❚ **Medicinal Chemistry & Drug Discovery**

An Efficient Lipase-Catalyzed Synthesis of Fatty Acid Derivatives of Vanillylamine with Antiherpetic Activity in Acyclovir-Resistant Strains

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A series of eleven N-fatty acid derivatives of vanillylamine was synthesized following an enzymatic approach, in excellent yield and a chemoselective way. The excellent results obtained by catalysis of *Thermomyces lanuginosus* lipase made the procedure very efficient, considering the low amount of enzyme required and its lower price in comparison with other commercial lipases. The influence of various reaction parameters in the lipase-catalyzed reactions, such as enzyme source, nucleophile/substrate ratio, enzyme/substrate ratio, solvent and temperature, was studied. In order to evaluate the influence of the fatty acid chain length and configuration on the reaction rate and yield, HPLC analysis and molecular modeling experiments were performed. The results showed that a chain length between 12–14 carbon atoms favors the activity of the enzyme, while insaturation had no effect on the reaction rate. These facts confirm the experimental results. In addition, some of the evaluated compounds exhibited antiviral activity against Herpes simplex virus type 1 (HSV-1) KOS strain (TK+) and Field and B2006 strains (TK-) in Vero cells.

Introduction

It is well recognized that N-alkylamides possessing a fatty acid residue are bioactive natural amides.[1] Capsaicinoids are characteristic alkylamides isolated from plants belonging to *Capsicum* genus. They are the active principle of the pungency, aroma, acidity and hot sensation in many peppers.[2–4] All capsaicinoids possess a N-vanillylamine residue (N-4-hydroxy-3-methoxybenzyl amine group) attached to a fatty acid core and differ in the length of the fatty acid chain and number of unsaturations. Besides its pungency, capsaicinoids exhibit health benefits, such as cancer prevention,[5] cardiovascular and gastrointestinal benefits,[6] antiarthritic, pain control, anti-inflammatory and antioxidant activities,[7,8] plasma lipids decrease[9] and weight loss properties.[10] Because of these multiple biological activities, the capsaicinoids are currently the subject of major researches.

On the other hand, the use of enzymes and whole cells of microorganisms in synthesis of pharmaceuticals derivatives is increasing in the last years.[11–13] It is recognized that enzymes are capable of accepting a wide array of substrates, and catalyze reactions in a chemo- regio- and stereoselective way. As a result, biocatalysis constitutes a good alternative to the synthesis of organic compounds through a Green Chemistry approach and allows carrying out different chemical transformations without the need for tedious protection and deprotection steps, especially in compounds with several functional groups.[11] Over the last years, the use of lipases in non-aqueous media has been widely used for several synthetic reactions such as esterification, transesterification, aminolysis, polymerization, etc.[11–18]

In our laboratory several studies have been accomplished on the use of lipases in reactions of esterification, transesterification and aminolysis of multiple substrates, obtaining a wide variety of biologically active novel compounds.[19–24] Recently, in the field of pharmaceuticals, we reported the synthesis of several compounds with application as potential antiparasitic,[20] and antitumoral agents.[21]

Keeping in mind the above data we based our investigations on template of N-acylvanillylamine derivatives. Thus, we report herein the synthesis of a series of eleven vanillylamides (1) derived from fatty acids by reaction of either the corresponding acid or their ethyl esters with vanillylamine through an enzymatic approach (Figure 1).

In addition, with the objective of finding an explanation on the effect of chain length, unsaturation and configuration of the fatty acid moiety on the enzymatic aminolysis, a kinetic study and computer simulation were carried out. Finally, all synthesized compounds were biologically evaluated as poten-

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1a-k
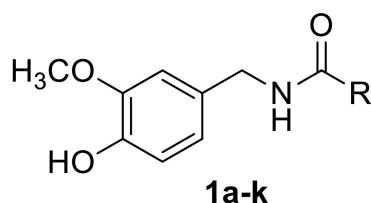
R: $CH_3(CH_2)_nCH_2$-, n = 3, 6, 9, 11, 13, 15
$CH_3(CH_2)_7CH=CH(CH_2)_6CH_2$-, *cis* and *trans*
$CH_3(CH_2)_4(CH=CHCH_2)_n(CH_2)_mCH_2$-, n = 2, 4 m = 1, 5
$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_6CH_2$-

Figure 1. Structure of obtained *N*-acylvanillylamines.

tial antiviral agents against TK+ and TK- strains of HSV-1, sensitive and resistant to acyclovir (ACV) treatment, respectively.

