http://dx.doi.org/10.21577/0103-5053.20190260

J. Braz. Chem. Soc., Vol. 00, No. 00, 1-10, 2019
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Article

Synthesis, *in silico* Study and Antimicrobial Evaluation of New Diesters Derived from Phthaloylglycine

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New diesters derived from phthaloylglycine (**7a**-**i**) were synthesized and their structures characterized by infrared, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The compounds were evaluated in an *in silico* study, which demonstrated positive features indicating a possible drug candidate. The diesters showed antifungal activity ranging from moderate to strong against strains of *Candida*. Compounds **7a**, **7b**, **7c**, **7e** and **7i** had a moderate minimum inhibitory concentration (MIC) of 1024 μg mL^{-1} against all fungal strains, while **7h** showed a very good MIC of 256 μg mL^{-1} against *Candida albicans*, *Candida parapsilosis* and *Candida krusei* and 64 μg mL^{-1} against *Candida tropicalis*. However, only **7h** and **7i** were able to inhibit bacterial growth of strains of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Escherichia coli* with an MIC of 1024 μg mL^{-1}.

Keywords: phthalimide, phthaloylglycine, antibacterial activity, antifungal activity

Introduction

Multidrug-resistance is posing a great threat to health care services worldwide, where infections caused by resistant bacteria and/or fungi are very difficult to treat, usually leading to therapeutic failure with high mortality rates. The development of new drugs is a prominent alternative in the control of these infections, aiming to prevent or decrease pathogen resistance to achieve better treatment outcomes.[1,2] Several heterocyclic compounds possess antimicrobial properties and have been studied and evaluated as potential drug candidates. Among such compounds is phthalimide, with a distinct and valuable structure for the design and development of new varieties of drugs.

Phthalimides have an imide ring, which is responsible for their biological activity.[3] These molecules have drawn attention because of their versatile range of biological applications including antibacterial, antifungal, analgesic, anti-inflammatory, antiviral, antitumor and anticonvulsant.[4]

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It is widely reported that phthalimide is an important biologically active pharmacophore and its derivatives have great antimicrobial activities.[5-7]

To counter the mechanisms of microbial resistance already known, it is necessary to employ molecular modification strategies such as molecular lipophilicity control, which influences the biological activity of new drug candidates.[8] This is achieved by altering the number of carbons in the alkyl chain of an ester, for example.

Due to these merits, nine diester compounds derived from phthalimide were synthesized as potential new drug candidates. The compounds initially went through a design stage and *in silico* evaluation, and they were then taken to the organic synthesis stage, and finally tested for antimicrobial activity.

Results and Discussion

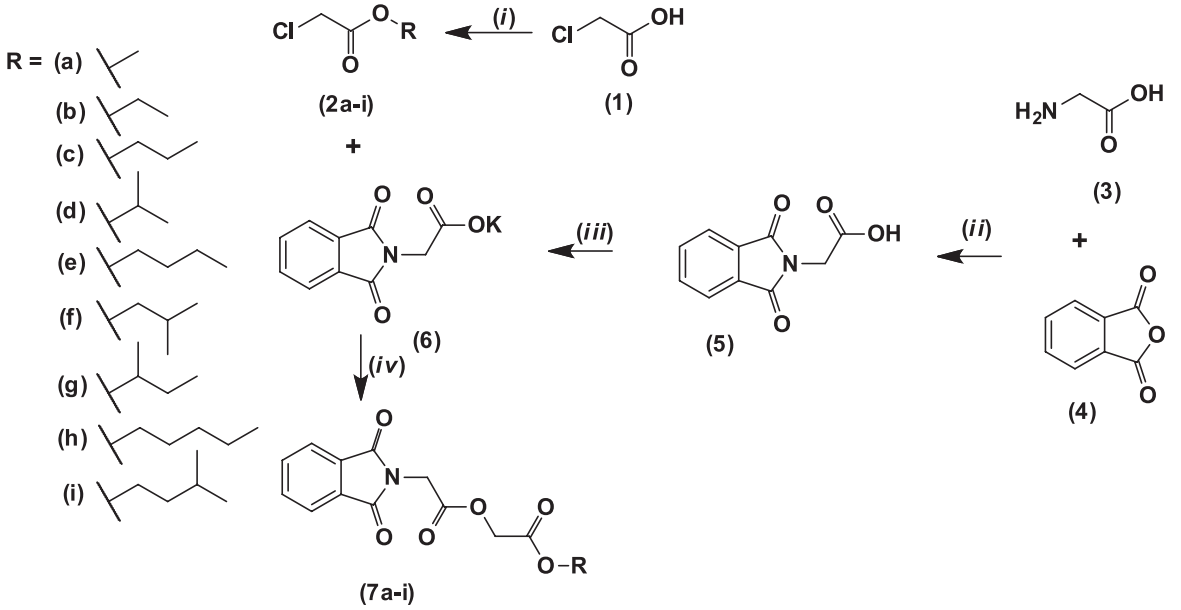
Chemical

The synthesis of the target molecules **7a**-**7i** involved four synthetic stages, which are described in Scheme 1.

The first step was the preparation of the 2-chloroacetate esters (**2a**-**2i**) via an esterification reaction between chloroacetic acid and the selected alcohols using Fisher's method, obtaining yields of 60-70%.[9] In the second step, phthaloylglycine (**5**) was prepared by the condensation reaction of the phthalic anhydride (**4**) with glycine (**3**) in glacial acetic acid as solvent.[10] Potassium phthaloylglycinate (**6**) was obtained by an acid-base reaction in an ethanolic solution of potassium hydroxide. The final diester products (**7a**-**7i**) were prepared from the nucleophilic substitution reaction S_N2 of the 2-chloroacetate esters (**2a**-**2i**) with potassium phthaloylglycinate (**6**) using dimethylformamide (DMF) as solvent, catalyzed by 1% sodium iodine in reflux, with yields ranging 38-75% at this stage.

The structures of the diesters were confirmed using infrared (IR) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, including the two-dimensional techniques ^{1}H-^{1}H-COSY (correlation spectroscopy) and ^{1}H-^{13}C HSQC (heteronuclear single quantum correlation) and HMBC (heteronuclear multiple bond correlation). In the NMR spectrum of compound **7b**, there were signals in the aromatic hydrogen region δ_H 7.88-7.74 ppm, referring to the 4 aromatic hydrogens. In the two-dimensional direct correlation spectrum (^{1}H-^{13}C HSQC), correlations were observed between the signal at δ_H 4.56 ppm, referring to the methylene hydrogen H-5, and the C-5 signal at δ_C 38.71 ppm and between the signal δ_H 4.68 ppm, referring to the methylene hydrogen H-7, and the carbon signal at δ_C 61.70 ppm (C-7).

