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SYNTHESIS AND ANTIMICROBIAL ACTIVITIES OF ELEVEN *N*-SUBSTITUTED MALEIMIDES

(Sintesis dan Kajian Antimikrob Terhadap Sebelas Terbitan Maleimida)

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Abstract

The antimicrobial activities of eleven maleimide derivatives were tested in this study. Out of the eleven samples, *N*-(4-fluorophenyl) maleimide, *N*-(4-chlorophenyl) maleimide and *N*-(4-bromophenyl) maleimide were synthesized by reacting maleic anhydride with 4-fluorophenylaniline, 4-chlorophenylaniline and 4-bromophenylaniline, respectively in the presence of acetic acid, by refluxing overnight. Crystals were successfully obtained after the products were recrystallized with different mixture of solvents. Their chemical structures were confirmed by infrared spectroscopy (IR), ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) and melting point determination. Subsequently, all the samples were screened for their biological activity using disc diffusion method. The bacteria chosen for this study were Gram-negative *Escherichia coli*, Gram-positive *Bacillus subtilis* and yeast *Saccharomyces cerevisae*. Positive control for bacteria was streptomycin and nystatin for the yeast. **M1** to **M6** compounds gave remarkable result at low concentrations whereas **M8, M9** and **M11** compounds are mostly inactive up to a high concentration. In contrast, the unsubstituted maleimide, **M7** was highly reactive towards both bacteria and yeast at low and high concentrations.

Keywords: *N*-substituted maleimide, antimicrobial, disc diffusion

Abstrak

Kajian antimikrob terhadap sebelas terbitan *N*-maleimida telah diuji dalam kajian ini. Tiga daripada sebelas sebatian maleimida yang diuji, iaitu *N*-(4-florofenil)maleimida, *N*-(4-klorofenil)maleimida dan *N*-(4-bromofenil)maleimida telah disintesis melalui tindakbalas maleik anhidrida dengan 4-florofenilanilina, 4-klorofenilanilina and 4-bromofenilanilina secara berasingan dalam kehadiran asid asetik, diikuti dengan refluks semalaman. Mendakan tidak berwarna telah diperolehi setelah penghabluran semula dilakukan ke atas hasil sintesis. Pencirian struktur sebatian dilakukan melalui kaedah spektroskopi Infra Merah (IR), ^{1}H dan ^{13}C Resonans Magnet Nukleus (NMR) dan penentuan takat lebur. Seterusnya, kajian antimikrob bagi kesemua sebelas sampel dilakukan melalui kaedah resapan cakera. Bakteria yang digunakan dalam kajian ini ialah *Escherichia coli* dan *Bacillus subtilis* manakala yis yang digunakan ialah *Saccharomyces cerevisae.* Kawalan positif untuk bakteria ialah *streptomisin* dan *nystatin* untuk yis. Sebatian **M1** ke **M6** mencatatkan keaktifan yang baik pada kepekatan rendah. Sebatian **M8**, **M9** dan **M11** kebanyakannya tidak menunjukkan keaktifan walaupun kepekatan dos ditingkatkan. Berbeza pula dengan sebatian **M7**, iaitu maleimida yang tidak mempunyai sebarang kumpulan gantian yang menunjukkan keaktifan yang tinggi terhadap kedua-dua bakteria dan yis pada kepekatan rendah dan tinggi.

Kata kunci: maleimida *N*-gantian, antimikrob, kaedah resapan cakera

Introduction

Microbe resistance has been a challenge for health practitioner and the pharmaceutical industry. The prescription of antibiotics are reported to be related to bacterial resistance. The most commonly found drug-resistant bacteria in hospitals are methicilin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* [1]. The resistance towards various drugs and medicines shown by bacteria and fungi has also become the main reason for failure in chemotherapy of infectious diseases. Therefore, the need for a novel anti microbe agent is highly desirable.

Recently, researchers are focusing on bacterial and fungal enzymes that are responsible for catalysing important biochemical reactions in microbe cells. Biologically active compounds capable of deactivating these enzymes and block the metabolic pathway of these bacteria and fungi selectively in human body are potential novel antimicrobial drugs [2]. Many biologically active compounds including most of the antibiotics are enzyme inhibitors. Compounds that are active towards the thiol group are able to inhibit cysteine protease and other proteins containing important cysteins. Maleimides for instance, portrayed the ability to inhibit cysteine protease and *N*-ethylmaleimide specifically, acts spontaneously and fast with the sulphydryl group.