Results and Discussion

Because of the great pharmacological potential exhibited by vanillylamides and the difficulty in achieving chemoselectivity in its acylation, the development of a simple and mild procedure to prepare them is of utter importance. Moreover, it would be desirable to obtain the derivatives in a single step and applying an easy isolation procedure.

Applying traditional synthetic reactions, a series of NVAs derived from the isoprenoid and unsaturated fatty acids has been chemically obtained by acylation of vanillylamine (VA) via mixed phosphoric anhydrides.[25]

Enzymatic methodology was also applied for the synthesis of some capsaicin analogues. They were prepared in very low yield, using CAL B as biocatalyst in a two phase system, at 70°C for 72 h or by transacylation of capsaicin with natural oils using CAL B in hexane at 70°C for 192 h.[26,27]

Another strategy was developed using lipases in organic solvents. In this case olvanil (vanillyloleamide) was selected as model compound for the synthesis of capsaicin derivatives using CAL B in hexane or 2-methyl-2-butanol as solvents.[28]

In all cases, vanillylamine was released from its hydrochloride at reaction media using a tertiary amine as *N,N*-diisopropylethylamine or triethylamine. The effect of the tertiary amine concentration in the synthesis rate and the total conversion was also reported.[30]

In this paper, we report an improved enzymatic procedure for the preparation of a series of *N*-acylvanillylamines from fatty acids and vanillylamine. Low cost, excellent chemoselectivity and products yield make this procedure an efficient way to obtain a broad range of acyl derivatives: short, medium and long chain, saturated and unsaturated, *cis* and *trans*, etc. (Figure 1).

Enzymatic Synthesis

Following our previous work on enzymatic amide synthesis, we applied a lipase catalyzed method,[21,23,24] which led to the obtention of eleven *N*-acylvanillylamines with various alkyl chain length and different number of unsaturations in the acyl moiety (Scheme 1).
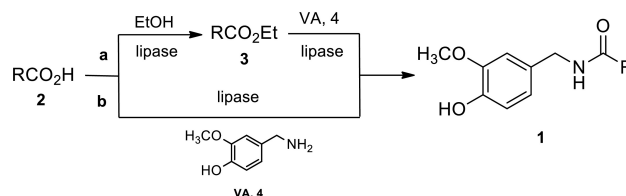
Scheme 1. Synthesis of *N*-acylvanillylamines.

The enzymatic strategy was performed through two different approaches. The first one involved two steps: i) the reaction of the fatty acid with ethanol to obtain the corresponding ethyl ester and ii) acylation of vanillylamine (VA, **4**) with the ester (Scheme 1, path a). In the second approach, the direct acylation of VA with the corresponding fatty acid was achieved (Scheme 1, path b). With the aim of achieving the optimal conditions, we studied the behavior of various lipases and some reaction parameters such as solvent, temperature, enzyme:substrate ratio (E/S) and acylating agent:substrate ratio (A/S). In every case stearic acid (**2f**) or ethyl stearate (**3**) were used as acylating agent.

Enzyme screening and solvent effect

In the present work, five lipases from several sources were evaluated: from the yeasts *Candida rugosa* (CRL) and *Candida antarctica* B (CAL B); Lipozyme from the fungus *Rhizomucor miehei* (RMIM) and *Thermomyces lanuginosus* (TLIM) and from plants: the naturally immobilized *Carica papaya* lipase (CPL), which is the remaining solid fraction of papaya latex after wash off of proteases.