Based on the analysis of compound **7b**, the other diesters (**7a**, **7c** and **7d**-**7i**) showed a methylene hydrogen singlet (–NC<u>H</u>$_2$CO$_2$/H-5) referring to phthaloylglycine with displacement at δ_H 4.71-4.66 ppm and a singlet of a methylene hydrogen (–CO$_2$C<u>H</u>$_2$CO$_2$–/H-7) referring to the part of the ester at δ_H 4.57-4.55 ppm. The signals of the aromatic ring hydrogens appeared at δ_H 7.92-7.67 ppm. The spectrum of compound **7a** displayed a singlet for 3 hydrogens with terminal CH_3 connected to the ester oxygen at δ_H 3.75 ppm (–CO$_2$C<u>H</u>$_3$). Compound **7c** showed a triplet for 2 methylene hydrogens (–CO$_2$C<u>H</u>$_2$–) at δ_H 4.13 ppm, multiplet for 2 methylene hydrogens (–CO$_2$CH$_2$C<u>H</u>$_2$–) at δ_H 1.67 ppm and a triplet for 3 methyl hydrogens (–CO$_2$CH$_2$CH$_2$CH$_3$) at δ_H 0.94 ppm. Compounds **7e** and **7h** showed a triplet for 2 methylene hydrogens (–CO$_2$C<u>H</u>$_2$–) at δ_H 4.13-4.18 ppm, one multiplet for 2 methylene hydrogens (–CO$_2$CH$_2$C<u>H</u>$_2$–) at δ_H 1.71-1.59 ppm, a multiplet for 2 methylene hydrogens (–CO$_2$CH$_2$CH$_2$C<u>H</u>$_2$–) at δ_H 1.42-1.29 ppm and a triplet for 3 methylic hydrogens at δ_H 1.26-0.90 ppm. Compound **7d** showed a multiplet for 1 methinic hydrogen at δ_H 5.09 ppm and one doublet for 6 methylene hydrogens at δ_H 1.26 ppm. Compound **7f** showed a multiplet for 1 methylene hydrogen at δ_H 1.95 ppm, a doublet for 2 methylene hydrogens at δ_H 3.96 ppm and one doublet for 6 methylic hydrogens at δ_H 0.93 ppm. Compound **7g** displayed a multiplet for 1 methinic hydrogen at δ_H 4.98-4.89 ppm, a multiplet for 2 methylene hydrogens at δ_H 1.67-1.53 ppm, a doublet for 3 methylic hydrogens at δ_H 1.23 ppm and a triplet for 3 methylic hydrogens at δ_H 0.88 ppm. Finally, compound **7i** showed a triplet for 2 methylene hydrogens at δ_H 4.20 ppm, a double doublet for 1 methinic hydrogen at δ_H 4.02 ppm, a quartet for 2 methylene hydrogens at δ_H 1.54 ppm and one doublet for 6 methylic hydrogens at δ_H 0.92 ppm.



Scheme 1. Synthetic route to obtain the target molecules. Reagents and conditions: (*i*) HO–R, H_2SO_4, reflux, 8 h, 85%; (*ii*) glacial acetic acid, 130 °C, 6 h, 80%; (*iii*) EtOH, KOH, room temperature, 2 h, 90%; (*iv*) DMF, NaI (1%), 100 °C, 24 h, 38-75%.

All nine **7a**-**7i** diesters had three characteristic signals attributed to carbonyl C-4 and C-4', C-6 and C-8 at δ_C 167.54-166.45 ppm. In the two-dimensional spectrum (HMBC) analysis of compound **7b**, it was possible to allocate the displacement of the carbonyl groups referring to the compound from the couplings between ^{13}C and ^{1}H distant 2 and 3 connections. $-CO_2C\underline{H}_2CH_3$ methylene hydrogens at δ_C 4.22 ppm showed coupling with a carbon in $-CO_2CH_2\underline{C}H_3$ at δ_C 14.18 ppm and with carbonyl carbon C-8 at δ_C 166.99 ppm. Methylene hydrogen H-7 at δ_C 4.68 ppm coupled with carbonyl carbons C-6 and C-8 at δ_C 167.00 and 166.99 ppm, respectively. Methylene hydrogen H-5 at δ_C 4.56 ppm showed coupling with carbonyl carbons C-4 and C-4' and C-6 at δ_C 167.34 and 167.00 ppm, respectively.

Based on the analysis of compound **7b**, the carbonyl compounds **7a**, **7c** and **7d**-**7i** can be seen in Table 1. The compounds showed two more characteristic signals referring to the methylene carbons (C-5 and C-7) at δ_C 38.61-38.73 and δ_C 61.56-61.83 ppm, respectively (Table 1). In all compounds, the signals attributed to the aromatic carbons were found at δ_C 123.67-134.45 ppm.

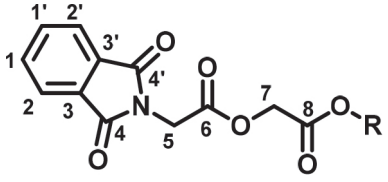
In the spectrum of compound **7a**, a signal was observed for the methyl group in the aliphatic region at δ_C 52.56 ppm. For compound **7c**, three signals were observed for the propyl group at δ_C 67.20, 21.85, 10.24 ppm. For compound **7e**, four signals were observed for the butyl group at δ_C 65.52, 30.44, 19.00 and 13.64 ppm. For compound **7h**, five signals were observed for the pentyl group at δ_C 65.80, 28.14, 27.89, 22.25 and 13.91 ppm. For compound **7d**, two signals were observed, one for the $-CO_2\underline{C}H-$ carbon at δ_C 69.62 ppm and another for the isopropyl group methyls at δ_C 21.67 ppm. For compound **7f**, three signals were observed, two for the $-CO_2\underline{C}H_2\underline{C}H-$ carbons at δ_C 71.59 and 27.63 ppm, and one for the isopropyl group methyls at δ_C 18.94 ppm. For compound **7i**, four signals were observed, three for the $-CO_2\underline{C}H_2CH_2\underline{C}H-$ carbons at δ_C 64.33, 37.09 and 24.98 ppm and one for the isopropyl group methyls at δ_C 22.38 ppm. For compound **7g**, four signals were observed, one for $-CO_2\underline{C}H-$ at δ_C 74.13 ppm, one for a methyl at δ_C 28.65 ppm and two signals for an ethyl at δ_C 19.30 and 9.56 ppm.

