Original Article

Insignificant level of *in vitro* cytotoxicity, anti-rotavirus, antibacterial, and antifungal activities of *N*-alkylmaleamic acids

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Abstract

By reacting maleic anhydride with amines, we synthesized the derivatives *N*-ethyl, *N*-(2-ethylamine), *N*-piperidinyl, *N*-phenyl, and *N*-phenylhydrazinyl maleamic acids. The purity of these products was initially verified by melting range and the presence of only one spot observed by thin layer chromatography. The chemical structures of the obtained *N*-alkyl maleamic acids were confirmed through infrared (IR) and hydrogen and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectrometry. Due to the already proven pharmacological activity of maleimides, maleic anhydride and its *N*-alkyl maleamic acids were subjected to *in vitro* assays to observe antiviral (SA-11 rotavirus), antibacterial (*Escherichia coli, Staphylococcus aureus,* and *Bacillus cereus*), antifungal (*Colletotrichum musae, Fusarium solani* f. sp. *phaseoli, Fusarium solani* f. sp. *piperis* Alb., and *Penicillium* sp.), and antiprotozoal (*Trichomonas vaginalis*, *Giardia lamblia*, and *Entamoeba histolytica*) effects. To study the anti-rotavirus properties, firstly the 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) method was used to establish the median cytotoxicity concentration (CC_{50}) of the compounds, using MA-104 cell line. Under the experimental conditions used, cytotoxic, anti-rotavirus, antibacterial, and antifungal properties were not observed for these compounds.

Key words: Antibacterial activity, antiprotozoal activity, anti-rotavirus, *N*-alkyl maleamic acid, MA-104 cytotoxicity

INTRODUCTION

Due to the occurrence of new diseases and concomitant acquisition of bacterial and fungus resistance to the currently drugs, there it becomes necessary to discover more bioactive compounds.[1,2] Obtaining new bioactive compounds is mainly from natural products and/or by organic synthesis. At the present time, organic synthesis is responsible for about 75% of the available drugs. The search for more viable synthetic alternatives led researchers to propose different routes of synthesis for a single molecule. In most cases, the wanted substance is synthesized in a few steps, with a high purity grade, and in a higher yield as possible. The major concern still remains the search for the adequate route to reach a particular

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synthetic compound, followed by experiments to prove the biological activity.

Cechinel Filho *et al.*[3] conducted a review of the literature data on the chemical and therapeutic potential of cyclic imides, *N*-alkyl maleamic acids, and succinimides. Amine derivatives of succinic acid have shown analgesic and other biological activities.[4] The anti-spasm and analgesic effects of these compounds and the antimicrobial activity of *N*-alkyl maleimides against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and other pathogenic bacteria were also reported, together with the activity of *N*-akyl aryl maleimides against *Microsporum canis*, *Candida albicans*, *Penicilium* sp., and other pathogenic fungus.[3] The maleic anhydride is a derivative produced through the cyclization of maleic acid, an *Z* isomer of butenoic acid. The more stable isomer *E* is the fumaric acid, a natural dicarboxylic acid which is part of the natural processes of cellular energy production involved in the Krebs cycle, and is produced by human skin during exposure to sunlight.[3] The maleic anhydride reacts with adequate amines, including substituted amines, producing the corresponding *N*-alkyl maleamic acids with good yields.[3]

In order to promote the discovery of promising molecules, five *N*-alkyl derivatives of maleamic acid were obtained by reacting maleic anhydride (1) with ethylamine, ethylenodiamine, piperidine, phenylamine, and phenylhydrazine, in accordance with the processes previously reported.[5-8] From these reactions were synthesized the derivatives *N*-ethylmaleamic acid (2), *N*-(2-ethylamine)-maleamic acid (3), *N*-piperidinyl maleamic acid (4), *N*-phenylhydracinylmaleamic acid (5), and *N*-phenylmaleamic acid (6), respectively [Figure 1]. The main objective of these syntheses was to subject maleic anhydride (1) and its *N*-alkyl maleamic acids (2-5) [Figure 1] to *in vitro* assays against pathogenic rotavirus, bacteria, and protozoals which have been associated with high incidence of dysenteries and other gastrointestinal diseases mainly in developing countries. Among these microorganisms, rotavirus and *Es. coli* have been responsible for the deaths of over half a million people in poor countries. Although vaccination with rhesus-human rotavirus reassortant-tetravalent vaccine (RRV-TV) confers 60% efficacy during the first year, it has been suspended in some countries due to the incidence of undesirable side effects.[9,10] For this reason, it is important to find drugs that can help prevent and/or treat diseases induced by rotavirus.