Although several studies have already been reported on the effect of organic solvents on lipase-catalyzed reactions,[17] it is still difficult to predict the solvent effect. Therefore the practical way is to select an appropriate solvent through screening experiments. The solvents tested were hexane, diisopropyl ether, toluene and acetone (Table 1). Taking into account the optimal reaction conditions for similar transformations, the reactions were carried out at 55°C using E/S: 10 and acylating agent:substrate ratio A/S: 1.2. Ethyl stearate was prepared according to conditions previously reported.[21]

It was observed that CAL B, TLIM and RMIM were active, whereas no product was detected with CRL or CPL for both **2f** and **3**. Conversion was determined at 24 h reaction. The most satisfactory results were obtained with TLIM using stearic acid as acylating agent in DIPE as solvent, affording **1f** (100% conversion) at 24 h of reaction (Table 1, entry 6). This lipase was

Table 1. Lipase-catalyzed synthesis of *N*-stearylvanillylamine (**1 f**).

Entry	Lipase	Solvent	Acylating agent	Conversion (%)
Lipase and solvent				
1	CAL B	Hexane	2f	49
2	CAL B	DIPE	2f	78
3	CAL B	Toluene	2f	NR
4	CAL B	Acetone	2f	NR
5	TLIM	Hexane	2f	73
6	**TLIM**	**DIPE**	**2f**	**100**
7	TLIM	Toluene	2f	25
8	TLIM	Acetone	2f	NR
9	RMIM	Hexane	2f	50
10	RMIM	DIPE	2f	89
11	RMIM	Toluene	2f	20
12	RMIM	Acetone	2f	NR
13	CAL B	Hexane	3	50
14	CAL B	DIPE	3	78
15	CAL B	Toluene	3	NR
16	CAL B	Acetone	3	NR
17	TLIM	Hexane	3	70
18	TLIM	DIPE	3	98
19	TLIM	Toluene	3	25
20	TLIM	Acetone	3	NR
21	RMIM	Hexane	3	40
22	RMIM	DIPE	3	78
23	RMIM	Toluene	3	20
24	RMIM	Acetone	3	NR
Temperature: 55 °C, time: 24 h, E/S: 10, A/S: 1.				

Table 2. Optimization of reaction parameters for lipase catalyzed synthesis of *N*-stearylvanillylamine (**1 f**).[a]

Entry	T (°C)	E/S[b]	A/S	t (h)	Conversion (%)
E/S					
1	55	10	1.2	3	100
2	55	5	1.2	3	100
3	55	2	1.2	3	100
4	55	1	1.2	3	100
5	55	0.5	1.2	3	97
6	55	0.2	1.2	3	89
7	55	0.2	1.2	8	100
8	55	0.1	1.2	8	78
Nu/S					
9	55	0.5	1.0	3	97
10	55	0.2	1.5	3	90
11	55	0.2	1.0	3	89
12	55	0.2	1.0	8	100
Temperature					
13	25	0.5	1.0	18	100
14	**25**	**0.2**	**1.0**	**18**	**100**
15	40	0.2	1.0	12	100

[a]Reactions conditions: Enzyme: TLIM. Solvent: DIPE [b]E/S: enzyme amount in mg/substrate amount in mg.

also active in hexane and toluene, but to a lesser extent (Table 1, entries 5 and 7). CAL B in DIPE showed a lower performance than TLIM showing 78% conversion after 24 h. In the absence of biocatalyst no product was obtained.

Once the enzyme and the solvent were chosen, the time needed to achieve 100% conversion was measured. It was observed that the conversion was completed after 3 hours.

Effect of enzyme:substrate ratio

The influence of E/S in the enzymatic acylation was evaluated using A/S: 1.2, DIPE as solvent at 55 °C and variable amounts of TLIM. From the obtained results, it was observed that $E/S = 1$ gave the best results (Table 2, entry 4). However, when working with $E/S = 0.2$, 100% conversion was obtained in a reasonable time of 8 h (Table 2, entry 7), therefore showing that TLIM was very efficient. Considering that, we concluded that it was preferable to use the lowest amount of enzyme, and $E/S = 0.2$ was the ratio of choice.

