Synthesis, toxicity towards brine shrimp (*Artemia salina* Leach) and antimicrobial activity evaluation of 3,5-diphenylchlorinated-1,2,4-oxadiazoles

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ABSTRACT. The known oxadiazoles 3,5-bis-(phenyl)-1,2,4-oxadiazole (**3a**); the 3-(4-chlorophenyl)-5-phenyl-1,2,4-oxadiazole (**3b**); and the new 3,5-diphenylchlorinated-1,2,4-oxadiazoles **3c-e** were synthesized from the reaction of benzamidoximes with an appropriated acid chloride and cyclisation of the resulting *O*-acylbenzamidoxime intermediate. The compounds synthesized were characterized on the basis of their IR, NMR (1D and 2D) and mass spectral data. Compounds **3a-e** were evaluated for their antimicrobial activity and for their toxicity towards brine shrimp (*Artemia salina* Leach).

Key words: 3,5-diphenylchlorinated-1,2,4-oxadiazoles, synthesis, NMR data, antimicrobial activity, toxicity on *Artemia salina* Leach.

RESUMO. Síntese, toxicidade frente a *Artemia salina* Leach e avaliação da atividade antimicrobiana de 1,2,4-oxadiazóis-3,5-difenilclorados. Os oxadiazóis 3,5-bis-(fenil)-1,2,4-oxadiazol (**3a**) e 3-(4-clorofenil)-5-fenil-1,2,4-oxadiazol (**3b**), já descritos na literatura, e, os 1,2,4-oxadiazóis-3,5-fenilclorados inéditos **3c-e**, foram sintetizados pela reação de bezamidoximas com um cloreto de ácido apropriado, seguido da ciclização do intermediário *O*-acilíco resultante. Os compostos obtidos foram caracterizados com base nas análises dos dados espectroscópicos de IV, EM e RMN (uni- e bidimensionais). Os compostos **3a-e** foram submetidos a bioensaios para avaliação de atividade antimicrobiana e, de toxicidade frente ao microcrustáceo *Artemia salina* Leach.

Palavras-chave: 1,2,4-oxadiazóis-3,5-difenilclorados, dados de RMN, atividade antimicrobiana, toxicidade em *Artemia salina* Leach.

Introduction

The 1,2,4-oxadiazole moiety can be found in a large number of biologically active heterocyclic compounds exhibiting pharmacological activities such as analgesic, anti-inflammatory, antimicrobial, and antiviral (Hemming, 2001). The 1,2,4-oxadiazole moieties have been used as bioisosters for esters and amides in biological studies. These unities are present in muscarinic agonists (Macor *et al.*, 1996), serotoninergic (5-HT_3), and receptor antagonists (Swain *et al.*, 1991; Weidner-Wells *et al.*, 2004).

Considering the potential of this class of compounds, in order to find new biologically actives oxadiazoles, we attempted to synthesize and to evaluate the toxicity and antimicrobial activity of 1,2,4-oxadiazoles containing phenylchlorinated substituents attached at C-3 and C-5. The known compounds 3,5-bis-(phenyl)-1,2,4-oxadiazole (**3a**); 3-(4-chlorophenyl)-5-pheny-1,2,4-oxadiazole (**3b**); the new 1,2,4-oxadiazoles 3-(4-chlorophenyl)-5-(3,4-dichlorophenyl)-1,2,4-oxadiazole (**3c**); 3-5-bis-(3,4-dichlorophenyl) 1,2,4-oxadiazole (**3d**); and 3-(3,4-dichlorophenyl)-5-phenyl-1,2,4-oxadiazole (**3e**) were synthesized and characterized on the basis of IR, MS, ^{1}H NMR, ^{13}C NMR / DEPT, ^{1}H-^{1}H-COSY, HMQC, and HMBC spectral data. The toxicities of the compounds **3a-e** were tested by using brine shrimp (*Artemia salina*) bioassay. Antimicrobial activities were evaluated against the bacteria *Staphylococcus aureus*, *Escherichia coli*, *Bacilus subtilis,* and *Pseudomonas aeruginosa*, and against the fungi *Candida albicans*, *C. krusei*, *C. parapsilosis,* and *C. tropicalis*.

