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LETTER ARTICLE

Synthesis and Antimicrobial Activity of Thiohydantoins Obtained from L-Amino Acids

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Abstract: ***Background*:** Thiohydantoins are an important class of heterocyclic compounds in drug discovery since they are related to a wide range of biological properties including antimicrobial activity.

***Objective*:** The objective of this study was to synthesize a series of thiohydantoins derived from L-aminoacids and to evaluated their inhibitory effect on the growth of Gram-negative and Gram-positive bacteria.

***Methods*:** All title compounds were synthetized by reaction of L-amino acids with thiourea or ammonium thiocyanate. Their antimicrobial activities were evaluated against bacterial strains by broth microdilution assays. The time-kill kinetics, the antibiofilm activity and the cytotoxicity to mammalian cells were determined for the compound that exhibited the best antimicrobial profile (1b).

***Results*:** Eleven thiohydantoins were readily obtained in good yields (52-95%). In general, thiohydantoins were more effective against Gram-positive bacteria. Compound 1b (derived from L-alanine) showed the best antibacterial activity against Staphylococcus epidermis ATCC 12228 and S. aureus BEC 9393 with MIC values of 940 and 1921 μM, respectively. The time-kill kinetics demonstrated time-dependent bactericidal effect in both strains for this derivative. Besides, 1b also exhibited antibacterial activity against biofilms of S. epidermidis ATCC 12228, leading to a 40% reduction in their metabolic activity compared to the untreated control. No cytotoxicity of 1b to mammalian cells was observed at MIC values.

***Conclusion*:** The data reported herein indicate relevant antimicrobial activity of thiohydantoins derived from L-aminoacid, mainly 1b, as potential pharmacophore to guide further chemical modification aiming at the search for new and improved antimicrobial agents.

Keywords: Synthesis, thiohydantoins, acylthioureas, acyl-thiohydantoins, antibacterial activity, antibiofilm activity.

1. INTRODUCTION

Microbial infections are associated with high morbidity and mortality among hospitalized patients worldwide. Healthcare-Associated Infections (HAI) lead to increased hospital stay and financial losses for the health system. The World Health Organization (WHO) emphasizes that out of every 100 hospitalized patients, 7 (in developed countries) and 10 (in developing countries) will acquire at least one HAI, which can be associated with high mortality rates [1, 2].

These infections become even more relevant when associated with antimicrobial resistant microorganisms in which treatments with the last resort antibiotics are ineffective Considering the negative impact of antibiotic resistance throughout the health system, the discovery and development of new efficient and safer drugs are needed. Thus, the screening for antimicrobial agents with different mechanisms of action is particularly promising, because of their potential for the establishment of new strategies to control antimicrobial resistant pathogens [3-5].

In drug discovery, thiohydantoins are an important class of heterocyclic compounds since these compounds are related to a wide range of biological properties, such as anticonvulsant, antiparasitic, antiviral, antitumoral, antioxidant, anticarcinogenic, anti-ulcer, anti-inflammatory, antithyroid, hypolipidemic, and antimicrobial activity [6-22]. Therefore, in our continuous program to the search for new candidates for antimicrobial agents, we proposed the synthesis of some thiohydantoins (**1a-i** and **2a-c**) by a

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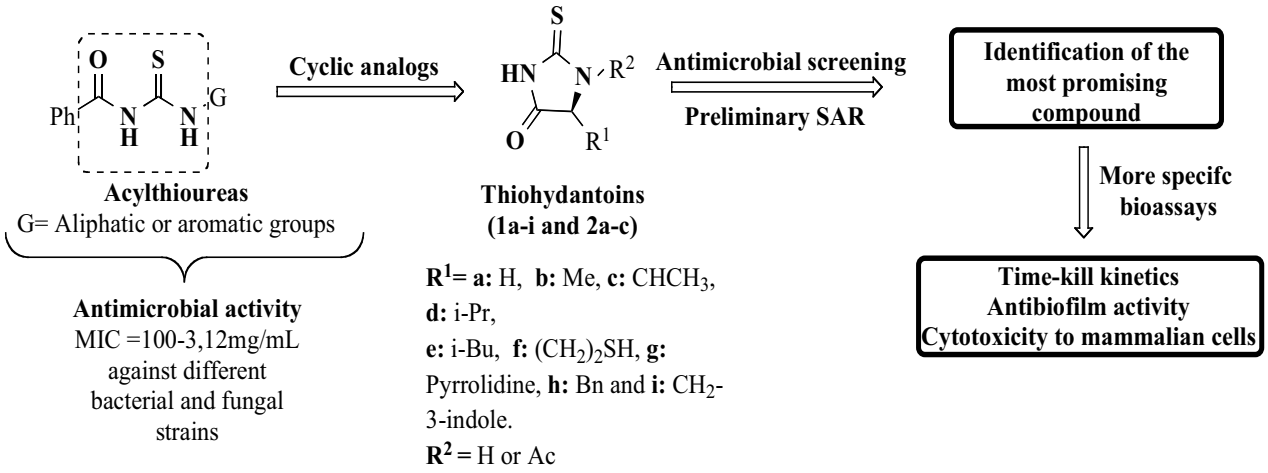