Effect of acylating agent:substrate ratio

The influence of A/S on acylation yield was evaluated in DIPE using TLIM at 55 °C. As shown in Table 2 (entries 9–12) an equimolar ratio was enough to achieve the best results (Table 2, entry 12).

Influence of temperature

With the aim of investigating the influence of temperature on the enzymatic acylation we performed it at 25 °C, 40 °C and 55 °C. The other reaction parameters were settled to their optimal values (TLIM, DIPE, E/S: 0.2 or 0.5 and A/S: 1.0). The results (Table 2, entries 13–15) show that at room temperature maximum conversion is also reached but at higher times. Although at higher temperatures the reaction times are reduced, at room temperature maximum conversion is obtained at a reasonable time. Taking into account that it is desirable to work at room temperature, 25 °C was the temperature of choice.

Based on these results, we have chosen as standard conditions for the enzymatic acylation of vanillylamine: TLIM as biocatalyst, DIPE as solvent, temperature: 25 °C, E/S: 0.2 and A/S: 1.0.

Once the experimental conditions were optimized, we applied them to a variety of fatty acids (**2a–e** and **2 g-k**). The results, expressed as yield of isolated product for *N*-acylvanillylamines **1a–k** are summarized in Table 3.

Table 3 shows the results at 24 h of reaction. Following the progress of each reaction it was noted that the lipase activity in the acylation of vanillylamine (**4**) is related to the chain length of the fatty acid. In all cases yield was excellent but the reaction time was variable, between 18 and 24 h. The best performance was achieved with a chain between twelve and sixteen carbon atoms (**2 c-2 e**) since the respective products (**1 c-1 e**) were obtained in almost quantitative yield at 3 h of reaction. Hence, we decided to carry out a reaction analysis to determine the influence of the fatty acid in the acylation of **4**, taking aliquots at different times and analyzing them by HPLC.

Table 3. Enzymatic synthesis of *N*-vanillylamines			
Entry	Fatty acid	Product	Yield
1	Caproic	**1 a**	90
2	Pelargonic	**1 b**	92
3	Lauric	**1 c**	99
4	Myristic	**1 d**	99
5	Palmitic	**1 e**	99
6	Stearic	**1 f**	96
7	Oleic	**1 g**	95
8	Elaidic	**1 h**	95
9	Linoleic	**1 i**	93
10	Linolenic	**1 j**	93
11	Arachidonic	**1 k**	92

Reaction conditions: Enzyme: TLIM; Solvent: DIPE; Temperature: 25 °C; E/S: 0.2; A/S: 1.0; t: 24 h

selection criteria (Figure 3). Since the electronic properties of the substrates are very similar, their steric characteristics are
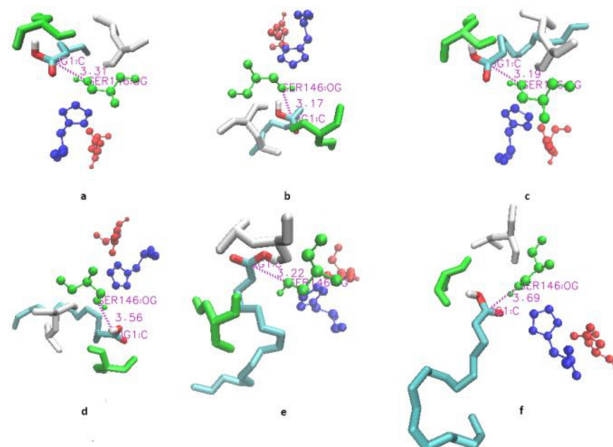

Figure 3. Docking results for TLIM with: (a) Caproic acid; (b) Pelargonic acid; (c) Lauric acid; (d); Myristic acid; (e) Palmitic acid; (f) Stearic acid. Distance in Å.