Thus, compounds 1-6 were subjected to antibacterial assays against gram-negative *Es. coli* (ATCC 8739) and gram-positive *S. aureus* (ATCC 25923) and *B. cereus* (ATCC 11778).

In agriculture, the extensive cultivation of certain plants suffers from the action of spoilage fungi like *Colletotrichum musae* (BERK and CURT) Arx and

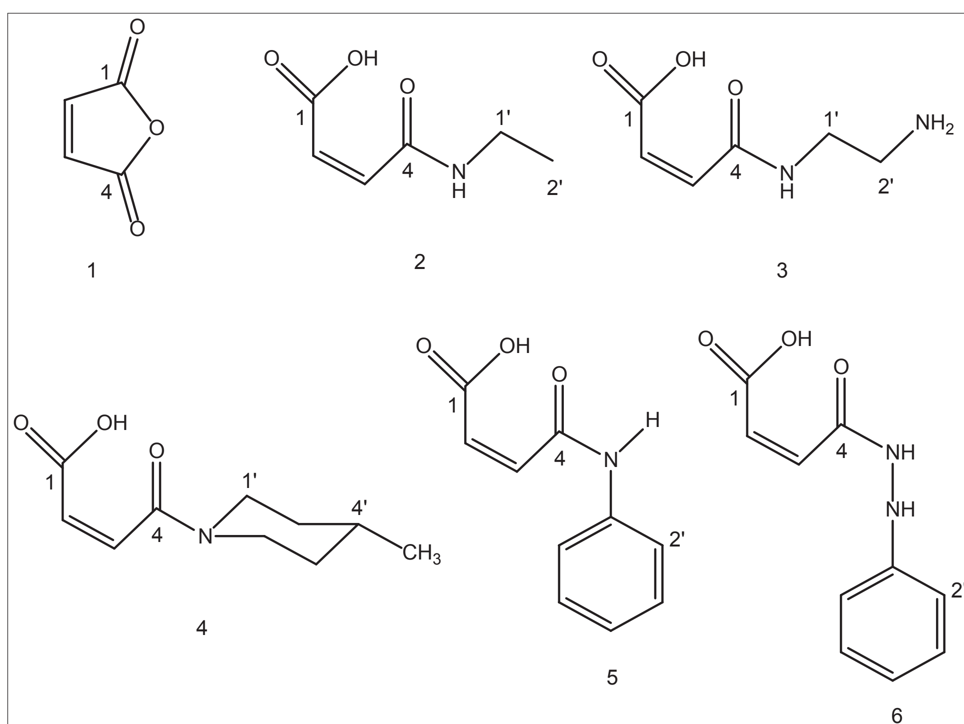

Figure 1: Chemical structures of maleic anhydride (1) and the *N*-alkyl maleamic acids 2-6

Penicillium sp., which are agents of post-harvest rot of banana and orange, respectively,[11] and *Fusarium solani* f. sp. *phaseoli* and *Fusarium solani* f. sp. *piperis* Alb., which are responsible for root rot of bean (*Phaseolus vulgaris*).[12] Due to the high incidence of this fungus in agriculture of Sâo Mateus City Espírito Santo, Brazil, the antifungal properties of compounds 1-6 were evaluated using wild strains of these phytopathogenic fungi.