Fig. (1). Design concept of thiohydantoins (**1a-i** and **2a-c**).

cyclization reaction using nine different *L*-amino acids and thiourea as starting material. The design concept used for proposing the synthesis and antimicrobial evaluation of thiohydantoins was supported by our previous results that showed the inhibitory effect of some acylthioureas on growth of several bacterial and fungal species [23]. Besides, the potential of the acylthioureas as antimicrobial agents also can be confirmed by several reports in the literature [24-27]. Hence, as thiohydantoins are cyclic analogues of acylthioureas, we decide to investigate their antibacterial potential against gram-positive and gram-negative bacteria, including antibacterial-resistant strains. Several lateral chains of amino acids were chosen to verify the influence of the different steric and electronic characteristics on the biological activity and, therefore, to carry out a preliminary structure-activity relationship study of this series of compounds. Moreover, the most promising compound was analyzed for bacterial killing-kinetics and antibiofilm activity, and cytotoxicity for mammalian cells (Fig. **1**).

2. EXPERIMENTAL

2.1. General

Analytical grade solvents were used as received. The Melting Points (MP) were determined on hot plate apparatus Microchemistry MQAPF 302. NMR spectra were obtained on a Bruker spectrometer (Model Ascend 400) operating at 400.13 MHz for ^{1}H and 100.13 MHz for ^{13}C equipped with a 5 mm broadband probe. NMR spectra were recorded using DMSO-d_6 or D_2O as a solvent. Chemical shifts (δ in ppm) were referenced for residual solvent signals (DMSO in DMSO at 2.50, HDO in D_2O-d_2 at 4.70). The multiplicity of the ^{1}H NMR signals are reported as follows: Singlet (s), doublet (d), double-doublet (dd), triplet (t), quartet (q), broad (br) and multiplet (m). The coupling constants (*J*) are reported in Hz.

2.2. Chemistry

2.2.1. Synthesis of Thiohydantoins (1d-i)

A mixture of a L-amino acid (60mmol) and thiourea (20mmol) was placed in a round bottomed flask and heated at 170-210ºC for 60 minutes under magnetic stirring. The mixture was allowed to cool down at room temperature and water (20 mL) was added. The resulting mixture was heated to dissolve all the solids present and then, stored at 5°C for 24 hours. The crystals were removed by vacuum filtration and the crude product was purified by recrystallization in water.

2.2.1.1. 5-Isopropyl-2-Thioxoimidazolidin-4-One (1d)

(L-valine) Yield: 196.00 mg (62%); Yellow solid; Mp. 134-136°C (Lit. Mp. 137-140ºC [28, 29]); ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.81 (d, *J*= 6.77 Hz, 3H), 0.95 (d, *J*= 6.77 Hz, 3H), 2.00-2.07 (m, 1H), 4.10 (d, *J*= 3.65 Hz, 1H), 10.02 (br, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 16.5 (CH_3), 18.7 (CH_3), 30.4 (CH), 66.2 (CH), 176.4 (C=O), 183.4 (C=S).

2.2.1.2. 5-Isobutyl-2-Thioxoimidazolidin-4-One (1e)

(L-leucine) Yield: 292.26 mg (85%); Yellow solid; Mp. 165-170°C (Lit. Mp. 177-178ºC [28, 29]); ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.88 (d, *J*= 6.71 Hz, 6H), 1.44-1.49 (m, 2H), 1.76-1.83 (m, 1H), 4.22 (dd, *J*= 5.17-8.33 Hz, 1H), 10.12 (s, 1H), 11.66 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 22.0 (CH_3), 23.4 (CH_3), 24.4 (CH), 59.7 (CH), 177.4 (C=O), 182.4 (C=S).