Material and methods

Equipments. IR spectra were recorded on a Bomem spectrophotometer model MB 100. ^{1}H (300 MHz), ^{13}C (75.5 MHz) and 2D (COSY, HMQC, HMBC) NMR spectra were recorded in a Varian spectrometer model Mercury plus BB 300MHz, using

$CDCl_3$ as solvent and TMS as internal standard. MS spectra were obtained in Hewllet Packard-GC/MS spectrometer model 5972, at 70 eV. Melting points were determined with a Microquimica-MQ APF-301 apparatus and are uncorrected. The purity of the compounds was confirmed by GC/MS analysis.

Synthesis

Preparation of the chlorobenzonitriles 1b and 1c. Chlorobenzonitriles **1b** and **1c** were prepared from 4-chlorobenzotrichloride and 3,4-dichlorobenzotrichloride, respectively. A mixture of 1 mol of chlorobenzotrichloride, ammonium chloride (1 mol) and 4-chlorobenzoic acid (0.07 mol) for **1b** or 3,4-dichlorobenzoil chloride (0.12 mol) for **1c** was stirred at 220ºC for 10 hours. The solid residue was purified by sublimation.

4-Chlorobenzonitrile (1b): 85% yield. ^{1}H NMR ($CDCl_3$): δ 7.47 (*d*, 2H, H-3 and H-5, J = 8.7 Hz), 7.61 (*d*, 2H, H-2 and H-6, J = 8.7 Hz). ^{13}C NMR ($CDCl_3$): δ 110.8 (C-1), 118.0 (CN), 129.7 (C-3 and C-5), 133.4 (C-2 and C-6), 139.6 (C-4). EI-MS (70 eV) *m/z* (int. rel. %): 137 ($M^{+\bullet}$, 100).

3,4-Dichlorobenzonitrile (1c): 97% yield. ^{1}H NMR ($CDCl_3$): δ7.51 (*dd*, 1H, H-6, J = 8.2 and 1.8 Hz), 7.59 (*d*, 1H, H-5, J = 8.2 Hz), 7.76 (*d*, 1H, H-2, J = 1.8 Hz). ^{13}C NMR ($CDCl_3$): δ 112.0 (C-1), 116.8 (CN), 131.1 (C-6), 131.5 (C-5), 133.8 (C-2), 134.1 (C-3), 138.3 (C-4). EI-MS (70 eV) *m/z* (int. rel. %): 171 ($M^{+\bullet}$, 100).

Preparation of the benzamidoximes 2a-c. To a stirred solution of commercial benzonitrile (**1a**) (1.23 g, 0.012 mol), 4-chlorobenzonitrile (**1b**) (1.64 g, 0.012 mol), or 3,4-dichlorobenzonitrile (**1c**) (2.05 g, 0.012 mol), in ethanol (10 mL), was added hydroxylamine hydrochloride (2.09 g, 0.03 mol) and triethyl amine (8 mL). The reaction mixture was maintained under reflux for 12 hours and the ethanol evaporated under vacuum. The mixture was poured in water (200 mL) and left to crystallize for 3h in refrigerator. The solid obtained was separated by vacuum filtration and dried at 55ºC.

Benzamidoxime (2a). 60% yield. EI-MS (70 eV) *m/z* (int. rel. %): 136 ($M^{+\bullet}$, 52), 103 (100).

4-Chlorobenzamidoxime (2b). 58% yield**.** Colorless crystals. Mp. 130-131ºC; lit. 128-130ºC (Ryu *et al*., 2001). ^{1}H NMR ($CDCl_3$): δ4.86 (*s*, 2H, NH_2), 7.37 (*d*, 2H, J = 8.7 Hz, H-3 and H-5), 7.57 (*d*, 2H, J = 8.7 Hz, H-2 and H-6). ^{13}C NMR ($CDCl_3$): δ127.2 (C-3 and C-5), 128.9 (C-2 and C-6), 130.9 (C-1), 136.1 (C-4), 151.0 (NH_2C=NOH). EI-MS (70 eV) *m/z* (int. rel. %): 170 ($M^{+\bullet}$, 90), 153 (100).

3,4-Dichlorobenzamidoxime (2c). 81% yield. Colorless crystals. Mp. 132-134ºC. EI-MS (70eV) *m/z* (int. rel. %): 203 ($M^{+\bullet}$, 64), 186 (100).