Compounds 1-6 were also tested against *Trichomonas vaginalis*, *Giardia lamblia*, and *Entamoeba histolytica* that are microaerophilic protists able to cause vaginitis, diarrhea and dysentery, respectively. Metronidazole, a first-line drug for the treatment of infections induced by *En. histolytica, G. lamblia*, and *T. vaginalis*, is reported to have unpleasant side effects like a metallic taste, headache, and dry mouth, and to a lesser extent nausea, glossits, urticaria, pruritis, and dark-colored urine, and others.[13] Another problem is the incidence of metronidazole-resistant strains.[14] Thus, the search for new active compounds that can be used against these protists, which present better activity, lower toxicity and more effective, keep a necessary goal.

MATERIALS AND METHODS

Synthesis of *N*-alkyl maleamic acids

N-alkyl maleamic acids were prepared as follows. To a solution of maleic anhydride (**1**) (0.10 mol) in dried dieythyl ether (100) was slowly added; the amine (ethylamine, ethylenodiamine, piperidine, phenylamine, or phenylhydrazine) (0.11 mol) and dissolved in dry ether (25 mL). The reaction mixture was cooled in an ice-water bath with shaking for 2 h (a time sufficient to precipate the product), a time sufficient to precipitate the product. The *N*-alkyl maleamic acid (2-5) obtained was filtered in a Büchner funnel, washed with anhydrous ethyl ether, and stored in glass desiccators for further analysis. The purity of each *N*-alkyl maleamic acid was verified by melting range and the presence of only one spot observed by thin layer chromatography (TLC). The chemical structures of compounds 2–5 were confirmed through infrared (IR) and hydrogen and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectrometry.

Cells, virus, and stock solution

Five-passage adherent epithelial cell cultures of green monkey *Cercopithecus aethiops* kidney (cellular line MA-104 ATCC CRL-2378.1) and a rotavirus (RV) strain SA-11 were kindly donated by Erna Geessien Kroon of Universidade Federal de Minas Gerais, Brazil, and used for cytotoxicity and antiviral assays. Cells are prepared from healthy, confluent MA-104 cells that are maintained by passing every 5 ± 2 days in Dulbecco's modified eagle medium (DMEM Sigma-Aldrich®) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 50 mg gentamicin, 100 U penicillin, and 5 mg of amphotericin B. The culture was maintained at 37°C in a humidified atmosphere containing 5% CO_2. No cells were used over the 10th passage at any point. Prior to the beginning of the trials, MA-104 cells were tested negative for *Mycoplasma*.[15] This cell line was chosen due to its known susceptibility to SA-11 rotavirus.[16,17]

Maleic anhydride (1) and the *N*-alkyl maleamic acids 2-6 were respectively weighed (~10 mg) and dissolved in distilled water. The solution was subjected to filter sterilization using nitrocellulose membrane (pore size, 0.2 μm) and stored at 4°C in sterile vials. The prepared stock solutions were used throughout all assays.

Viral titration

A six-well plate was seeded 300 μl/well with rhesus monkey kidney cells (MA-104 epithelial). The cells were seeded at a density of 3×10^6 cells/well that would produce 90-100% confluence after 2 days of incubation in DMEM. Then, the medium was discarded and replaced by 200 μl of DMEM with 10 μg/ml of trypsin, without FBS, and containing a 10-fold dilution of the virus in each well. A well containing mock-infected cells was used as cell control. After 1 h incubation, the medium was replaced with an overlay of DMEM containing 0.7% agarose and 1.0 μg/ml of trypsin, followed by an incubation period of 5 days at 37°C in a humidified atmosphere containing 5% CO_2. Then, the cells were fixed with formaldehyde 10% (v/v) for 60 min, washed with distilled water, and stained with crystal violet 10%. The dilution that presented between 15 and 40 plaques was chosen to calculate the virus titer using the formula: Plaque Forming Units (PFU)/ml = $n \times CF \times 10^i$, where n = number of plaques, CF = correction factor (1000/volume used to infect with rotavirus), and i = inverse of the dilution factor.[18]