2.2.1.3. 5-(2-(methylthio)Ethyl)-2-Thioxoimidazolidin-4-One (1f)

(L-methionine) Yield: 252.67 mg (72%); Yellow solid; Mp. 139-145°C (Lit. Mp. 147-149°C [28, 29]); ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.77-1.88 (m, 1H), 1.90-1.99 (m, 1H), 2.04 (s, 3H), 2.49-2.59 (m, 2H), 4.30 (dd, *J*= 4.89-7.75 Hz, 1H), 10.08 (br, 1H), 11.69 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 14.9 (CH_3), 29.1 (CH_2), 30.7 (CH_2), 60.0 (CH), 176.8 (C=O), 182.9 (C=S).

2.2.1.4. 3-Thioxohexahydro-1H-Pyrrolo [1, 2-c]Imidazol-1-one (1g)

(L-proline) Yield: 114.57 mg (37%); Yellow solid; Mp. 153-155°C (Lit. Mp. 161-163ºC [29, 30]); ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.60-1.71 (m, 1H), 2.03-2.18 (m, 4H), 3.66-3.73 (m, 1H), 4.39 (dd, *J*= 6.19-10.06 Hz, 1H), 11.73

(br, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 26.3 (CH_2), 27.3 (CH_2), 47.8 (CH_2), 66.8 (CH), 175.8 (C=O), 186.5 (C=S).

2.2.1.5. 5-Benzyl-2-Thioxoimidazolidin-4-One (1h)

(L-phenylalanine) Yield: 290.91 mg (71%); Yellow solid; Mp. 165-167°C (Lit. Mp. 178-180ºC [28, 29]); ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.98 (dd, *J* = 4.94-14.36 Hz, 2H), 4.55 (t, *J*= 4.94 Hz, 1H), 7.16-7.29 (m, 5H), 10.06 (s, 1H), 11.43 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 36.1 (CH_2), 61.8 (CH), 127.3 (CH), 128.6 (CH), 130.1 (CH), 135.5 (C), 176.1 (C=O), 182.7 (C=S).

2.2.1.6. 5-((1H-indol-3-yl) Methyl)-2-Thioxoimidazolidin-4-One (1i)

(L-tryptophan) Yield: 162.03 mg (33%); Yellow solid; Mp. 166, 5-168,2°C (Lit. Mp. 190-192ºC [28, 29]); ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.08-3.19 (m, 2H), 4.53 (dd, *J*= 4.24-4.91 Hz, 1H), 6.95-7.00 (m, 1H), 7.04-7.08 (m, 1H), 7.11 (d, *J*= 2.37 Hz, 1H), 7.31-7.33 (m, 1H), 7.55 (d, *J*= 7.94 Hz, 1H), 10.06 (s, 1H) 10.91 (s, 1H), 11.37 (s, 1 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 26.1 (CH_2), 61.8 (CH), 107.8 (CH), 111.7 (CH), 118.8 (CH), 121.3 (CH), 124.6 (C), 127.8 (C), 136.3 (C), 176.7 (C=O), 182.8 (C=S).

2.2.2. General Procedure for the Synthesis of Acyl-thiohydantoins (2a-b) and Thiohydantoin (2c)

A mixture of the appropriate L-amino acid (13.3 mmol) and ammonium thiocyanate (13.3 mmol) was added to acetic anhydride (79.3 mmol) and was placed in a round bottomed flask and heated at 100ºC for 30 minutes under magnetic stirring. The mixture was allowed to cool down at room temperature and cold water (20 mL) was added. Then, the mixture was stored at 5ºC for 24 hours, the crystals were removed by vacuum filtration and the crude product was purified by recrystallization in water.

2.2.2.1. 1-Acetyl-2-Thioxoimidazolidin-4-One (2a)

(L-glycine) Yield: 110.50 mg (52%); Orange solid; Mp. 168-170°C (Lit. Mp. 173-174ºC [31]); ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.68 (s, 3H), 4.40 (s, 1H), 12.57 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 27.0 (CH_3), 52.6 (CH_2), 169.8 (C=O), 170.8 (C=O), 182.9 (C=S).

