ethyl acetate 2:1) to give product **4** as an oil (575 mg, 1.83 mmol, 63%). Compounds **5**, **6** and **7** were prepared according to the same procedure.

5.7. Antiviral activity

The experiments were performed by adding different concentrations (1.0 and 0.5 μg/mL) of compounds to the in vitro WISH cells. After an incubation period of 24 h, the WISH cells were infected with HSV-2 or HSV-1 at a multiplicity of infection (M.O.I.) 0.1 PFU/mL cell. After 1 h, unabsorbed viruses were removed by washing twice with phosphate buffer solution (PBS) and cell cultures were further incubated in the medium containing the molecules at concentrations of 1.0 and 0.5 μg/mL. After 24 h, total yield of virus (cell-associated and surnatant) was determined by plaque titration on WISH cells and expressed as plaque forming unit per mL (PFU/mL).

5.8. Cytotoxicity test

WISH cells in RPMI 1640 containing 5% heat-inactivated fetal serum, were seeded at 3×10^4 per well in 96-well micro plates and incubated overnight at 37 °C in an atmosphere of 5% CO_2 in air. The compounds were diluted in EtOH at a concentration of 1.0 μg/mL, and the further dilutions of the compounds were made in RPMI-1640 cell medium; 100 μL of each dilution (1.0 and 0.5 μg/mL) was added in triplicate to the cells. The next day, cell survival was assessed by fixing and staining the cells with crystal violet (0.2% in 20% methanol). After washing and drying, 100 μL of 1% sodium dodecylsulphate were added to each well to solubilize the stained cells. The absorbance of each well was read at 490 nm with Micro Elisa Auto reader. Percentage cytotoxicity was calculated as $1-(A_{490}$ of sample/A_{490} of control) ×100.

5.9. Viruses

Herpes simplex virus type-2 (HSV-2) and Herpes simplex virus type-1 (HSV-1) were prepared on WISH cells. Virus titer was determined by assaying for PFU/mL on WISH cells.

5.10. U937 cells culture and differentiation

The human monocytic cell line U937 was a kind gift from Prof. J.P. Liautard, INSERM, University of Montpellier II, France.

The cells were maintained in culture at 37 °C in 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 50 mM 2-mercaptoethanol, 1 mM pyruvate, 1 mM non-essential aminoacids, 1 mM HEPES, gentamycin (50 mcg/mL) and 10% heat inactivated FCS (all reagents from Seromed, Milan, Italy). The cells (3×10^5 per well) were differentiated for 72 h with 100 nM 1α,25-dihydroxyvitamin D3 (VD) (Calbiochem, Milan, Italy) plus 100 nM all-transretinoic acid (RA) (Sigma, Milan, Italy) in tissue culture multi-well plates (Sigma, Milan, Italy). VD and RA were dissolved in absolute ethanol and stored at −80 °C at an initial concentration of 10^{-3} M. The final concentration of ethanol had no effects on cell growth and differentiation. After 72 h of incubation with VD-RA, differentiation was complete, and most cells were adherent. Differentiation was confirmed by growth inhibition, morphological changes, increased phagocytosis of *C. albicans*. Culture media and reagents tested for the presence of endotoxin by E-Toxate kit (Sigma) were found to contain 10 pg of endotoxin per mL. All media were free of Mycoplasma.

5.11. IFN-α/β titer

WISH cell lines and vesicular stomatitis virus (VSV) were used to measure the levels of IFN-α/β in all the U937 cell supernatants. All IFN titers were corrected against standard (recombinant human IFN-α/β, specific activity: 1 10^7 U/mg, Genzyme, Milan, Italy). One unit of IFN-α/β was defined as the reciprocal value of the highest dilution reducing the cytopathic effect by 50%.[28]

5.12. Statistical evaluation

Results are expressed as the mean of four experiments ± standard deviation (SD). Data were analysed by one-way analysis of variance (ANOVA) and Student–Newman–Keuls test.

5.13. Anticancer in vitro pre-screen

The in vitro disease-oriented primary antitumor screening performed in the US-National Cancer Institute (NCI) Laboratories of Bethesda—Maryland,[24] consists of the evaluation of the effects of each compound against 3 tumoral cell lines: MCF7 (breast), NCI-H460 (lung) and SF-268 (CNS). These 3-cell lines, one-dose assay have been in use by Developmental Therapeutics Program (DTP) for several years for the evaluation of combinatorial libraries and has proven to be an effective pre-screen.

In the current protocol, each cell line is inoculated and preincubated on a microliter plate. Test agents are then added at a single concentration (10^{-4} M) and the culture incubated for 48 h. End point determinations are made with sulforhodamine B, a protein-binding dye. Results for each test agent are reported as the percent of growth of the treated cells when compared to the untreated control cells. Compounds which reduce the growth of any one of the cell lines to 32% or less (negative numbers indicate cell-kill) are passed for evaluation in the full panel at 60 cell lines over a 5-log dose range.