likely to influence the outcome of TLIM-catalyzed reactions. A correlation between the length of the fatty acids and mean binding energy of the intermediate was observed in those clusters as shown in Figure 4.
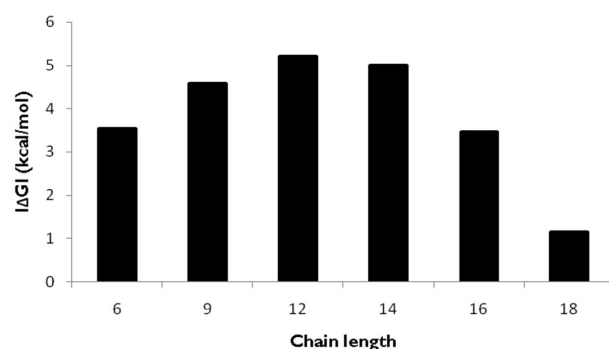
In order to illustrate this tendence, Figure 2 shows the HPLC peak area vs. number of carbon atoms of fatty acids after

Figure 2. HPLC peak area vs chain length after 4 h of reaction.

Figure 4. Docking results: binding energy vs chain length.

4 h of reaction. It can be observed a correlation between enzyme activity and the effect of the chain length of the acylating agent considering saturated fatty acids. While a short or long chain requires longer reaction times, an intermediate chain favors the reaction, being observed a maximum for lauric acid. In addition, it was observed that neither presence nor number of unsaturations influenced reactivity.

Molecular modeling

To shed light to the molecular determinants of the enzymatic acylation of **4**, we examined the reaction using molecular modeling. This approach allowed us to understand the relation between the substrate structure and reaction rate. Then, molecular docking studies have been performed on acids **2a–f** with TLIM and the ability of the catalytic pocket to accommodate these substrates was evaluated.

The analysis of the results led to the selection of a possible conformation for each fatty acid, considering the interaction with the amino acids of the catalytic site (Asp201-His258-Ser146), binding energy and the population of clusters as

These results provided an insight about interaction between catalyst and acylating agent, therefore giving information at a molecular level about reaction kinetics. It was observed that lauric and myristic acid shown the highest binding energy in the clusters with the lowest interaction distance. This might explain why they have the highest reaction rate of all the fatty acids studied, in agreement with our experimental results

Biological evaluation

To investigate a potential antiviral effect, we first performed a qualitative screening of eleven vanillylamides. Vero cell mono-

layers grown in 96-well plates were infected with HSV-1 KOS strain (multiplicity of infection (m.o.i.) = 0.1) and then treated or not with different concentrations of vanillylamides (from 1 to 100 μM). After 24 h of incubation at 37 °C, cytopathic effect was observed under microscope, and compared to that corresponding to control virus, in which cell death due to HSV-1 multiplication reached 100%. Only vanillylamides **1 b** and **1 d** proved to reduce 50–60% of the cytopathic effect (data not shown) and, hence, the corresponding supernatants containing free and cell associated virus were collected and titrated in Vero cells. Thus, Vero cells grown in 24 well plates were infected with serial 10-fold dilutions of viral yields. After 48 h incubation at 37 °C, cells were fixed, stained with Crystal Violet, and plaque forming units were counted.

A dose-dependent inhibition of viral replication was observed in drug-treated cultures. We established that **1 b** and **1 d** prevented HSV-1 multiplication in Vero cells when added after infection, exhibiting 50% inhibition of virus yields (EC_{50}) at concentrations of 61.69 μM and 26.45 μM for **1 b** and **1 d**, respectively (Figure 5).
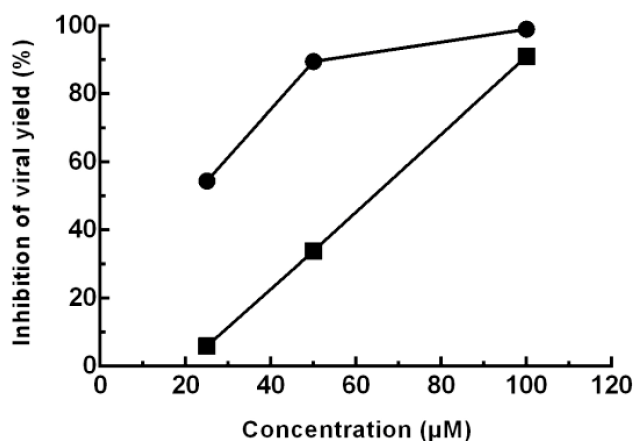

Figure 5. Antiviral activity of compounds **1 b** (■) and **1 d** (●). Vero cells grown in 96-well plates were infected with HSV-1 KOS strain (m.o.i. = 0.1) and mock-treated or treated with vanillylamides. After 24 h of incubation at 37 °C, free and cell-associated virus corresponding to treatment with compounds **1 b** (■) and **1 d** (●), as well as control virus yield, was collected and titrated in Vero cells.