In the IR spectrum, a stretch band referring to C=O was observed, a notable feature in the structures of diesters. All **7a**-**7i** compounds showed absorption bands ascribed to the functional group $-NCH_2\underline{CO}O-$ closest to the phthalimide between 1755 and 1728 cm^{-1}, functional group $-OCH_2\underline{CO}O-$ referring to the terminal ester between 1776 and 1747 cm^{-1} and functional group $-N(\underline{CO})_2$ related to phthalimide between 1720 and 1706 cm^{-1}. For all compounds, the stretches of the aromatic hydrogens of phthalimide ranged from 3111 to 3043 cm^{-1}. Two stretching bands of C–O, a strong and weak one in the range of 1193-1107 cm^{-1}, were also observed.

In silico study

The stages of developing new drug candidates demand a high cost of resources and time. To reduce these costs, theoretical studies have been of fundamental importance in the indication of factors that qualify new chemical compounds as potential drugs. Several authors[11,12] highlight the importance of the pharmacokinetic parameters absorption, distribution, metabolism and excretion (ADME), which give information about the permeability and concentration of certain compounds in therapeutic targets and their consequent elimination by the body. ADME parameters can be checked by *in silico* studies on the basis of calculations of physicochemical properties

Table 1. Data of ^{13}C NMR (101 and 126 MHz) of the diesters **7a**-**7i**, phthaloylglycine derivatives, in $CDCl_3$



C	δ_c / ppm								
	7a	**7b**	**7c**	**7d**	**7e**	**7f**	**7g**	**7h**	**7i**
4-4'	167.54	167.34	167.25	167.28	167.26	167.26	167.26	167.24	167.22
5	38.73	38.71	38.64	38.63	38.62	38.61	38.62	38.64	38.63
6	167.40	167.00	166.99	166.91	166.90	167.00	166.91	166.99	166.98
7	61.56	61.70	61.60	61.83	61.60	61.57	61.78	61.60	61.61
8	167.03	166.99	166.88	166.45	167.02	166.90	166.60	166.88	166.88

such as lipophilicity (clog P), water solubility (log S) and molecular weight (MW).

In the 1990s, Lipinski *et al.*[13] presented a relationship between pharmacokinetic and physicochemical parameters, indicating that the molecules with high potential to become a drug were those that resembled existing drugs in certain measured properties. Their study resulted in "Lipinski's rule of 5", which has only four factors (whose values are multiples of five): molar mass ≤ 500 g mol^{-1}, log P ≤ 5, number of hydrogen bond acceptors ≤ 10 (accounted for as a function of N or O atoms in the molecule), and number of hydrogen bond donors ≤ 5 (represented as a function of the NH or OH groups in the molecule).

In this work, the *in silico* study of the **7a**-**7i** diesters was performed to determine the Lipinski parameters using OSIRIS Property Explorer[14] and Swiss ADME[15] software. In addition to these, other parameters such as rotating bonds (Rb), topological polar surface area (TPSA), absorption percentage (ABS), drug-likeness and drug score were included in the study, since they are important parameters in the design of new drug candidates. ABS was calculated using the equation ABS(%) = 109 – (0.345 × TPSA) according to Zhao *et al.*[16] The values determined in this study are shown in Table 2.

The *in silico* results displayed in Table 2 showed that all **7a**-**7i** diesters were in line with Lipinski's rule of 5, indicating that these compounds may show good oral availability. The TPSA values of all the **7a**-**7i** diesters were 89.98 Å^2, indicating good permeability in the plasma membrane of cells and a moderate absorption percentage of 77.95%. The number of Rb ranged from 6 to 10 for compounds **7a**-**7i**, which indicated, along with a TPSA below 140 Å^2, a high probability of good oral bioavailability.[11]

The log S (Ali method) of the diesters **7a**-**7g** and **7i** showed values between −2.21 and −3.96, indicating that the compounds were soluble, while diester **7h** showed a value of −4.07 and was described as moderately soluble. More than 80% of drugs on the market show values higher than −4.00.[14]

The drug-likeness value of the **7a**-**7i** diesters varied between −8.1 and −17.6, where the highest value was found for **7i** and the lowest for **7h**. When this value is closer to being positive, the molecule contains more moieties that are often present in commercial drugs; ideally, the drug-likeness value should be positive. The drug score value combines clog P, log S, molecular weight and toxicity risks and varies from 0.0 to 1.0 which can be used to predict the overall potential of a compound to be a new drug candidate. The values obtained with this synthesis approach ranged from 0.21 for diester **7i** to 0.38 for diesters **7a** and **7b**, suggesting that the series of diesters **7a**-**7i** has the potential to include new drug candidates.

Biological studies

Antibacterial activity

The *in vitro* antibacterial activity of the compounds **7a**-**7i** was evaluated by the microdilution method with four strains of pathogenic bacteria, *Staphylococcus aureus* ATCC-6538, *Staphylococcus epidermidis* ATCC-12228, *Escherichia coli* ATCC-25922 and *Pseudomonas aeruginosa* ATCC-9027, using gentamicin (64 μg mL^{-1}) as the standard control drug

Table 2. *In silico* studies evaluating Lipinski's rule of five, topological surface area (TPSA), solubility (log S), adsorption percentage (ABS), rotating bonds, drug-likeness and drug score of the compounds **7a**-**7i**

Compound	Lipinski parameter					Rb	TPSA / Å^2	ABS / %	Ali log S	Ali class	Drug-likeness	Drug score
	MW	HBD	HBA	clog P	nV							
7a	277.23	0	6	0.11	0	6	89.98	77.95	−2.21	soluble	−10.6	0.38
7b	291.26	0	6	0.52	0	7	89.98	77.95	−2.59	soluble	−12.1	0.38
7c	305.28	0	6	0.97	0	8	89.98	77.95	−3.13	soluble	−8.5	0.37
7d	305.28	0	6	0.87	0	7	89.98	77.95	−3.04	soluble	−10.9	0.37
7e	319.31	0	6	1.42	0	9	89.98	77.95	−3.51	soluble	−13.3	0.22
7f	319.31	0	6	1.19	0	8	89.98	77.95	−3.59	soluble	−8.5	0.22
7g	319.31	0	6	1.33	0	8	89.98	77.95	−3.59	soluble	−9.9	0.27
7h	333.34	0	6	1.88	0	10	89.98	77.95	−4.07	moderately	−17.6	0.23
7i	333.34	0	6	1.64	0	9	89.98	77.95	−3.96	soluble	−8.1	0.21

Physicochemical properties: MW: molecular weight; HBD: hydrogen bonding donor; HBA: hydrogen bonding acceptor; clog P: octanol/water partition coefficient based on Molinspiration milog P model; nV: number of violations; Rb: rotatable bonds; TPSA: total polar surface area; ABS: adsorption percentage; Ali log S: coefficient of solubility determined by the Ali method calculated on SwissADME;[15] Ali class: insoluble < −10 < poor < −6 < moderately < −4 < soluble < −2 < very < 0 < highly.