Acknowledgements

This work was financially supported by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST-Rome). We thank Prof. Cristina Nativi for her helpful suggestions during the work preparation.

References and notes

1. Osorio, Y.; Ghiasi, H. *J. Virol.* **2003**, *77*, 5774.
2. Goodman & Gilman, '*Le basi farmacologiche della terapia*', Zanichelli Ed.: Bologna, 1996, IX ed., cap. 51, pp 1203–1254.
3. Arcamone, F. *Doxorubicin — Anticancer Antibiotics*; Academic Press: New York, 1981.
4. Wang, H. K.; Morris-Natschke, S. L.; Lee, K. H. *Med. Res. Rev.* **1997**, *17*, 367.
5. Pullman, B. Molecular mechanisms of specificity in DNA-antitumor drug interactions. In *Advances in Drug Research, vol. 18*; Academic Press: New York, 1989; p 74–76.
6. Sainz, B.; Halford, W. P. *J. Virol.* **2002**, *76*, 11541.
7. Samuel, C. E. *Curr. Top. Microbiol. Immunol.* **1998**, *233*, 125.
8. Vilcek, J.; Sen, J. Interferons and other cytokines. In *Virology*; Fields, B. N., Knipe, D. M., Howley, P. M., Eds.; Raven Publishers: Philadelphia, Pa, 1996; p 375–400.
9. Fischer, J. *Biochem. J.* **1995**, *312*, 215.
10. (a) Fisher, J. *Gen. Pharmacol.* **1996**, *27*, 1317. (b) Lullmann-Rauch, R. *Exp. Toxicol. Pathol.* **1994**, *46*, 315. (c) Prokopek, M. *Biochem. Pharmacol.* **1991**, *42*, 2187.
11. (a) Fisher, J. *Biochem. J.* **1996**, *315*, 369. (b) Lullmann-Rauch, R. *Biochem. Pharmacol.* **1995**, *49*, 1223.
12. Fenick, D. J. *J. Med. Chem.* **1997**, *40*, 2452.
13. Fischer, J. F.; Harrison, A. W.; Bundy, G. L.; Wilkinson, K. F.; Rush, B. D.; Ruwart, M. J. *J. Med. Chem.* **1991**, *34*, 4140.
14. (a) Kennedy, J. F.; White, C. A. Bioactive Carbohydrates. In *Chemistry, Biochemistry and Biology*; John Wiley & Sons: New York, 1983 (b) Musser, J. H.; Fügedi, P.; Anderson, M. B. Carbohydrate-based therapeutics. In *Burger's Medicinal Chemistry and Drug Discovery, vol. I,* 4th ed.; John Wiley & Sons: New York, 1995; p 902–947.
15. Simmons, A. *J. Infect. Dis.* **2002**, *186*, 71.
16. Hanessian, S.; Haskell, T. H. Antibiotics containing sugars. In *The carbohydrates — Chemistry and Biochemistry,* 2nd ed.; Academic Press: London, 1970; p 139–200.

17. (a) Greene, T. W.; Wuts, P. G. M. *Protective groups in Organic Synthesis*; John Wiley & Sons: New York, 1991. (b) Kocienski, P. J. *Protecting groups*; Thieme: Stuttgart, 1994.
18. Koenigs, W.; Knorr, E. *Ber. Dtsch. Chem. Ges.* **1901**, *34*, 957.
19. (a) Schmidt, R. R. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 212. (b) Schmidt, R.et al. In *Advances in Carbohydrate Chemistry and Biochemistry*; Horton, D., Ed.; Academic Press: London, 1994; p 21–123 50.
20. Schmidt, R. R. Synthesis of glycosides. In *Comprehensive Organic Synthesis*; Pergamon Press: Oxford, 1991; p 49–58.
21. (a) Smits, E.et al. *J. Chem. Soc., Perkin Trans. I* **1996**, 2873. (b) Jensen, K. J., *et al. J. Chem. Soc., Perkin Trans. I* **1993**, 2119.
22. Olah, G. A.; Narang, S. C. *Tetrahedron* **1982**, *38*, 2225.
23. Uchiyama, T.; Hindsgaul, O. *Synlett* **1996**, 499.
24. . *J. Natl. Cancer Inst.* **1991**, *83*, 757.
25. Still, W. C.; Tempczyk, A.; Hawley, R. C.; Hendrickson, T. *J. Am. Chem. Soc.* **1990**, *112*, 6127.
26. Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440.
27. Private communication.
28. Havell, E. A.; Vilcek, J. *Antimicrob. Agents and Chemother.* **1972**, *2*, 476.