Synthesis of the 1,2,4-oxadiazoles 3a-e. Compounds **3a-e** were obtained from the reaction of the bezamidoximes **2a-c**, in pyridine, with an appropriated acid chloride (Figure 1). To a solution of benzamidoxime (**2a**) (0.817 g, 6.0 mmol); 4-chlorobenzamidoxime (**2b**) (1.02 g, 6.0 mmol); or 3,4-dichlorobenzamidoxime (**2c**) (1.22 g, 6.0 mmol), in pyridine (10 mL), the appropriated acid chloride was added (0.02 mol). The mixture was stirred at room temperature for 4 hours. The pyridine was removed under vacuum and the solid purified by column chromatography on silica gel with mixtures of hexane/ethyl acetate as solvent.

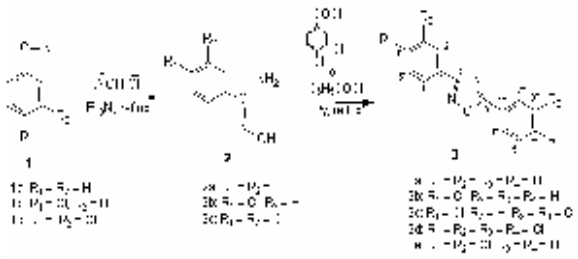

Figure 1. Synthetic route for preparation of the 1,2,4-oxadiazoles **3a-e**.

3,5-Bis-(phenyl)-1,2,4-oxadiazole (3a). 40% yield. Colorless crystals. Mp. 107-109ºC; lit. 107-108ºC (Molina *et al*., 1986). Characterized by comparison of its NMR data with those of literature (Poulain *et al*., 2001); ^{1}H NMR ($CDCl_3$): δ 7.47 – 8.20 (*m*, H aromatic). ^{13}C NMR ($CDCl_3$): δ168.9 (C-3), 175.6 (C-5), 126.9 (C-1'), 127.5 (C-2' and C-6'), 128.8 (C-3' and C-5'), 131.1 (C-4'), 124.2 (C-1"), 128.1 (C-2" and C-6"), 129.0 (C-3" and C-5"), 132.5 (C-4"). EI-MS (70eV) *m/z* (int. rel. %): 222 ($M^{+\bullet}$, 34), 119 (100, $[C_6H_5CNO]^{+\bullet}$), 103 (82, $[C_6H_5CN]^{+\bullet}$).

3-(4-Chlorophenyl)-5-(phenyl)-1,2,4-oxadiazole (3b). 40% yield. Colorless crystals. Mp. 108-110ºC; lit. 106-108ºC (Ryu *et al*., 2001). Characterized by comparison of its NMR data with those of literature (Ryu *et al*., 2001); ^{1}H NMR ($CDCl_3$): δ 8.12 (*d*, 2H, J =8.7 Hz, H-2' and 6'), 7.48 (*d*, 2H, J = 8.7 Hz, H-3' and H-5'), 8.20 (*dd*, 1H, J = 8.2 and 1.8 Hz, H-2" and H-6"), 7.56 – 7.66 (*m*, 3H, H-3", H-4" and H-5"). ^{13}C NMR ($CDCl_3$): δ168.1 (C-3), 175.9 (C-5), 125.5 (C-1'), 128.8 (C-2' and C-6'), 129.2 (C-3' and C-5'), 137.3 (C-4'), 124.1 (C-1"), 128.2 (C-2" and C-6"), 129.1 (C-3" and C-5"), 132.8 (C-4"). EI-MS (70eV) *m/z* (int. rel. %): 256 ($M^{+\bullet}$, 100), 151 (96, $[4\text{-}ClC_6H_4CN_2]^+$), 123 (47, $[4\text{-}ClC_6H_4C]^+$).

3-(4-Chlorophenyl)-5-(3,4-dichlorophenyl)-1,2,4-oxadiazole (3c). 50% yield. Colorless crystals. Mp: 151-153ºC. EI-MS (70eV) *m/z* (int. rel. %): 326 ($M^{+\bullet}$, 24), 171 (77, $[3,4\text{-di-}ClC_6H_3CN]^{+\bullet}$), 137 (100, $[4\text{-}ClC_6H_4C]^{+\bullet}$). ^{1}H and ^{13}C NMR data: See Table 1.