(Table 3). The antibacterial activity of the products was interpreted and considered as active or inactive, according to the following minimum inhibitory concentration (MIC) criteria: below 600 μg mL^{-1} = strong/optimum activity; 600-1500 μg mL^{-1} = moderate activity; above 1500 μg mL^{-1} = weak activity or inactive product.[17-19]

Only **7h** and **7i**, diesters with the longest alkyl chain, showed moderate antibacterial activity, with an MIC of 1024 μg mL^{-1} against strains of *S. aureus* ATCC-6538, *S. epidermidis* ATCC-12228, *E. coli* ATCC-25922 and *P. aeruginosa* ATCC-9027 (Table 3). Studies have shown that the activity of compounds with antibacterial properties against Gram-positive and Gram-negative bacteria is improved by increasing their lipophilicity.[20-22] However, further studies should be conducted to identify what makes **7h** and **7i** substances able to act against both types of bacteria. The other diester compounds, **7a**, **7b**, **7c**, **7d**, **7e**, **7f** and **7g**, showed no inhibition on bacterial growth of the strains used.

Antifungal activity

The *in vitro* antifungal activity of compounds **7a**-**7i** was evaluated by the microdilution method with eight strains of pathogenic yeasts, *Candida albicans* ATCC-76645 and LM-111, *Candida tropicalis* ATCC-13803 and LM-07, *Candida parapsilosis* ATCC-22019 and LM-302, *Candida krusei* ATCC-6258 and LM-656, using amphotericin B (32 μg mL^{-1}) as the standard control drug. The antifungal activity of the products was interpreted and considered as active or inactive, according to the following MIC criteria: below 600 μg mL^{-1} = strong/optimum activity; 600-1500 μg mL^{-1} = moderate activity; above 1500 μg mL^{-1} = weak activity or inactive product.[17-19] Of the nine diesters tested, only compounds **7d**, **7f**, and **7g** did not show any antifungal activity, while **7a**, **7b**, **7c**, **7e**, **7h** and **7i** exerted 100% inhibition against all *Candida* strains tested (Table 4).

The compounds **7a**, **7b**, **7c**, **7e** and **7i** presented antifungal activity with a minimum inhibitory concentration (MIC) of 1024 μg mL^{-1} against all *Candida* strains. The compound **7h** showed antifungal activity with an MIC of 256 μg mL^{-1} against the strains *C. albicans* ATCC-76645, *C. albicans* LM-111, *C. tropicalis* ATCC-13803, *C. parapsilosis* ATCC-22019, *C. parapsilosis* LM-302, *C. krusei* ATCC-6258 and *C. krusei* LM-656; and 64 μg mL^{-1} against *C. tropicalis* LM-07. Comparing the experimental (biological) results with the theoretical (*in silico*), in relation to the increase in alkyl chain length of the terminal esters of compounds **7a**-**7i**, we observed that MIC was inversely proportional to lipophilicity. In relation to the isomers, we observed a decrease in MIC of the compounds **7c**, **7e** and **7h** with *N*-alkyl chains, in relation to the compounds **7d**, **7f**, **7g** and **7i** with branched chains. The results are in accordance with the literature,[20,23,24] which reports better activities and smaller MICs for compounds with longer chain and consequently greater lipophilicity. The results (Table 4) were considered moderate for diester compounds **7a**, **7b**, **7c**, **7e** and **7i** and strong for **7h**, in terms of antifungal activity.

Table 3. Minimum inhibitory concentration (MIC) of diesters against bacterial strains used

Compound	Minimum inhibitory concentration / (μg mL^{-1})			
	Bacterial strain			
	S. aureus ATCC-6538	*S. epidermidis* ATCC-12228	*P. aeruginosa* ATCC-9027	*E. coli* ATCC-25922
7a	+	+	+	+
7b	+	+	+	+
7c	+	+	+	+
7d	+	+	+	+
7e	+	+	+	+
7f	+	+	+	+
7g	+	+	+	+
7h	1024	1024	1024	1024
7i	1024	1024	1024	1024
Control				
Culture medium	–	–	–	–
Microorganism	+	+	+	+
Gentamicin	–	–	–	–

+: microbial growth present; –: no microbial growth.

Table 4. Minimum inhibitory concentration (MIC) of diesters against fungal strains tested

Compound	Minimum inhibitory concentration / (μg mL^{-1})							
	Fungal strain							
	C. albicans ATCC-76645	*C. albicans* LM-111	*C. tropicalis* ATCC-13803	*C. tropicalis* LM-07	*C. parapsilosis* ATCC-22019	*C. parapsilosis* LM-302	*C. krusei* ATCC-6258	*C. krusei* LM-656
7a	1024	1024	1024	1024	1024	1024	1024	1024
7b	1024	1024	1024	1024	1024	1024	1024	1024
7c	1024	1024	1024	1024	1024	1024	1024	1024
7d	+	+	+	+	+	+	+	+
7e	1024	1024	1024	1024	1024	1024	1024	1024
7f	+	+	+	+	+	+	+	+
7g	+	+	+	+	+	+	+	+
7h	256	256	256	64	256	256	256	256
7i	1024	1024	1024	1024	1024	1024	1024	1024
Control								
Culture medium	–	–	–	–	–	–	–	–
Microorganism	+	+	+	+	+	+	+	+
Amphotericin B	–	–	–	–	–	–	–	–

+: microbial growth present; –: no microbial growth.

Conclusions

Nine new diesters were synthesized and characterized using IR, ^{1}H and ^{13}C NMR spectroscopic techniques. The *in silico* study showed that all synthesized diesters were in line with Lipinski's rule of 5, indicating good oral bioavailability with drug administration, thus being a good new drug candidate. In the antibacterial activity study, only diesters **7h** and **7i** showed moderate antibacterial activity (MIC of 1024 μg mL^{-1}) against all strains tested. In the antifungal activity study, diesters **7a**-**7c**, **7e** and **7i** also had moderate activity (MIC of 1024 μg mL^{-1}) against all *Candida* strains, while **7h** displayed strong activity (MIC of 64-256 μg mL^{-1}) against all *Candida* strains. The results indicate that both the increase in the linear alkyl chain of the terminal esters and their different geometric arrangements have an influence on biological activity. Future studies involving the synthesis of new diesters with alkyl chains longer than five carbons will be carried out to determine to what extent alkyl chain length influences biological activity.