2.2.2.2. 1-Acetyl-5-Methyl-2-Thioxoimidazolidin-4-one (2b)

(L-alanine) Yield: 210.70 mg (92%); White solid; Mp. 160-162°C (Lit. Mp. 164-166ºC [31]); ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.43 (d, *J*= 7.00 Hz, 3H), 2.71 (s, 3H), 4.68 (q, *J*= 7.00 Hz, 1H), 12.63 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 16.3 (CH_3), 27.8 (CH_3), 59.2 (CH), 170.1 (C=O), 174.4 (C=O), 182.7 (C=S).

2.2.2.3. (Z)-5-Ethylidene-2-Thioxoimidazolidin-4-one (2c)

(L-threonine) Yield: 105.06 mg (56%); Yellow solid; Mp. 252-255°C (Lit. Mp. 264ºC [28, 32]); ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.86 (d, *J*= 7.68 Hz, 3H), 5.71 (q, *J*= 7.68 Hz, 1H), 11.92 (s, 1H), 12.06 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.0 (CH_3), 112.2 (CH), 132.2 (C), 164.6 (C=O), 178.4 (C=S).

2.2.3. General Procedure for Deacetylation. Synthesis of Thiohydantoins 1a-b

Corresponding acyl-thiohydantoin (1 mmol) was added at 10 mL of hydrochloric acid solution (5M) and was placed in a round bottomed flask and heated at 110ºC for 60 minutes under magnetic stirring. The mixture was allowed to cool down at room temperature and then, the resulting clear yellow solution was extracted with 3 x 10 mL of ethyl acetate. The combined organic layers were dried over Na_2SO_4, concentrated, and dried under vacuum to afford the thiohydantoins of interesting.

2.2.3.1. 2-Thioxoimidazolidin-4-One (1a)

(L-glycine) Yield: 6.38 mg (55%); Orange solid; Mp. 204-206°C (Lit. Mp. 229-231ºC [31, 33]); ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.08 (d, 2H), 9.84 (s, 1H), 11.64 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 50.8 (CH_2), 175.1 (C=O), 183.9 (C=S).

2.2.3.2. 5-Methyl-2-Thioxoimidazolidin-4-One (1b)

(L-alanine) Yield: 12.36 mg (95%); White solid; Mp. 158–160°C (Lit. Mp. 165-166ºC [31-34]); ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.24 (d, *J* = 7.16 Hz, 3H), 4.22 (dd, *J*= 0.86-6.97 Hz, 1H), 9.99 (s, 1H), 11.62 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 16.5 (CH_3), 56.7 (CH), 177.7 (C=O), 182.5 (C=S).

2.3. Antimicrobial Activity

2.3.1. Bacteria and Growth Conditions

Staphylococcus epidermidis ATCC 12228, *S. epidermidis* ATCC 1E4248, *Streptococcus pyogenes* ATCC 19615, *Klebsiella pneumoniae* ATCC 700603, *K. pneumoniae* ATCC 10031, *Proteus mirabilis* ATCC 7002, *Escherichia coli* ATCC 8739, *Enterococcus faecium* ATCC 6569, *Streptococcus agalactiae* ATCC 13813, and methicillin-resistant *S. aureus* (MRSA) BEC9393 were included in this study. Bacteria were grown in Brain Heart Infusion (BHI, Difco) agar at 37^{o}C for 24 h. Before the experiments, colony forming units (CFU) of each bacterium were transferred to BHI Broth to achieve a turbidity equivalent to a 0.5 McFarland standard using the DensiCHECK™ PLUS colorimeter (bioMérieux), which corresponded to approximately 1.0 to 2.0 x 10^8 CFU/mL (standard bacterial suspension). Bacteria were stored in BHI containing 20% glycerol at -80^{o}C.

2.3.2. Determination of Minimum Inhibitory Concentration

The inhibitory activity of the thiohydantoins on the growth of all bacteria was determined by broth microdilution assays according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [35]. A standard bacterial suspension of each bacterial species was added to wells of 96-wells U-bottom microtiter plates (Techno Plastic Products, Switzerland) containing twofold serial dilutions of each compound in Muller-Hinton Broth (MHB, Difco®). Concentrations ranging from 62.5 to 1000 µg/mL were evaluated. Wells containing medium or medium plus 1% DMSO and wells without bacterial cells in each plate served as growth and sterility controls, respectively. The minimum

inhibitory concentration of the thiohydantoins was determined at total inhibition of visual growth after 24 h at 37 ^{o}C compared to untreated cells. The assays were carried in duplicate on three different occasions.