Cytotoxicity assay

After reaching about 90% confluence, but still at exponential growth phase, cells were detached from a 175-cm^2 vented cell culture flask and counted using 0.4% trypan blue stain. A 96 flat-bottom well plate was seeded with 4.0×10^4 MA-104 cells per well and maintained overnight for accession. After confirming that a confluent monolayer has been formed, the medium was discarded and 150 μl of DMEM without FBS and with twofold dilutions of compounds 1-6

was added in the respective well. Eight wells were left without any compound to serve as cell control and the other eight wells with cells treated only with DMSO 0.02% solution (130 μl/well) were used as solvent control. The cells were incubated for 72 h at 37°C under humidified atmosphere containing 5% CO_2. Cell viability was assayed by using 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) method.[19] The optical density at 570 nm ($OD_{570\ nm}$) was measured on STAT FAX 2100 Microplate Reader. Lower cell viability induces little reduction of the MTT and consequent lower optical density. All assays were realized in triplicate and the results expressed as a median, and were performed following ATCC's MTT cell proliferation assay previously reported.[20]

Antiviral assay

As described for the cytotoxicity assay, 4.0×10^4 cells were seeded per well in a 96 flat-bottom well plate. After a confluent monolayer was formed, the medium was replaced by 100 μl DMEM (1.0 μg/ml of trypsin, without FBS) with SA-11 rotavirus at a multiplicity of infection (MOI) of 2.0 (8). Immediately, 100 μl of DMEM (1.0 μg/ml of trypsin, without FBS) was added, which contained four serial dilutions of compounds **1-6**, respectively. The starting concentration was the previously determined CC_{50}. Eight wells were used for cell and for solvent (DMSO 0.02% solution) controls. The final MOI was 1.0. The plate was incubated for 3 days at 37°C in an atmosphere of 5.0% CO_2 with high humidity. The cell viability was evaluated following ATCC's MTT cell proliferation assay according to Twentyman and Luscombe.[20]

Antiprotozoals assay

Solutions of compounds 1-6 were diluted in YI-S medium[21] for *En. histolytica* and *T. vaginalis* or in TYI-S-33[22] for *G. lamblia* to a final volume of 6 ml and a final concentration of 100.0 μM. They were placed in individual vials containing axenic trophozoite cultures (2.4×10^5 *En. histolytica*, 6×10^4 *T. vaginalis*, and 1.2×10^5 *G. lamblia* inoculums) in log growth phase. The vials were incubated for 48 h at 37°C. All assays were performed in triplicate and repeated twice. Three vials were used as negative control (inoculum + medium) and three as positive control (metronidazole, Sigma-Aldrich).

Protozoans' viability was qualitatively measured using an inverted microscope Olympus IX70 to observe trophozoites' motility and adherence by comparing with the positive and negative controls.

Antibacterial assay

Assay of antibacterial property of compounds 1-6 was performed in 96-well plate using Mueller-Hinton broth microdilutions, in accordance with the procedures published by Clinical and Laboratory Standards Institute (CLSI).[23] The concentration of compounds 1-6 ranged from 100 to 0.39 μg/ml. Vancomycin and ceftriaxone were used as positive controls; there were also mock-infected wells to determine whether the samples were contaminated.

Compounds 1-6 were respectively added to the wells with gram-negative *Es. coli* (ATCC 8739), and gram-positive *S. aureus* (ATCC 25923) and *B. cereus* (ATCC 11778). The 96-well plate was incubated for 24 h at 35 ± 2°C and then analyzed to observe the antibacterial activity.

Antifungal assay

Antifungal property of compounds 1-6 was measured using Sabouraud dextrose broth and agar in accordance with the procedures published by CLSI.[23] The concentration of compounds 1-6 ranged from 1000 to 3.9 μg/ml. Ketoconazole (30.0 μg/ml) and itraconazole (30.0 μg/ml) were used as positive controls. Mock-infected wells were used as negative control. After incubation for 48-72 h at 27 ± 1°C, the results were obtained observing the wells through optical microscopy (Nikon, Model Eclipse E200).

Statistical analysis

Cytotoxicity was evaluated as the compound concentration that caused a 50% cell viability reduction (CC_{50}). For that, mock-infected cells' viability was considered 100%. Antiviral and antiprotozoal properties were evaluated as the concentration that led to 50% of growth inhibition (EC_{50}). Linear regression, obtained using the software GraphPad Prism® version 5.0, was used to establish the CC_{50} and EC_{50}.