Cyclic imides such as succinimides, maleimides, phthalimides and their derivatives have an imide ring and the –CO-N(R)-CO- structure that gives them neutrality and hydrophobicity. Previous studies have shown that various kinds of biological activities such as antibacterial, antifungal and antitumor are related to these compounds and their derivatives as shown in Table 1 [3]. Maleimide compounds in specific have shown antifungal and antibacterial properties [4], ability to inhibit Protein Kinase C and antitumor property [5], and analgesic activity [6]. Li research group mentioned about the antifungal activity of simple compounds with dimethylmaleimide structure against *Botrytis cinerea,* which revealed that natural products containing maleimide structure has high biological activity while non-natural products having maleimide structure also shows antifungal activity [7].

Table 1. Antimicrobial activity of *N*-substituted maleimide

Types of Maleimide	**Types of Microbe**		
	E. coli	***B. subtilis***	***S. cerevisiae***
N-(4-chlorophenyl) maleimide	-	-	MIC: 3.90 µg/mL [10]
N-(4-florophenyl) maleimide	-	-	MIC: 3.90 µg/mL [10]
N-(4-bromophenyl) maleimide	MIC: 128 µg/mL [2]	MIC: 64 µg/mL [2]	MIC: 1.0 µg/mL [2]
			MIC: 7.81 µg/mL [10]
N-(2-methylphenyl) maleimide	-	-	MIC: 7.81 µg/mL [10]
N-(4-methylphenyl) maleimide	-	-	MIC: 3.90 µg/mL [10]
N-(*tert*-butyl) maleimide	MIC: 32 µg/mL [2]	MIC: 2.0 µg/mL [2]	MIC: 2.0 µg/mL [2]
N-methylmaleimide	-	-	MIC: 7.81 µg/mL [2]

Studies have also reported that some of the maleimide derivatives that is biologically active is neither haemolytic nor cytotoxic towards the human cancer cell, which indicated the selectivity of action of maleimides towards fungal pathogen [8]. Zentz et al. has reported that *N*-substituted maleimides, *N*-(*tert*-butyl)maleimide in particular shows antibacterial properties towards *Escherichia coli*. [9].

Maleimide compounds are Michael acceptors prone to be attacked at the double bond in the maleimide ring, by nucleophilic species such as the thiol group as shown in Figure 1. The maleimides react specifically on biological molecules containing thiol group and form stable conjugate protein through thioether bond [11]. The thioether bond formed has strong C-S bond which is often irreversible and cannot be reduced by reducing agents. This reaction takes place specifically at pH 6.5 – 7.5. in alkaline condition when pH exceeds 8.5, hydrolysis of maleimide to maleimic acid is prone to take place [12].

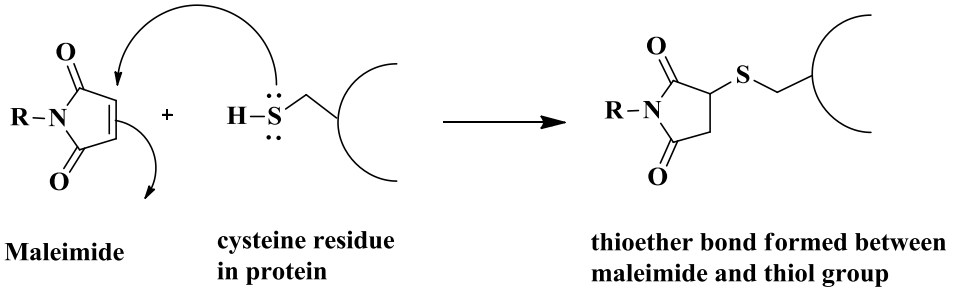


Figure 1. Reaction between maleimide and thiol group

Many types of active protein and enzymes can be found on bacteria cell membrane. When these enzymes react with foreign substances such as drugs, their enzyme activity will change and subsequently inhibit its growth [13].

In this study, we focused on *N*-substituted maleimides, which refer to any maleimide compounds that has substituent groups such as alkyl or halides attached to the nitrogen atom in the maleimide ring. Since biological activitiy of each maleimide derivatives varies from one another, the structure activity relationship display distinct properties that will affect the reactivity of different maleimide compounds. Some of the factors responsible for the different reactivities of maleimide compounds are the presence of aryl group on the nitrogen atom in the maleimide ring [5], the length of alkyl chain on the alkylphenyl group attached to the nitrogen atom in the maleimide ring [7], the lipophilicity and bulkiness of the compound molecule [2] and the presence of any substituents on the maleimide ring [14].