Experimental

Chemical

All reagents and solvents were purchased from commercial sources (Sigma-Aldrich, Brazil) and used without further purification. The progress of the reactions was monitored by thin layer chromatography (TLC) on silica gel plates. The compounds were purified by recrystallization in ethanol and confirmed by determining the melting point (mp) range on an MQAPF-3 brand hotplate. Fourier transform infrared (FTIR) spectra were obtained on a Shimadzu Prestige-21 spectrometer using attenuated total reflectance (ATR). ^{1}H and ^{13}C NMR spectra were obtained on two different instruments: a Bruker Avance Ultrashield™ (400 MHz for ^{1}H and 101 MHz for ^{13}C) and Bruker Avance Ultrashield™ (500 MHz for ^{1}H and 126 MHz for ^{13}C). Deuterated chloroform ($CDCl_3$) and deuterated dimethyl sulfoxide (DMSO-d_6) were used as solvent, and tetramethylsilane (TMS) was used for the internal standard. Chemical shifts (δ) were measured in parts *per* million (ppm), and the coupling constants (J), in hertz (Hz).

Preparation of 2-chloroacetate esters (**2a-2i**)

In a 100-mL round-bottomed flask equipped with a condenser, a mixture of chloroacetic acid (10 mmol), alcohol (methyl, ethyl, propyl, butyl and isopentyl) (50 mL) and concentrated sulfuric acid (1 mL) was heated under reflux conditions for 4 h. Afterwards, excess solvent was rotary evaporated and the residue poured into cold water. The residue was transferred to a separation funnel containing 250 mL of water and 50 mL of ethyl ether were then added. The organic phase was separated and washed repeatedly with 10% sodium

bicarbonate to neutral pH and then dried with anhydrous $MgSO_4$. The ethyl ether was evaporated in a rotary evaporator, yielding the respective esters, which were used in the next step of the synthesis.

Methyl 2-chloroacetate (**2a**)

Colorless liquid; yield: 80%; IR (ATR) ν / cm^{-1} 2957 (CH_{Aliph}), 1753 (C=O), 1299 and 1002 (O–C_{Aliph}), 789 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.08 (s, 2H, C$\underline{H}_{2Aliph}$), 3.80 (s, 3H, OC$\underline{H}_{3Aliph}$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.7, 53.0, 40.6.

Ethyl 2-chloroacetate (**2b**)

Colorless liquid; yield: 93%; IR (ATR) ν / cm^{-1} 2985, 2942 (CH_{Aliph}), 1735 (C=O), 1287 and 1024 (O–C_{Aliph}), 781 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.24 (q, 2H, OC$\underline{H}_{2Aliph}$), 4.05 (s, 2H, C$\underline{H}_{2Aliph}$), 1.30 (t, 3H, C$\underline{H}_{3Aliph}$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.3, 62.2, 40.9, 14.0.

Propyl 2-chloroacetate (**2c**)

Colorless liquid; yield: 92%; IR (ATR) ν / cm^{-1} 2970, 2881 (CH_{Aliph}), 1737 (C=O), 1290 and 1056 (O–C_{Aliph}), 792 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.15 (t, 2H, OC$\underline{H}_{2Aliph}$), 4.06 (s, 2H, C$\underline{H}_{2Aliph}$), 1.75-1.64 (hex, 2H, C$\underline{H}_{2Aliph}$), 0.96 (t, 3H, C$\underline{H}_{3Aliph}$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.3, 67.7, 40.9, 21.8, 10.3.

Isopropyl 2-chloroacetate (**2d**)

Colorless liquid; yield: 80%; IR (ATR) ν / cm^{-1} 2983, 2939 (CH_{Aliph}), 1732 (C=O), 1375 (isopropyl), 1287 and 1024 (O–C_{Aliph}), 792 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.09 (m, 1H, OC$\underline{H}_{Aliph}$), 4.02 (t, 2H, C$\underline{H}_{2Aliph}$), 1.28 [d, 6H, (C$\underline{H}_{3Aliph}$)$_2$]; ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.8, 70.1, 41.2, 21.6.

Butyl 2-chloroacetate (**2e**)

Colorless liquid; yield: 80%; IR (ATR) ν / cm^{-1} 2960, 2936, 2873 (CH_{Aliph}), 1737 (C=O), 1288 and 1020 (O–C_{Aliph}), 785 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.20 (t, 2H, OC$\underline{H}_{2Aliph}$), 4.06 (s, 2H, C$\underline{H}_{2Aliph}$), 1.70-1.61 (m, 2H, C$\underline{H}_{2Aliph}$), 1.40 (sext, 2H, C$\underline{H}_{2Aliph}$), 0.94 (t, 3H, C$\underline{H}_{3Aliph}$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.4, 66.1, 40.9, 30.4, 18.9, 13.6.

Isobutyl 2-chloroacetate (**2f**)

Colorless liquid; yield: 78%; IR (ATR) ν / cm^{-1} 2962, 2875 (CH_{Aliph}), 1736 (C=O), 1378 (isobutyl), 1288 and 1174 (O–C_{Aliph}), 788 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.08 (s, 2H, C$\underline{H}_{2Aliph}$), 3.98 (d, 2H, OC$\underline{H}_{2Aliph}$), 1.98 (m, 1H, C$\underline{H}_{Aliph}$), 0.95 [d, 6H, (C$\underline{H}_{3Aliph}$)$_2$]; ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.4, 72.1, 40.9, 27.6, 18.9.

sec-Butyl 2-chloroacetate (**2g**)

Colorless liquid; yield: 65%; IR (ATR) ν / cm^{-1} 2976, 2939, 2881 (CH_{Aliph}), 1732 (C=O), 1381 (*sec*-butyl), 1288 and 1190 (O–C_{Aliph}), 792 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.99-4.88 (t, 1H, OC$\underline{H}_{Aliph}$), 4.04 (s, 2H, C$\underline{H}_{2Aliph}$), 1.72-1.52 (m, 2H, C$\underline{H}_{2Aliph}$), 1.26 (d, 3H, C$\underline{H}_{3Aliph}$), 0.92 (t, 3H, C$\underline{H}_{3Aliph}$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.01, 74.69, 41.23, 28.66, 19.31, 9.59.

Pentyl 2-chloroacetate (**2h**)

Colorless liquid; yield: 90%; IR (ATR) ν / cm^{-1} 2958, 2933, 2862 (CH_{Aliph}), 1737 (C=O), 1180 and 1045 (O–C_{Aliph}), 792 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.19 (t, 2H, OC$\underline{H}_{2Aliph}$), 4.06 (s, 2H, C$\underline{H}_{2Aliph}$), 1.72-1.62 (qt, 2H, C$\underline{H}_{2Aliph}$), 1.40-1.30 [m, 4H, (C$\underline{H}_{2Aliph}$)], 0.92 (t, 3$\underline{H}$, CH_{3Aliph}); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.4, 66.3, 40.9, 28.1, 27.8, 22.2, 13.8.