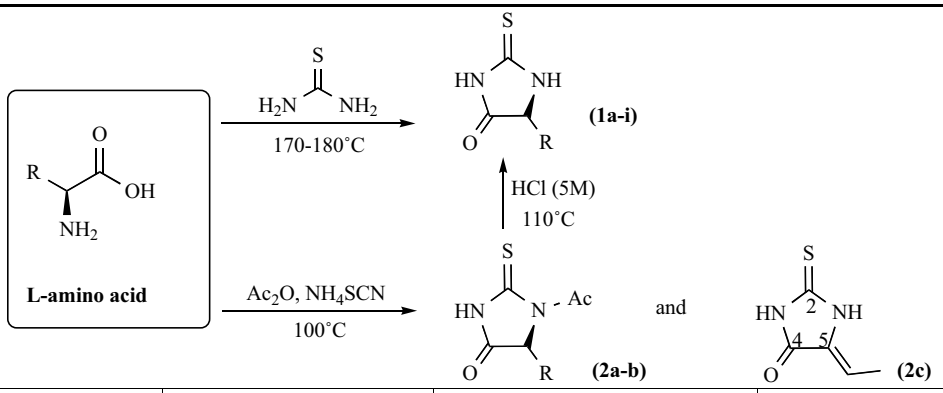
2.3.3. Time Kill Kinetics

The bacterial killing kinetics in presence of thiohydantoin **1b** was analyzed by time-kill assay [36]. Bacterial cells (5 x 10^6 CFU/mL) were added in MHB containing the compound at MIC values and incubated at 37^{o}C. At specific time points (0, 2, 4, 7, 10 and 24 h), 20 µL were removed from each well and diluted tenfold in 0.15 M phosphate-buffered saline pH 7.2 (PBS). An aliquot of 100 µL of each dilution was inoculated on plates of MH Agar and the CFU counts were determined after incubation at 37ºC for 24 h. Data of two independent experiments were averaged and plotted as log10 CFU/mL *versus* time (h).

2.3.4. Effect of Compound 1b on Staphylococcus Epidermidis Biofilm

The effect of thiohydantoin **1b** was evaluated against established biofilm of *S. epidermidis* ATCC 12228. Biofilm was formed on polystyrene surface of flat-bottomed 96-well microtiter plates (Techno Plastic Products, Switzerland) according to Stepanovic *et al*. (2007) with modifications [37]. Briefly, 20 µL of standard bacterial suspension was placed in each well containing 180 µL of MHB, and the plates were incubated statically at 37^{o}C for 24 h. After the incubation, the medium was aspirated off and sessile cells were gently washed with sterile PBS. Fresh MHB (200µL) containing thiohydantoin **1b** at MIC concentration (125 µg/mL) was added and the plate was incubated for further 24 h. Thiohydantoin-free wells and biofilm-free wells were included as controls. Metabolic activities of treated sessile cells were compared to untreated cells using the 2,3-bis(2 methoxy 4-nitro-5-sulfo-phenyl)-5- [(phenylamino) carbo-nyl]-2*H*-tetrazolium hydroxide (XTT)- reduction assay [38]. A 200 μL-aliquot of XTT-menadione [1 mg/mL XTT, 0.4 mM menadione (Sigma Chemical Co., USA)] was added to each well, and the plates were incubated in the dark at 37°C for 3 h, after which the optical density was measured at 492 nm with a microtiter plate reader (Synergy HT, Biotek). Experiments were carried out in quintuplicate on two different occasions.

Table 1. Results for the synthesis of thiohydantoins (1a-i) and (2c), and acyl-thiohydantoins (2a-b).



Entry	L-amino Acid	Thiohydantoin	R	Yield (%)
1	Glycine	1a[a]	H	55
2	Alanine	1b[a]	Me	95
3	Threonine	1c	$OHCHCH_3$	-
4	Valine	1d	*i*-Pr	62
5	Leucine	1e	*i*-Bu	85
6	Methionine	1f	$(CH_2)_2SCH_3$	72
7	Proline	1g	Pyrrolidine	37
8	Phenylalanine	1h	Bn	71
9	Tryptophan	1i	CH_2-3-indole	33
10	Glycine	2a	H	52
11	Alanine	2b	Me	92
12	Threonine	2c[b]	$CHCH_3$	56

[a]Yield obtained from hydrolysis of corresponding acetylated derivative. [b]Product resultant by elimination process.