In view of this, 11 samples of *N*-substituted maleimides (**M1** to **M11**) have been tested on three different microbes, namely *Escherichia coli* (Gram negative), *Bacillus subtilis* (Gram positive) and *Saccharomyces cerevisiae* through the disc diffusion method to study the inhibition ability of these samples.

Materials and Methods

Synthesis of *N*-(2-methylphenyl)maleimide (M1)

Maleic anhydride (44.0 mmol) was reacted with *o*-toluidine (42.3 mmol) by dissolving the mixture in THF (50 mL). The reaction mixture was stirred overnight at room temperature (RT) under nitrogen atmosphere. The precipitate formed was filtered and used in next reaction without further purification. The reaction mixture (1.71 mmol) was suspended in dry acetonitrile (40 mL) before the addition of zinc bromide (1.71 mmol) and HMDS (8.55 mmol) and heated at 90 °C for 2 hours before it was allowed to cool to RT and filtered. Subsequently, water (25 mL) was added and acidified with 1M HCl until pH 1. The mixture was washed with ethyl acetate (3 x 50 mL) and the organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo* to obtain black solution. The solution was recrystallized to yield colourless crystalline solid (35%).

Synthesis of N-(3-methylphenyl)maleimide (M2)

Maleic anhydride (44.0 mmol) was reacted with *m*-toluidine (42.3 mmol) by dissolving the mixture in THF (50 mL). The reaction mixture was stirred overnight at RT under nitrogen atmosphere. The precipitate formed was filtered and used in next reaction without further purification. The reaction mixture (1.71 mmol) was suspended in dry acetonitrile (40 mL) before the addition of zinc bromide (1.71 mmol) and HMDS (8.55 mmol) and heated at 90 °C for 2 hours before it was allowed to cool to RT and filtered. Subsequently, water (25 mL) was added and acidified with 1M HCl until pH 1. The mixture was washed with ethyl acetate (3 x 50 mL) and the organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo* to yield yellowish crystalline solid (28%).

Synthesis of *N*-(4-methylphenyl)maleimide (M3)
Maleic anhydride (44.0 mmol) was reacted with *p*-toluidine (42.3 mmol) by dissolving the mixture in THF (50 mL). The reaction mixture was stirred overnight at RT under nitrogen atmosphere. The precipitate formed was filtered and used in next reaction without further purification. The reaction mixture (1.71 mmol) was suspended in dry acetonitrile (40 mL) before the addition of zinc bromide (1.71 mmol) and HMDS (8.55 mmol) and heated at 90 °C for 2 hours before it was allowed to cool to RT and filtered. Subsequently, water (25 mL) was added and acidified with 1M HCl until pH 1. The mixture was washed with ethyl acetate (3 x 50 mL) and the organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo* to obtain yellow powder. The solution was recrystallized to yield yellow needle-like crystalline solid (84%).

Synthesis of *N*-(4-florophenyl)maleimide (M4)
Maleic anhydride (9.0 mmol) was reacted with equimolar of 4-fluoroaniline by dissolving the mixture in 50 mL acetic acid at RT. The reactants are stirred for 6.5 hours. The resulting suspension was refluxed overnight for 17.5 hours at 130 °C and then cooled to room temperature. The resulting solution of maleimide was extracted with ethyl acetate (3 x 50 mL). Extracts were combined and recrystallized (40%).

Synthesis of *N*-(4-chlorophenyl)maleimide (M5)
Maleic anhydride (7.84 mmol) was reacted with equimolar of 4-chloroaniline by dissolving the mixture in 50 mL acetic acid at RT. The reactants are stirred for 5.5 hours at 130 °C and then cooled to room temperature. The resulting solution of maleimide was extracted with ethyl acetate (3 x 50 mL). Extracts were combined and recrystallized (45%).

Synthesis of *N*-(4-bromophenyl)maleimide (M6)
Maleic anhydride (5.81 mmol) was reacted with equimolar of 4-bromoaniline by dissolving the mixture in 50 mL acetic acid at RT.The reactants are stirred for 5.5 hours at 130 °C and then cooled to room temperature. The resulting solution of maleimide was extracted with ethyl acetate (3 x 50 mL). Extracts were combined and recrystallized (51%).