Isopentyl 2-chloroacetate (**2i**)

Colorless liquid; yield: 75%; IR (ATR) ν / cm^{-1} 2960, 2873 (CH_{Aliph}), 1736 (C=O), 1386 (isopentyl), 1184 and 1047 (O–C_{Aliph}), 784 (C–Cl); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.22 (s, 2H, C$\underline{H}_{2Aliph}$), 4.05 (t, 2H, OC$\underline{H}_{2Aliph}$), 1.75-1.63 (m, 1H, C$\underline{H}_{Aliph}$), 1.56 (q, 2H, C$\underline{H}_{2Aliph}$), 0.93 [d, *J* 6.7 Hz, 6H, (C$\underline{H}_{3Aliph}$)$_2$]; ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.3, 64.9, 40.9, 37.0, 24.9, 22.3.

Preparation of 2-(1,3-dioxoisoindolin-2-yl) acetic acid (**5**)

A solution of phthalic anhydride (5 mmol) and glycine (5 mmol) in glacial acetic acid was stirred under reflux for 6 h. Afterwards, the solvent was evaporated under reduced pressure, and the solid residue was washed with distilled water, filtered, dried and recrystallized. Yield: 80%; mp 189-190 °C; IR (ATR) ν / cm^{-1} 3475 (OH), 3099-3051 (CH_{Ar}), 1612-1467 (C=C_{Ar}), 2985-2872 (CH_{Aliph}), 1770-1718 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.25 (s, 1H, O$\underline{H}$), 7.92 (m, 2H), 7.89-7.85 (m, 2H), 4.32 (s, 2H, CH_{2Aliph}); ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.92, 167.26, 134.83, 131.44, 123.42, 38.92.

Preparation of potassium acetate 2-(1,3-dioxoisoindolin-2-yl) (**6**)

An ethanolic solution (50 mL) of potassium hydroxide (10 mmol) was added slowly to an ethanolic solution of phthaloylglycine (10 mmol). The reaction mixture was stirred for 3 h. Afterwards, the solvent was evaporated under low pressure and the precipitate dried. Yield: 90%; mp > 250 °C; IR (ATR) ν / cm^{-1} 3097-3041 (CH_{Ar}), 1537-1466 (C=C_{Ar}), 2987-2927 (CH_{Aliph}), 1743-1714 (C=O).

Preparation of derivatives of phthaloylglycine (**7a**-**7i**)

The potassium salt of phthaloylglycine (10 mmol) was placed in a flask with 10 mmol alkyl chloroacetate in 10 mL of DMF. The mixture was stirred under reflux for 24 h. After 24 h of reaction time, the product was allowed to cool at room temperature. After 10 min, cold distilled water was added and the mixture was then transferred to a separation funnel containing 250 mL of water, followed by the addition of 50 mL of ethyl ether. The organic phase was separated then dried with anhydrous $MgSO_4$. The ethyl ether was evaporated in a rotary evaporator, yielding the respective ester derivatives.

2-Methoxy-2-oxoethyl 2-(1,3-dioxoisoindolin-2-yl)acetate (**7a**)

White solid; yield: 75%; mp 95-96 °C; IR (ATR) ν / cm^{-1} 3074 (CH_{Ar}), 2995-2948 (CH_{Aliph}), 1759 (C=O), 1728 (C=O), 1708 (C=O), 1185 and 1107 (O–C_{Aliph}); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92-7.83 (m, 2H, H-1 and H-1'), 7.77-7.67 (m, 2H, H-2 and H-2'), 4.69 (s, 2H, H-7), 4.55 (s, 2H, H-5), 3.75 (s, 3H, OC$\underline{H}_{3Aliph}$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.54 (C-4 and C-4'), 167.40 (C-6), 167.03 (C-8), 134.45 (C-1 and C-1'), 132.07 (C-2 and C-2'), 123.82 (C-3 and C-3'), 61.56 (C-7), 52.56 (O$\underline{C}H_{3Aliph}$), 38.73 (C-5).

2-Ethoxy-2-oxoethyl 2-(1,3-dioxoisoindolin-2-yl)acetate (**7b**)

White solid; yield: 75%; mp 85-86 °C; IR (ATR) ν / cm^{-1} 3068 (CH_{Ar}), 2987-2935 (CH_{Aliph}), 1747 (C=O), 1747 (C=O), 1718 (C=O), 1182 and 1114 (O–C_{Aliph}); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.87 (dd, *J* 5.2, 3.1 Hz, 2H, H-1 and H-1'), 7.73 (dd, *J* 5.2, 3.1 Hz, 2H, H-2 and H-2'), 4.67 (s, 2H, H-7), 4.55 (s, 2H, H-5), 4.21 (q, *J* 7.1 Hz, 2H, OC$\underline{H}_{2Aliph}$), 1.26 (t, *J* 7.1 Hz, 3H, C$\underline{H}_{3Aliph}$); ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.34 (C-4 and C-4'), 167.00 (C-6), 166.99 (C-3 and C-3'), 134.38 (C-1 and C-1'), 132.05 (C-2 and C-2'), 123.76 (C-3 and C-3'), 61.76 (O$\underline{C}H_{2Aliph}$), 61.70 (C-7), 38.71 (C-5), 14.09 ($\underline{C}H_{3Aliph}$).

2-Oxo-2-propoxyethyl 2-(1,3-dioxoisoindolin-2-yl)acetate (**7c**)

White solid; yield: 72%; mp 76-77 °C; IR (ATR) ν / cm^{-1} 3111-3076 (CH_{Ar}), 2980-2928 (CH_{Aliph}), 1754 (C=O), 1754 (C=O), 1714 (C=O), 1170 and 1118 (O–C_{Aliph}); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.89 (dd, *J* 5.5, 3.1 Hz, 2H, H-1 and H-1'), 7.75 (dd, *J* 5.5, 3.1 Hz, 2H, H-2 and H-2'), 4.69 (s, 2H, H-7), 4.56 (s, 2H, H-5), 4.13 (t, *J* 6.7 Hz, 2H, OC$\underline{H}_{2Aliph}$), 1.71-1.62 (m, 2H, C$\underline{H}_{2Aliph}$), 0.93 (t, *J* 7.4 Hz, 3H, C$\underline{H}_{3Aliph}$); ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.25 (C-4 and C-4'), 166.99 (C-6), 166.88 (C-8), 134.27 (C-1 and C-1'), 132.02 (C-2 and C-2'), 123.69 (C-3 and C-3'), 67.20 (O$\underline{C}H_{2Aliph}$), 61.60 (C-7), 38.64 (C-5), 21.85 ($\underline{C}H_{2Aliph}$), 10.24 ($\underline{C}H_{3Aliph}$).