2.3.5. Cytotoxicity Assay

The cytotoxicity of compound **1b** (1000 to 31.5 μg/mL) was evaluated on LLC-MK2 cells (*Macaca mulata* kidney epithelial cells, ATCC® CCL-7™). Epithelial cells were grown in Roswell Park Memorial Institute (RPMI) medium 1640 (Invitrogen-Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL amphotericin B. The cells were grown in a flat-bottomed 96-well microtiter plate at a density of 2.5 x10^4 cells/well for 24 h in 5% CO_2 at 37°C. At confluence, non-adherent cells were removed by washing with sterile PBS. The medium (200 μL), containing different concentrations of the tested compounds (1000 to 31.5µg/mL), was added to each well and the plates were incubated for 48 h. Cell viability was determined by the dimethylthiazol diphenyl tetrazolium bromide (MTT, Sigma Chemical Co., USA) reduction method according to the manufacturer's recommendations. The assays were carried out in triplicate on two separate occasions.

3. RESULTS AND DISCUSSION

3.1. Chemistry

A series of eleven thiohydantoin derivatives were synthesized by three synthesis methods. The first involved a condensation reaction between thiourea and nine L-amino acids (glycine, alanine, threonine, valine, leucine, methionine, proline, phenylalanine and tryptophan). Thiohydantoins **1d-f** and **1h** derived from amino acids with non-polar side chains were obtained in good yields (62 to 85%) by direct condensation between L-amino acids and thiourea in absence of solvent [29] and readily isolated by precipitation from water (Table **1**). The derivatives **1g** and **1i** were obtained from proline and tryptophan in lower yields 37% and 33%, respectively using this methodology (Table **1**, entries **7** and **9**). The thiohydantoins resulting respectively from alanine, glycine and threonine were not obtained or isolated under these reaction conditions.

Alternatively, the derivatives **1a** and **1b**, were obtained in good yields utilizing the second method by treatment of corresponded amino acid with acetic anhydride and ammonium thiocyanate to give the corresponding acyl-thiohydantoins **2a** and **2b**, which were hydrolyzed in acidic media resulting on the compounds **1a** and **1b** with 55% and 95% yield respectively (Table **1**) [39, 40]. Conversely, under the same acetylation conditions, the compound **2c** was obtained directly as an elimination product with a C5/C6 double bond (Table **1**) (Supplementary Material).

The ^{1}H NMR spectra showed the characteristic signal for the NH proton at 9.90-12.7 ppm and the ^{13}C NMR spectra showed the signal for C = O and C = S endocyclic carbon resonances at 173.0-179.1 and 182.4-184.3 ppm, respectively. The signals corresponding to the CH_3 protons of the acetyl-thiohydantoins were observed in the ^{1}H NMR spectra at 2.68-2.71 ppm and the acetyl C=O signal for on spectra at 169.7-170.1 ppm in the ^{13}C NMR spectra. The ^{13}C NMR spectra to the compound **2c** showed the signals for C = O and C = S endocyclic carbon resonances at 164.6 and

Table 2. The *in vitro* activity of thiohydantoins derivatives 1a-i and 2a-c against four different strains of sensitive gram-positive bacteria.

Compounds	R	Gram-positive bacteria MIC (µM*)				
		S. epidermidis **ATCC 12228**	***S. pyogenes*** **ATCC 19615**	***S. agalactiae*** **ATCC 13813**	***E. faecium*** **ATCC 6569**	***S. aureus*** **BEC 9393**
1a	H	R	R	R	8610	R
1b	Me	940	R	R	R	1921
1d	*i*-Pr	R	R	R	6320	R
1e	*i*-Bu	726	2903	2903	5806	R
1f	$(CH_2)_2SCH_3$	R	R	R	R	2628
1g	Pyrrolidine	R	R	R	6402	R
1h	Bn	606	R	R	4848	2424
1i	CH_2-3-indole	1019	2038	2038	R	2038
2a	H	R	1581	1581	R	1581
2b	Me	2904	2904	2904	5807	2904
2c	$CHCH_3$	1758	3517	3517	7033	R
Erythromycin	--------	0.25	0.01	0.12	-	-
Vancomycin	--------	-	-	-	0.5	0.5

R = MIC > 1000 µg/mL, which indicates that the strain is resistant to tested substance. *MIC were determined as µg/mL, but the values of thiohydantoin compounds were converted in µM to perform an adequate structure-relationship study. -: Not determined.