Table 1. ^{1}H and ^{13}C NMR data ($\delta CDCl_3$, 300 MHz) of the1,2,4-oxadiazoles **3c-e.**

	3c		3d		3e	
H/C	δ_H (mult. *J* in Hz)	δ_C	δ_H (mult. *J* in Hz)	δ_C	δ_H (mult. *J* in Hz)	δ_C
3	-	168.3	-	167.7	-	167.2
5	-	173.9	-	174.4	-	176.1
1'	-	125.0	-	126.5	-	126.9
2'	8.08 (d 8.7)	128.8	8.27 (d 2.0)	129.5	8.26 (d 1.8)	129.3
3'	7.48 (d 8.7)	129.2	-	133.6	-	133.3
4'	-	137.6	-	135.9	-	135.4
5'	7.48 (d 8.7)	129.2	7.60 (d 8.4)	131.2	7.56 (d 8.4)	130.9
6'	8.08 (d 8.7)	128.8	7.99 (dd 8.4 and 2.0)	126.6	7.98 (dd. 8.1 and 2.1)	126.5
1"	-	123.8	-	123.7	-	123.9
2"	8.28 (d 1.8)	129.9	8.32 (d 2.0)	130.1	8.18 (dd 8.4 and 1.4)	128.1
3"	-	133.9	-	134.1	7.58 – 7.66 (m)	129.1
4"	-	137.6	-	137.9	7.58 – 7.66 (m)	133.0
5"	7.64 (d 8.4)	131.3	7.66 (d 8.4)	131.5	7.58– 7.66 (m)	129.1
6"	8.02 (dd 8.4 and 2.1)	127.0	8.04 (dd 8.4 and 2.0)	127.2	8.18 (dd 8.4 and 1.4)	128.1

3-5-Bis-(3,4-dichlorophenyl)-1,2,4-oxadiazole (3d): 45% yield. Colorless crystals. Mp: 199-201°C. EI-MS (70 eV) *m/z* (int. rel. %): 360 ($M^{+\bullet}$, 100), 187 (31, [3,4-di-$ClC_6H_3CNO]^{+\bullet}$). 1H and 13C NMR data: See Table 1.

3-(3,4-Dichlorophenyl)-5-phenyl-1,2,4-oxadiazole (3e): 30% yield. Colorless crystals. Mp: 128-130°C. EI-MS (70eV) *m/z* (int. rel. %): 290 ($M^{+\bullet}$, 77), 187 (100, [3,4-di-$ClC_6H_3CNO]^{+\bullet}$). 1H and 13C NMR data: See Table 1.

Antimicrobial bioassay. The antimicrobial activity and the minimum inhibitory concentration (MIC) of the compounds **3a-e** were evaluated by the dilution method using Mueller-Hinton and RPMI-1640 medium for antibacterial and antifungal assays, respectively (NCCLS, 1997; 2000). The stock solution of each compound in DMSO was diluted to serial two-fold dilutions, which were added to each medium resulting in concentrations ranging 10 to 100 μg/mL. The following microorganisms were used for detecting antimicrobial activities: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 15442, *Bacillus subtilis* ATCC 6623, *Candida albicans*, *C. parapsilosis*, *C. krusei* and *C. tropicalis*. Micostatin, vancomycin, tetracycline and penicillin were used as reference antibiotics.

Brine shrimp bioassay. Brine shrimps (*Artemia salina*) eggs were placed, for hatching, in a side of a tank divided by a net and containing a 3% (w/v) sodium chloride solution. A light source was placed in the other side of the tank to attract the nauplii. After 48h ten nauplii were added to a series of vials containing different concentrations of the test compounds. The vials were maintained under light and after 24h the number of survivors was counted. The bioassays were carried out in triplicate and expressed with 95% confidence limit (Mc Laughlin, 1991).

Results and discussion

Synthesis and characterization of the oxadiazoles 3a-e

Although several methodologies have been reported for the synthesis of 1,2,4-oxadiazoles, the reaction of an amidoxime with an acid carboxylic derivative, a carbonic acid derivative or related species remains the most commonly employed methodology for the synthesis of 1,2,4-oxadiazoles (Hemming, 2001).