RESULTS AND DISCUSSION

In the screening of drugs, the antiviral assays firstly have been realized *in vitro* based on the cytopathogenic effect (CPE) in cell culture and the activity is expressed by the 50% endpoint titration technique (EPTT). Recently, the colorimetric MTT assay, in which the MTT dye is reduced by viable cells, has been commonly used. This assay is semi-automated, rapid, requires only a small amount of test sample, and directly assesses cell viability.[19,24] Reduction of the tetrazolium by mitochondrial reductases of viable cells makes it possible to observe the antiviral action.

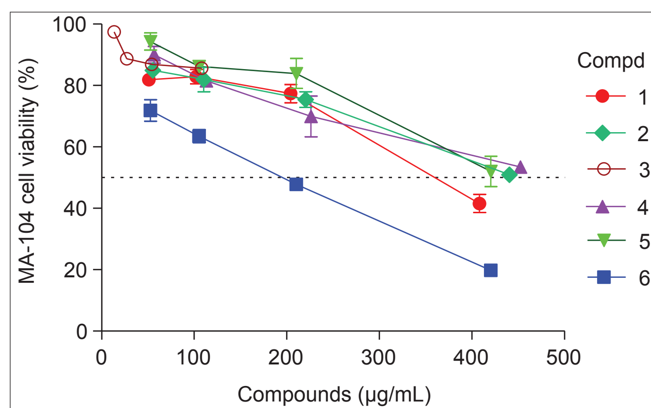

Figure 2: MA-104 cell viability in function of the treatment (µg/ml) with maleamic anhydride (1) and its *N*-alkyl maleamic acids (2-6)

Through the MTT method, the median cytotoxic concentration (CC_{50}) on MA-104 cell line was established for compounds 1 (476.4), 2 (471.6), 3 (378.7), 4 (375.3), 5 (440.3), and 6 (201.1 µg/ml) [Figure 2]. The median value of CC_{50} observed for treated cells was 390.5 µg/ml, the highest value being observed for maleic anhydride (1) (476.4 µg/ml) and the lowest for *N*-phenylmaleamic acid (6) (201.1 µg/ml).

In the antiviral assays MTT readings was observed that $OD_{570\,nm}$ of mock-infected cells were normal (above 0.8). All experimental wells showed lower optical density than the infected cells without any amine derivatives. Some of those presented $OD_{570\,nm}$ close to blank control (around 0.01), indicating near absence of cells.

The results of $OD_{570\,nm}$ observed for infected and untreated cells, infected cells, and for infected cells treated with different concentrations of compounds showed that the readings of optical density were around 0.01. The results indicate that maleic anhydride (1) and its *N*-acids (2-6) offer no protection against the cytopathic effect caused by the infection with rotavirus. For these compounds were not observed cytotoxic effects on MA-104 cells [Figure 2].

In all assays, the readings of $OD_{570\,nm}$ showed that both in infected untreated cells and the infected treated cells with compounds 1-6, the results were similar ($OD_{570\,nm} \leq 0.8$). The test results showed that antiviral substance has no detectable activity against rotavirus SA-11. For all concentrations of compounds 1-6, lower cell viability in the wells with cells, viruses, and substance was observed. Thus, the wells only with cells and virus were omitted from the data and graph related to this phase of the experiment, since the viability results from wells with virus and extracts showed $OD_{570\,nm}$ similar to referential background (wells where there was no cell). Then no analysis was applied to this situation. From the results it was observed that these compounds did not induce observable protection against the cytopathic effect triggered by rotavirus infection, through the protocols used in the assays.

In relation to antiprotozoal assay, after 48 h incubation period, compounds 1-6 presented median inhibition concentration (IC_{50}) greater than 100.0 µM. In comparison to metronidazole that presents activity against *En. histolytica* (1.26 µM), *G. lamblia* (1.96 µM), and *T. vaginalis* (0.068 µM), compounds 1-6 had no observable effects against these trophozoites, in the experimental conditions used in the assays.