Table 3. The *in vitro* activity of thiohydantoins derivatives 1a-i and 2a-c against three different strains of sensitive gram-negative bacteria.

Compounds	R	Sensitive Gram-negative Bacteria MIC (μM*)		
		***K. pneumoniae* ATCC 10031**	***P. mirabilis* ATCC 7002**	***E. coli* ATCC8739**
1a	H	R	R	R
1b	Me	R	1921	R
1d	*i*-Pr	R	R	R
1e	*i*-Bu	R	R	2903
1f	$(CH_2)_2SCH_3$	R	2628	R
1g	Pyrrolidine	R	R	R
1h	Bn	R	R	R
1i	CH_2-3-indole	2038	R	R
2a	H	R	R	R
2b	Me	R	R	R
2c	$CHCH_3$	3517	R	R
Ciprofloxacin	----------	0.06	0.12	0.06

R = MIC > 1000 μg/mL, which indicates that the strain is resistant to tested substance. * MIC were determined as μg/mL, but the values of thiohydantoin compounds were converted in μM to perform an adequate structure-relationship study.

178.4 ppm respectively, as also was observed the characteristic signals for the unsaturated carbons corresponding to the C5/C6 double bond at 132.2 and 112.2 ppm respectively (Table **1**). The ^{1}H NMR spectra showed the characteristic signal for the NH proton at 11.9-12.1 ppm and the signal corresponding to the proton α-carbonyl was not observed, confirming the formation of elimination product **2c**. Furthermore, all detected ^{1}H and ^{13}C NMR signals were consistent with the presence of the corresponding aliphatic or aromatic group in each compound of the series and compared with the data published from the literature.

3.2. Antimicrobial Activity

Firstly, the antibacterial activity of all thiohydantoins derivatives **1a-i** and **2a-c,** and reference antimicrobials were evaluated against Gram-positive and Gram-negative bacteria, and the MIC values are showed in Tables **2** and **3**, respectively. As expected, all bacteria were sensitive to reference antimicrobials according to the CLSI breakpoints.

In general, the thiohydantoins were more effective against Gram-positive bacteria. Remarkably, *S. epidermidis* seems to be more sensitive to thiohydantoins derivatives, as judged by the MIC values, which ranged from 606 to 2904 μM. Except for **1b**, the presence of aromatic groups (**1h** and **1i**) or bulky aliphatic groups (**1e** and **2c**) in the side chains led to an improvement of antimicrobial activity. A decrease of antimicrobial activity resulting by the presence of an acetyl group at R^2 is another important trend in this series of compounds (**1b** *versus* **2a** and **1a** *versus* **2b**).

Interestingly, thiohydantoin derivatives also exhibited a good inhibitory activity on the growth of MRSA BEC 9393 strain. MRSA is one of the leading causes of human infections worldwide [41]. The acquisition of *mecA* gene, which encodes a penicillin-binding protein (known as PBP2a) with lower affinity to beta-lactam antibiotics, is the main mechanism of methicillin-resistance. Crucially, besides beta-lactams this bacterium has become resistant to nearly all commercially available antibacterial agents, limiting therapeutic options [42]. In this study, the thiohydantoin **1f** inhibited the planktonic growth only of MRSA strain. In addition, substances **1h, 1i, 1b** and **2a** were also active against this resistant strain, exhibiting MIC values equal to or higher than those of *S. epidermidis*. The presence of aromatic groups (**1h** and **1i**) or a methyl group (**1b**) in the side chain was also important to the biological activity against MRSA. However, the presence of acetyl group at R^2 did not reduce the antimicrobial activity as occurred against Gram-positive sensitive strains (Table **2**), contrariwise, there was an improvement in the potency of the acetylated derivatives.

After these considerations about the preliminary SAR study, the compound **1b** was selected to the time-kill assay since it showed good inhibitory activity against both *S. epidermidis* ATCC 12228 and MRSA BEC 9393. Thus, planktonic cells of both strains were grown in the presence of compound **1b** at MIC (125 μg/mL and 250 μg/mL, respectively) and CFU counts were determined at time-intervals during 24 h (Fig. **2**).