2-Isopropoxy-2-oxoethyl 2-(1,3-dioxoisoindolin-2-yl)acetate (**7d**)

White solid; yield: 38%; mp 74-75 °C; IR (ATR) ν / cm^{-1} 3105-3078 (CH_{Ar}), 2984-2943 (CH_{Aliph}), 1768 (C=O), 1753 (C=O), 1720 (C=O), 1179 and 1115 (O–C_{Aliph}); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92-7.88 (m, 2H, H-1 and H-1'), 7.77-7.74 (m, 2H, H-2 and H-2'), 5.09 (m, 1H, OC$\underline{H}_{Aliph}$), 4.66 (s, 2H, H-7), 4.57 (s, 2H, H-5), 1.26 [(d, *J* 6.3 Hz, 6H, ($C\underline{H}_{3Aliph}$)$_2$]; ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.28 (C-4 and C-4'), 166.91 (C-6), 166.45 (C-8), 134.29 (C-1 and C-1'), 132.00 (C-2 and C-2'), 123.69 (C-3 and C-3'), 69.62 ($\underline{C}H_{Aliph}$), 61.83 (C-7), 38.63 (C-5), 21.67 ($\underline{C}H_{3Aliph}$).

2-Butoxy-2-oxoethyl 2-(1,3-dioxoisoindolin-2-yl)acetate (**7e**)

White solid; yield: 68%; mp 58-59 °C; IR (ATR) ν / cm^{-1} 3101-3068 (CH_{Ar}), 2964-2943 (CH_{Aliph}), 1763 (C=O), 1748 (C=O), 1716 (C=O), 1187 and 1114 (O–C_{Aliph}); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91-7.88 (m, 2H, H-1 and H-1'), 7.76 (m, 2H, H-2 and H-2'), 4.70 (s, 2H, H-7), 4.57 (s, 2H, H-5), 4.18 (t, *J* 6.7 Hz, 2H, OC$\underline{H}_{2Aliph}$), 1.67-1.59 (m, 2H, C$\underline{H}_{2Aliph}$), 1.42-1.32 (m, 2H, C$\underline{H}_{2Aliph}$), 0.93 (t, *J* 7.4 Hz, 3H, C$\underline{H}_{3Aliph}$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.26 (C-4 and C-4'), 167.02 (C-6), 166.90 (C-8), 134.30 (C-1 and C-1'), 131.99 (C-2 and C-2'), 123.69 (C-3 and C-3'), 65.52 (O$\underline{C}H_{2Aliph}$), 61.60 (C-7), 38.62 (C-5), 30.44 ($\underline{C}H_{2Aliph}$), 19.00 ($\underline{C}H_{2Aliph}$), 13.64 ($\underline{C}H_{3Aliph}$).

2-Isobutoxy-2-oxoethyl 2-(1,3-dioxoisoindolin-2-yl)acetate (**7f**)

White solid; yield: 66%; mp 69-70 °C; IR (ATR) ν / cm^{-1} 3099-3079 (CH_{Ar}), 2965-2935 (CH_{Aliph}), 1750 (C=O), 1750 (C=O), 1707 (C=O), 1178 and 1114 (O–C_{Aliph}); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (dd, *J* 5.5, 3.0 Hz, 2H, H-1 and H-1'), 7.75 (dd, *J* 5.5, 3.0 Hz, 2H, H-2 and H-2'), 4.71 (s, 2H, H-7), 4.57 (s, 2H, H-5), 3.96 (d, *J* 6.7 Hz, 2H, OC$\underline{H}_{2Aliph}$), 1.95 (m, 1H, C$\underline{H}_{Aliph}$), 0.93 [(d, *J* 6.7 Hz, 6H, ($C\underline{H}_{3Aliph}$)$_2$]; ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.26 (C-4 and C-4'), 167.00 (C-6), 166.90 (C-8), 134.30 (C-1 and C-1'), 131.99 (C-2 and C-2'), 123.69 (C-3 and C-3'), 71.59 ($\underline{C}H_{Aliph}$), 61.57 (C-7), 38.61 (C-5), 27.63 (O$\underline{C}H_{2Aliph}$), 18.94 ($\underline{C}H_{3Aliph}$).

2-(*sec*-Butoxy)-2-oxoethyl 2-(1,3-dioxoisoindolin-2-yl) acetate (**7g**)

White solid; yield: 60%; mp 68-69 °C; IR (ATR) ν / cm^{-1} 3099-3082 (CH_{Ar}), 2974-2939 (CH_{Aliph}), 1776 (C=O), 1755 (C=O), 1707 (C=O), 1179 and 1114 (O–C_{Aliph});

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (dd, *J* 5.5, 3.0 Hz, 2H, H-1 and H-1'), 7.75 (dd, *J* 5.5, 3.0 Hz, 2H, H-2 and H-2'), 4.98-4.89 (m, 1H, OC$\underline{H}_{Aliph}$), 4.67 (s, 2H, H-7), 4.57 (s, 2H, H-5), 1.67-1.53 (m, 2H, C$\underline{H}_{2Aliph}$), 1.23 (d, *J* 6.3 Hz, 3H, C$\underline{H}_{3Aliph}$), 0.88 (t, *J* 7.5 Hz, 3H, C$\underline{H}_{3Aliph}$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.26 (C-4 and C-4'), 166.91 (C-6), 166.60 (C-8), 134.29 (C-1 and C-1'), 132.00 (C-2 and C-2'), 123.68 (C-3 and C-3'), 74.13 (O$\underline{C}H_{Aliph}$), 61.78 (C-7), 38.62 (C-5), 28.65 ($\underline{C}H_{2Aliph}$), 19.30 ($\underline{C}H_{3Aliph}$), 9.56 ($\underline{C}H_{3Aliph}$).