In the antibacterial assays, vancomycin and ceftriaxone showed a minimum inhibition concentration (MIC) of 7.81 and 3.90 µg/ml, respectively. Using a range from 100 to 0.39 µg/ml, none of the compounds 1-6 induced some observable activity against the proliferation of *Es. coli* (ATCC 8739), *S. aureus* (ATCC 25923), or *B. cereus* (ATCC 11778). The positive controls ketoconazole and itraconazole exhibited an MIC of 7.81 µg/ml.

It was observed that compound 1, a cyclic anhydride, that presents the lowest molecular mass, showed the lowest cytotoxicity. Determining the cytotoxicity of compounds 1-6 was important for the anti-rotavirus assays. The results obtained revealed that there was no detectable anti-rotavirus property in any tested substance. Nevertheless, it does not mean that it is not worth to keep searching for antiviral activity as this study concentrated on a specific virus strain.

Compounds 1-6 showed no detectable effect against the bacteria, fungi, and parasites subjected to protocols used in this work.

Physical and chemical data

Maleic anhydride (1)

White solid. Melting range: 53-55°C. TLC: R_f 0.24 [diethyl ether-dichloromethane (2:3)]. IR: (KBr, cm^{-1}): 3500, 3330, 1700, 1660, 1600, 1550, 1500, 1390, 1365, 1220, 1080, 900, and 790. ^{1}H NMR (200 MHz, D_2O) δ_H: 4.71 (s, 4H, H-2, and H-3). ^{13}C NMR (50 MHz, D_2O) δ_C: 172.15 (C-1 and C-4) and 135.69 (C-2 and C-3).

N-ethylmaleamic acid (2)

White solid. Melting range: 212-215°C. TLC: Rf 0.32 [diethyl ether–dichloromethane (2:3)]. IR: IV (KBr) $\bar{\eta}_{max}$, cm^{-1}: 3400, 3030, 3000, 2960, 1700, 1640, 1580, 1420, 1310, 1260, 1220, 990, 900, 860, and 780. ^{1}H

NMR (200 MHz, MeOH) δ_H: δH: 1.29 (t, 3H, *J*2',1' = 7.2 Hz, H-2'), 2.99 (q, 2H, $J_{1'-2'}$=7.2 Hz, H-1′), 6.33 (d, 2H, H-2, and H-3), 6.76 (s, 2H, N-H, and O-H). ^{13}C NMR (50 MHz, MeOH) δ_C: 168.07 (C-4), 167.04 (C-1), 134.16 (C-3), 130.81 (C-2), 35.05 (C-1′), and 11.89 (C-2′).

N-(2-aminoethyl) maleamic acid (3)

Incolor solid. Melting range: 134-135°C. TLC: R_f = 0.29 [diethyl ether–dichloromethane (2:3)]. IR: (KBr, cm^{-1}): 3280, 3080, 2980, 1685, 1600, 1560, 1500, 1440, 1385, 1310, 1260, 1230, 1205, 1070, 950, 900, 840, 805, 800, 705, 620, and 600. ^{1}H NMR (400 MHz, MeOH) δ_H: 2.49 (t, 2H, NH_2), 3.28 (m, 2H, H-2′), 3.56 (m, 2H, H-1′), 6.02 (s, 1H, N-H), 6.21 (d, 1H, $J_{3,2}$ = 12.4 Hz, H-3), 6.35 (d, 1H, $J_{2,3}$ = 12.4 Hz, H-2), and 8.99 (s, 1H, O-H). ^{13}C NMR (100 MHz, MeOH) δ_C: 166.99 (C-4), 165.47 (C-1), 132.13 (C-3), 131.96 (C-2), 38.58 (C-1′), and 36.97 (C-2′).