In this work, 1,2,4-oxadiazoles (**3a-e**) were prepared by *O*-acylation of the benzamidoximes (**2a-c**) with appropriated carboxylic acid chlorides, in pyridine, and cyclisation of the resulting *O*-acylbenzamidoximes as shown in Scheme 1. The benzamidoximes **2a-c** were available from the reaction of the nitriles **1a-c** with hydroxylamine hydrochloride, in triethyl amine (Deegan *et al.*, 1999). Nitriles **1b** and **1c** were obtained from the reaction of 4-chloro-benzotrichlorides or 4-chlorobenzoic acid or 3,4-dichlorobenzoyl chlorides with ammonium chloride.

The IR spectra of the compounds **3a-e** showed absorptions bands at 1606, 1555, 1490, 1410, 1360, 1090 and 740 cm^{-1}, consistent with the presence of an oxadiazole moiety and phenyl chlorinated groups (Ryu *et al.*, 2001). Molecular ion peaks ($M^{+\bullet}$) consistent with the expected structures and fragmentation characteristics for aryl-1, 2, 4-oxadiazoles were observed for all compounds in the mass spectra (Tyrkov *et al*., 2004). The formation of the 1,2,4-oxadiazole systems was confirmed by the signals at δ 167.2–168.7 and δ 173.9–176.1 in the ^{13}C NMR spectra, which were assigned to C-3 and C-5, respectively (Table 1). The ^{1}H and ^{13}C NMR data showed typical signals for di and tri-substituted aromatic groups containing the atom of chlorine. The signals for the carbons bearing chlorine atoms appear in the region of δ 132.8 – 137.6. The NMR data for the new compounds **3c-e** are presented in

Table 1. The chemical shifts assignments of the oxadiazoles **3a-e** were based on analysis of the correlations observed in the 1H-1H-COSY, HMQC and HMBC spectrum. From the correlations in 1H-1H-COSY spectra it was possible to confirm the connectivity between the aromatic hydrogens. The HMBC spectra showed correlations of the signals at δ 167.2 –168.7 (C-3) and at δ 173.9 – 176.1 (C-5) with those of H-2'/ H-6' and H-2"/ H-6", respectively. From the correlations of the signals for C-1' and C-1" with those of aromatic hydrogens it was possible to confirm the positions of the hydrogens and, consequently, of the chlorine atoms in the aromatic rings. These data, together with the ^{1}H-^{13}C correlations in the HMQC spectra permitted the chemical shifts assignments of the hydrogens and carbons, and to confirm the chlorine atoms positions in the aromatic rings for the synthesized oxadiazoles.

Biological assays

The antimicrobial bioassays showed that all compounds were inactive against the microorganisms tested with a MIC > 100 μg/mL.

The toxicities of the compounds **3a-e** were tested in *Artemia salina* larvae. The lethal dose (LD_{50}), which corresponds to the minimum concentration that causes 50% of the larvae mortality, was determined for all compounds. The results indicated that the oxadiazoles **3a** (LD_{50} = 2.13 μg/mL; 1.59-2.66 μg/mL, 95% confidence limits) and **3b** (LD_{50} = 4.57 μg/mL; 3.62-6.52 μg/mL, 95% confidence limits) exhibited significant toxicity. On the other hand, compounds **3c-e** were inactive (LD_{50} > 500 μ/mL), indicating that the increasing of the number of chlorine atoms did not result in increasing of the toxicity. The brine shrimp assay has been used as a convenient and rapid assay to discover new cytotoxic compounds. Thus, the oxadiazoles **3a** and **3b** are potential cytoxoxic agents and their activity on cancer cell lines will be further tested.

Conclusion

In the present work we have synthesized and evaluated the toxicity towards *Artemia salina* and the antimicrobial activity of 1,2,4-oxadiazoles **3a-e** containing phenylchlorinated substituents attached at C-3 and C-5. The oxadiazoles **3a** and **3b** exhibited significant toxicity towards *Artemia salina* with LD_{50} of 2.13 and 4.57 μg/mL, respectively. The antimicrobial bioassays showed that all compounds were inactive against the microorganisms tested with a MIC > 100 μg/mL.

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Received on July 20, 2005.
Accepted on November 23, 2005.