2-Oxo-2-(pentyloxy)ethyl 2-(1,3-dioxoisoindolin-2-yl)acetate (**7h**)

Brown solid; yield: 67%; mp 39-40 °C; IR (ATR) ν / cm^{-1} 3100-3043 (CH_{Ar}), 2959-2929 (CH_{Aliph}), 1774 (C=O), 1752 (C=O), 1706 (C=O), 1193 and 1115 (O–C_{Aliph}); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.91-7.87 (m, 2H, H-1 and H-1'), 7.77-7.73 (m, 2H, H-2 and H-2'), 4.69 (s, 2H, H-7), 4.57 (s, 2H, H-5), 4.16 (t, *J* 6.8 Hz, 2H, OC$\underline{H}_{2Aliph}$), 1.68-1.61 (m, 2H, C$\underline{H}_{2Aliph}$), 1.36-1.29 (m, 2H, C$\underline{H}_{2Aliph}$), 0.90 (t, *J* 7.0 Hz, 3H, C$\underline{H}_{3Aliph}$); ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.24 (C-4 and C-4'), 166.99 (C-6), 166.88 (C-8), 134.27 (C-1 and C-1'), 132.02 (C-2 and C-2'), 123.68 (C-3 and C-3'), 65.80 (O$\underline{C}H_{2Aliph}$), 61.60 (C-7), 38.64 (C-5), 28.13 ($\underline{C}H_{2Aliph}$), 27.89 ($\underline{C}H_{2Aliph}$), 22.25 ($\underline{C}H_{2Aliph}$), 13.91 ($\underline{C}H_{3Aliph}$).

Isopentyl 2-(2-(1,3-dioxoisoindolin-2-yl)acetoxy)acetate (**7i**)

Yellow liquid; yield: 64%; IR (ATR) ν / cm^{-1} 3097-3080 (CH_{Ar}), 2958 (CH_{Aliph}), 1753 (C=O), 1753 (C=O), 1716 (C=O), 1176 and 1114 (O–C_{Aliph}); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.89 (dd, *J* 5.3, 3.1 Hz, 2H, H-1 and H-1'), 7.75 (dd, *J* 5.3, 3.1 Hz, 2H, H-2 and H-2'), 4.69 (s, 2H, H-7), 4.57 (s, 2H, H-5), 4.20 (t, *J* 6.9 Hz, 2H, OC$\underline{H}_{2Aliph}$), 4.02 (m, 1H, C$\underline{H}_{Aliph}$), 1.54 (q, *J* 6.9 Hz, 2H, C$\underline{H}_{2Aliph}$), 0.92 [d, *J* 6.6 Hz, 6H, ($C\underline{H}_{3Aliph}$)$_2$]; ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.22 (C-4 and C-4'), 166.98 (C-6), 166.88 (C-8), 134.28 (C-1 and C-1'), 132.00 (C-2 and C-2'), 123.67 (C-3 and C-3'), 64.33 ($\underline{C}H_{Aliph}$), 61.61 (C-7), 38.63 (C-5), 37.09 (O$\underline{C}H_{2Aliph}$), 24.98 ($\underline{C}H_{2Aliph}$), 22.38 ($\underline{C}H_{3Aliph}$).

Antimicrobial activity

Test substance

Solutions of the synthesized compounds **7a**-**7i** were prepared at the time of the tests by dissolving the compound in 5% DMSO and 2% Tween 80 (Sigma-Aldrich, São Paulo, Brazil), and completing the final volume with sterile distilled water.[25,26]

Culture media

The fungal and bacterial strains were maintained in Sabouraud dextrose agar (SDA) and brain heart infusion (BHI) medium (Difco Laboratories Ltd., USA, France), respectively. For biological activity assays, BHI broth and Roswell Park Memorial Institute (RPMI)-1640 medium with L-glutamine and no sodium bicarbonate (Difco Laboratories Ltd., USA, France and INLAB, São Paulo, Brazil) were used for tests with bacteria and fungi, respectively. The culture media were prepared according to the manufacturer's instructions and sterilized by autoclaving at 121 °C and 1 atm for 15 min.

Microorganisms

The following strains were used for antimicrobial activity assays: *Staphylococcus aureus* ATCC-6538, *Staphylococcus epidermidis* ATCC 12228, *Pseudomonas aeruginosa* ATCC-9027, *Escherichia coli* ATCC-25922, *Candida albicans* ATCC-76645 and LM-111, *C. tropicalis* ATCC-13803 and LM-07, *C. parapsilosis* ATCC-22019 and LM-302, *C. krusei* ATCC-6258 and LM-656. The microorganisms were obtained from the Micoteca (collection) of the Mycology Laboratory, Department of Pharmaceutical Sciences (DCF), Health Sciences Center (CCS) of the Federal University of Paraíba (UFPB), Brazil. The fungal and bacterial strains were maintained at 4 °C in SAD and BHI, respectively. For use in the assays, the fungi and bacteria were harvested in SAD and BHI, respectively, and incubated at 35 ± 2 °C for 24-48 h. The microorganism suspension was prepared according to the 0.5 McFarland scale tube and was adjusted by the use of a spectrophotometer (Leitz-Phtometer 340-800) to 90% T (530 nm), corresponding to approximately 10^6 colony-forming unit (CFU) mL^{-1} for fungi and 10^8 CFU mL^{-1} for bacteria.[27,28]

Determination of minimum inhibitory concentration (MIC)

The determination of the MIC of the products in bacterial and fungal strains was performed using the broth microdilution method with 96-well round-bottom microplates (TPP, Switzerland) containing. Initially, 100 µL of RPMI/BHI broth were distributed in the wells of the microdilution plates. Next, 100 µL of the substances were dispensed in the first column of wells, and through a twofold serial dilution, concentrations of 1024 µg mL^{-1} down to 64 µg mL^{-1} were obtained. Finally, 10 µL of the bacterial and fungal suspensions were added to the wells. In parallel, controls were included: microorganisms (BHI + bacteria and RPMI + yeasts) and culture medium (RPMI/BHI), to assure the strains' viability and sterility of the medium, respectively; and negative control with the antimicrobials gentamicin (100 µg mL^{-1}) for bacteria and amphotericin B (100 µg mL^{-1}) for fungi. The prepared

plates were aseptically closed and incubated at 35 ± 2 °C for 24-48 h.

In the biological assay with bacteria, after 24 h of incubation, 20 μL of 0.01% resazurin dye indicator (INLAB), a colorimetric redox, were added.[29] A change in dye color from blue to red indicated microbial growth, and if the color remained blue, it meant the absence of microbial growth. The MIC for each product was defined as the lowest concentration capable of visually inhibiting microbial growth with no dye color change.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

This work was supported by the Brazilian agencies CNPq and CAPES. Dr A. Leyva (USA) provided English editing of the manuscript.

Author Contributions

R. F. O., H. D. S. S. and P. F. A.-F. conceived and designed the experiment; R. F. O., F. S. A., A. P. S., E. O. T. and P. S. V. L. performed the experiments; R. F. O., H. D. S. S. and M.-F. N. H., *in silico* study and data analyses; L. V. C., H. D. N. and E. O. L. performed the antimicrobial study; R. F. O., H. D. S. S., P. F. A.-F. and J. M. B.-F. wrote the paper.

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Submitted: August 13, 2019
Published online: November 19, 2019