N-(4-methylpiperidinyl) maleamic acid (4)

White solid. Melting range: 139-140°C. TLC: R_f 0.27 [diethyl ether–dichloromethane (2:3)]. IR (KBr) $\bar{n}_{max}$, cm^{-1}: 3500, 3350, 2960, 2880, 2860, 1730, 1670, 1605, 1505, 1480, 1450, 1390, 1360, 1250, 1200, 1080, 950, 900, 860, 805, and 780. ^{1}H NMR (200 MHz, DMSO) δ_H: 0.88 (d, 3H, $J_{1',3'}$=6.2 Hz, H-1′), 1.27 (m, 2H, H-4′), 1.61 (m, 1H, H-3′), 1.67 (m, 2H, H-2′), 2.80 (t, 2H, $J_{5',4'}$=12.6 Hz, H-5′), 3.22 (t, 2H, $J_{1',2'}$=12.6 Hz, H-1′), 3.62 (s, 2H, N-H, and O-H), 6.06 (d, 2H, H-2, and H-3). ^{13}C NMR (50 MHz, DMSO) δ_C: 167.80 (C-1 and C-4), 136.30 (C-2 and C-3), 43.44 (C-1′ and C-5′), 30.40 (C-2′and C-4′), 28.30 (C-3′), and 21.52 (C-1′).

N-phenylmaleamic acid (5)

White solid. Melting range: 199-200°C. TLC: R_f 0.35 [diethyl ether–dichloromethane (2:3)]. IR (KBr) $\bar{n}_{max}$, cm^{-1}: 3300, 3200, 3080, 3040, 2990, 1700, 1600, 1585, 1540, 1480, 1440, 1405, 1310, 1300, 1260, 1200, 1190, 1000, 980, 900, 840, 800, 750, 690, and 600. ^{1}H NMR (200 MHz, DMSO) δ_H: 6.31 (d, 1H, $J_{2',3'}$ =12.0 Hz, H-2), 6.49 (d, 1H, $J_{3',2'}$=12.0 Hz, H-3), 7.09 (t, 1H, $J_{4',3'}$= $J_{4',5'}$=7.4 Hz, H-4′), 7.33 (t, 2H, $J_{3',2'}$ = $J_{3',4'}$ = $J_{5',4'}$ = $J_{5',6'}$=7.4 Hz, H-3′ and H-5′), 7.63 (d, 2H, $J_{2',3'}$ = $J_{6'5'}$=7,4 Hz, H-2′, and H-6′), 10.40 (s, 2H, N-H, and O-H). ^{13}C NMR (50 MHz, DMSO) δ_C: 166.56 (C-1), 162.95 (C-4), 138.20 (C-1′), 131.41 (C-3′), 130.12 (C-2), 128.50 (C-3′ and C-5′), 123.59 (C-4′), and 119.24 (C-2′and C-6′).

Phenylhydrazinylmaleamic acid (6)

Yellowish solid. Melting range: 138-139°C. TLC: R_f 0.38 [diethyl ether–dichloromethane (2:3)]. IR (KBr) $\bar{n}_{max}$, cm^{-1}: 3260, 3030, 2990, 1690, 1600, 1590, 1540, 1510, 1490, 1400, 1310, 1300, 1230, 1210, 1100, 1030, 980, 900, 860, 750, 730, and 690. ^{1}H NMR (200 MHz, DMSO) δ_H: 6.08 (d, 1H, $J_{NH,NH}$ = 12.4 Hz, N-H), 6.27 (d, 1H, $J_{2,3}$ = 12.0 Hz, H-2), 6.41 (d, 1H, $J_{3,2}$ = 12.0 Hz, H-3), 6.57 (d, 1H, $J_{NH,NH}$ = 12.4 Hz, N-H), 6.71 (t, 1H, $J_{4',3'}$ = $J_{4',5'}$=7.4 Hz, H-4′), 6.77 (d, 2H, $J_{2',3'}$ = $J_{6',5'}$=7.4 Hz, H-2′, and H-6′), 7.13 (t, 2H, $J_{3',2'}$ = $J_{3',4'}$ = $J_{5',4'}$ = $J_{5',6'}$=7.4 Hz, H-3′ and H-5′), 7.91 (s, 1H, O-H). ^{13}C NMR (50 MHz, DMSO) δ_C: 169.93 (C-1), 163.34 (C-4), 148.34 (C-1′), 130.92 (C-3), 130.04 (C-2), 128.54 (C-3′ and C-5′), 118.71 (C-2′ and C-6′), and 112.27 (C-4′).

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