The thiohydantoin **1b** showed a time-dependent bactericidal effect in both strains (Fig, **2**). After a lag phase

of about 2 h, control cultures (without **1b**) showed a gradual increase in the number of CFUs over 24 h. In contrast, for *S. epidermidis* ATCC 12228, the presence of compound **1b** at MIC reduced the planktonic cell population approximately by 3 and 6 log10 CFU/mL ($P < 0.05$) after 4 h and 7 h of incubation, respectively, compared to the initial inoculum. Similarly, **1b** provoked a reduction of approximately 3 log10 CFU/mL ($P < 0.05$) for *S. aureus* BEC 9393, after 4 h of incubation, and no CFUs were detected after 7 h of incubation.

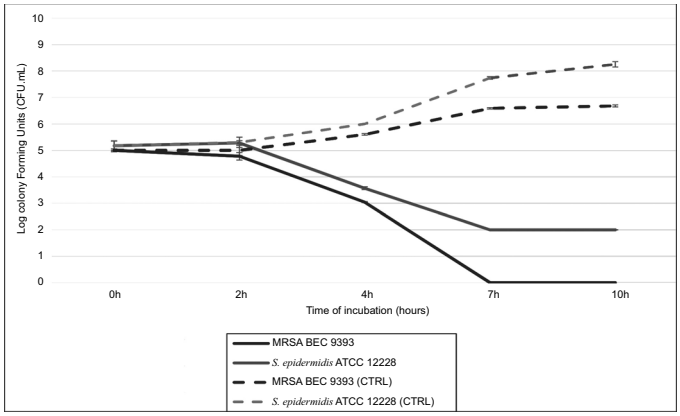


Fig. (2). Bactericidal activity of thiohydantoin **1b** against *Staphylococcus* species.

In this study, thiohydantoin **1b** also exhibited an antibiofilm activity against *S. epidermidis* ATCC 12228. It is well known that most *S. epidermidis* infections are associated with its capacity of adhering and forming biofilm on implanted medical devices [43]. Microbial biofilms consist of a community of surface-attached sessile cells embedded in a self-produced extracellular polymeric substance [44]. Notably, sessile cells display reduced sensitivity to antimicrobials and host defense mechanisms compared to that of planktonic cells [43, 44]. The treatment of established (after 24 h of formation) with **1b** at MIC (940 μM) decreased significantly ($P < 0.05$) the viability of sessile cells. A decrease of 40% in metabolic activity of the treated biofilm was observed compared to the untreated control (biofilm in absence of **1b**).

3.3. Cytotoxicity of Thiohydantoin 1b to Mammalian Cells

To assess the cytotoxic effect of compound **1b** to mammalian cells, LLC-MK2 cells were exposed to increasing concentrations of the compound. In presence of bactericidal concentrations of **1b** for *S. epidermidis* ATCC (MIC = 125 μg/mL) and MRSA BEC 9393 (MIC = 250 μg/mL), 96 and 92% of mammalian viable cells were detected by the MTT reduction assay, after 48 h of incubation (Table **4**). The calculated concentration of **1b** that was cytotoxic to 50% of the cells (CC50) was 935.7 μg/mL, indicating the potential of this compound as a good prototype in the search of new antimicrobial drugs.

Table 4. Effect of thiohydantoin 1b on viability of LLC-MK2 cells (*Macaca mulata* kidney epithelial cells, ATCC® CCL-7™).

Concentration of 1b (μg/mL)	1000	500	250	125	62.5	31.2
% Viable Cell*	41	97	92	96	100	100

*LLC-MK2 cells were treated for 48 h with different concentrations of compound **1b**. Cell viability was determined by the MTT reduction method according to the manufacturer's recommendations.

CONCLUSION

A facile synthesis of eleven thiohydantoins in good yields (52-95%) was reported using simple and convenient methodology. In general, the thiohydantoins were more effective against gram-positive bacteria. Compound **1b** showed better results against sensible *S. epidermidis* ATCC 12228 (MIC = 940 μM) and resistant *S. aureus* BEC 9393 (MIC = 1921 μM). This compound exhibited a time-dependent bactericidal effect in planktonic cells of both strains. Furthermore, this compound also inhibited the biofilm of *S. epidermidis* ATCC 12228, promoting a decrease of 40% on metabolic activity of treated biofilm. No cytotoxicity of **1b** to mammalian cells was observed at MIC values. These data indicate the potential of **1b** as a prototype to guide further chemical modification in the search for new and better antimicrobial agents.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by Committee on Animal Experimentation of the State University of Londrina: 12417.2016.25, Brazil.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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