To rule out an eventual cytotoxic effect, the 50% cytotoxic concentration (CC_{50}) was determined. For that purpose, Vero cells grown 96-well plates were treated or not with vanillylamides **1 b** and **1 d** in concentrations ranging from 3 to 200 μM. After 24 h incubation at 37 °C a MTT colorimetric assay was performed.

As shown in Figure 6, vainillylamides exhibited CC_{50} of 440 μM and 80 μM, respectively, which were higher than those corresponding to EC_{50} values. The selectivity index ($SI = CC_{50}/EC_{50}$) were 7.13 and 3.02 after treatment with **1 b** and **1 d.**

The conventional therapy for the management of HSV-1 infections mainly comprises acyclovir (ACV) and other nucleoside analogues. HSV-1 infections are characterized by recurrent
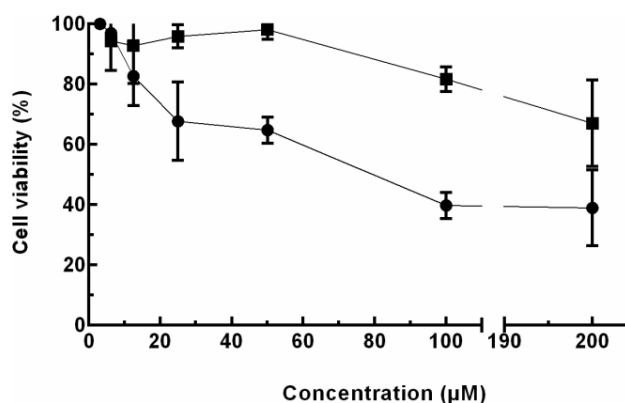

Figure 6. Cytotoxicity of compounds **1 b** (■) and **1 d** (●). Vero cells grown in 96-well plates were treated with 3.125 - 200 μM of both vanillylamides for cytotoxicity assays. After 24 h of incubation at 37 °C, a MTT colorimetric assay was performed.

episodes of disease and a common outcome of repeated treatments with ACV is the emergence of resistant viral strains, particularly when immunosuppressed patients are involved.[29] Therefore, we investigated whether both vanillylamides showed an inhibitory effect on HSV-1 ACV-resistant strains spread.

For that purpose, Vero cell monolayers grown in 96-well plates were infected with HSV-1 Field and B2006 strains (TK-) (m.o.i. = 0.1) and then treated with different concentrations of **1 b** and **1 d.** After 24 h of incubation at 37 °C, cytopathic effect was observed under microscope, and compared to that corresponding to control virus. From those wells where a reduction of cytopathic effect was observed, supernatants containing free and cell associated virus were collected and titrated in Vero cells. As shown in Figure 7, a dose-response effect was also observed. In the case of infection with HSV-1 Field strain, EC_{50} values obtained were 65 μM and 4.4 μM for **1 b** and **1 d**, respectively, whereas EC_{50} values of 44 μM and 4.8 μM were obtained for **1 b** and **1 d** when infection was performed with HSV-1 B2006 strain. In each case, SI of 10 and 16.7 were estimated for Field and B2006 strains, respectively.

Conclusions

This work describes the enzymatic synthesis of eleven fatty acid vanillylamides by direct acylation of vanillylamine. Among the evaluated enzymes, the lipase from the fungus *Thermomyces lanuginosus* (TLIM) gave the best results. *N*-vanillylamides were obtained as the only products, showing a high chemoselective behavior of the lipase.

In summary, the described enzymatic strategy offers an excellent alternative to synthesize *N*-fatty acylvanillylamines in comparison with methods previously reported, which have the disadvantage of using polluting catalysts or reagents such as metals, carbodiimides, strong basic media, etc. The lipase-catalyzed procedure uses less toxic and pollutant reactants, and is performed at room temperature using a very small amount of catalyst and a more volatile solvent. The use of very
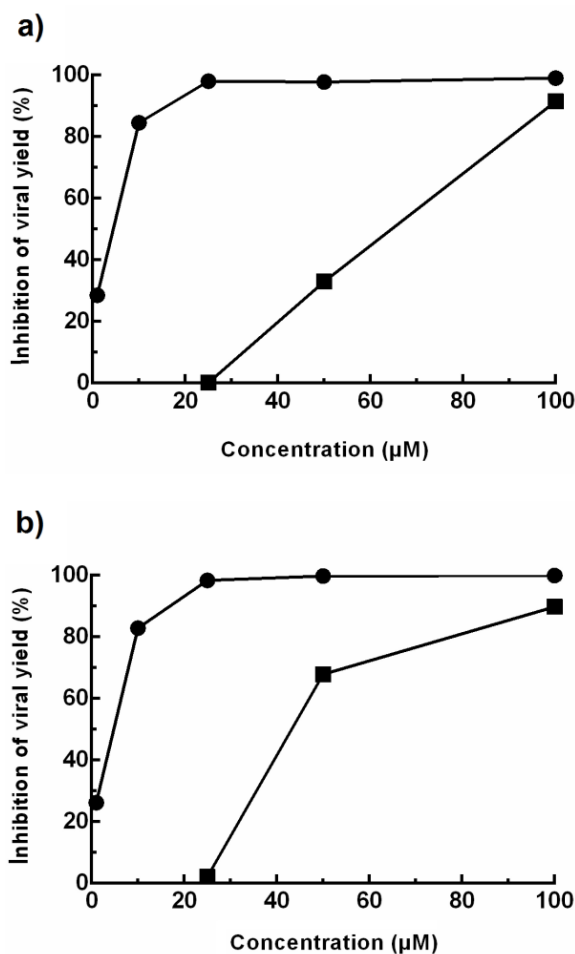
Figure 7. Antiviral activity of compounds **1 b** (■) and **1 d** (●) against HSV-1 ACV-resistant strains. Vero cells grown in 96-well plates were infected with HSV-1 Field (**A**) and B2006 (**B**) strains (m.o.i. = 0.1) and mock-treated or treated with vanillylamides. After 24 h of incubation at 37 °C, free and cell-associated virus corresponding to treatment with compounds **1 b** (■) and **1 d** (●), as well as control virus yield, was collected and titrated in Vero cells.

low quantity of enzyme, added to its low cost compared with CAL B make this procedure very advantageous. The lipase is biodegradable and, consequently, more friendly to the environment than chemical catalysts. Besides, because the enzyme is insoluble in the reaction medium, it is easily removed by filtration and can be reused. In this reaction, TLIM retained 75 % activity after five reaction cycles.

The observed difference in reactivity of fatty acids prompted us to carry out further analysis, which involved HPLC studies of reaction kinetics, showing that substrates with a chain length of 12–14 carbon atoms react faster. In addition, computer simulation results led to the conclusion that the observed difference in reactivity is determined mainly by differences in binding energy between fatty acids and the serine residue in the catalytic triad of the enzyme.

On the other hand, the present report examines the effect of the synthesized derivatives as antiviral agents. From eleven vanillylamides assayed, two of them proved to exert an

inhibitory effect on HSV-1 infection in vitro conditions. As expected, SI values obtained for **1 b** and **1 d** were considerably lower than those reported with ACV when KOS strain of HSV-1 was used. Both **1 b** and **1 d** significantly restrained ACV-resistant strains Field and B2006 replication, with SI > 10. Thus, these derivatives of vanillylamine described herein could represent a starting point for the development of new antiherpetic drugs.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest.

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