Synthesis of 1-(3,5-bis(trifloromethyl)phenyl)-3-(4-(3-(2,5-dioxo-2H-pirol-1(5H)-yl)phenetyl)phenyl)urea (M8)
4'-ethylene dianiline (1.08 g, 5.1 mmol) in dry CH_2Cl_2 (20 mL) was slowly reacted with 3,5-dimethyl phenylisocyanate (0.5 g, 3.4 mmol) at −5°C and allowed to stir at ambient temperature for 16 hours. The solvent was removed under reduced pressure. The residue was then purified by column chromatography (CH_2Cl_2:EtOAc, 8:1). The product was obtained as yellow solid was dissolved in THF (10 mL) before reacted with maleic anhydride (82 mg, 0.84 mmol). The reaction mixture was stirred at ambient temperature under a Ar atmosphere for 3 hours. The precipitate was filtered and the compound obtained (460 mg, 98%) was carried out to the next step without further purification. The acid derivative (0.20 g, 0.35 mmol) was dissolved in dry CH_3CN (10 mL) and refluxed for 1 hour after the subsequent addition of $ZnBr_2$ (80 mg, 0.35 mmol) and hexamethyldisilazone (0.38 mL, 1.77 mmol). The reaction mixture was filtered and the filtrate was reduced to 10%. The solution was acidified with 0.5M HCI until pH 1 and extracted into CH_2CI_2. The organic layer were combined and dried over $MgSO_4$. The solvent was evaporated *in vacuo* to give the maleimide **M8** as yellow solid (78%).

Synthesis of 1-(4-(4-(2,5-dioxo-2H-pirol-1(5H)-yl)phenetyl)phenyl)-3-(3,5-dimethylphenyl)urea (M9)
A solution of 3,5-dimethylbenzoic acid (2.0 g, 13.3 mmol) in toluene (50 mL) and thionyl chloride (1.1 mL, 14.6 mmol) was heated to reflux at 80 °C for 16 h. The excess thionyl chloride was distilled off by the azeotropic distillation with toluene under reduced pressure, affording 3,5-dimethylbenzoyl chloride as brownish oil quantitatively. The product was used in the next step without further purification. The acid derivative (2.24 g, 13.3 mmol) in dry CH2Cl2 (20 mL) was added dropwise over 1.5 h to a solution of 4,4'-ethylenedianiline (6.20 g, 29.2 mmol) and Et_3N (3.7 mL, 26.6 mmol) in dry CH_2Cl_2 (50 mL) at ambient temperature under N_2 atmosphere. The reaction mixture was stirred at ambient temperature for 16 hours. The organic phase was washed successively with sat. solution of $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated by evaporation under reduced pressure. The residue was purified by column chromatography (Hexane:EtOAc, 2:1), affording a yellow solid. The solid (0.5 g, 1.45 mmol) was treated with maleic anhydride (0.14 g, 1.45 mmol) in THF (15 mL) and stirred at ambient

temperature under N_2 atmosphere for 3 hours. The yellow precipitate (66%) was filtered and carried out to the next step without further purification. The acid derivative obtained (0.34 g, 0.78 mmol) was dissolved in dry CH_3CN (20 mL) and refluxed for 2 h after the subsequent addition of $ZnBr_2$ (0.18 g, 10.78 mmol) and hexamethyldisilazone (0.83 mL,3.91 mmol). The reaction mixture was filtered and the filtrate was reduced to 10%. The solution was acidified with 0.5M HCl until pH 1 and extracted into CH_2Cl_2. The organic layers were combined and dried over $MgSO_4$. The solvent was evaporated to give the desired product as yellow crystalline solid (96%).

Synthesis of 2-(4-(2,5-dioxo-2H-pirol-1(5H)-yl)phenyll) acetic acid (M10)
Maleic anhydride (6.05 g, 40 mmol, 1 eq) and 4-aminophenyl acetic acid (3.92 g, 40 mmol, 1 eq) were dissolved in acetic acid (200 mL) and stirred under N_2 at RT for 2 hours before refluxing for 2 hours. The solvent was then evaporated under vacuum and the residue was dissolved in 5% acetic acid in CH_2Cl_2. The solution was passed through a plug of silica eluted with 5% acetic acid in CH_2Cl_2. The fractions containing product as identified by tlc were combined and the solvent removed under vacuum. The residue was then purified by recrystallisation by using chloroform to yield the product as a yellow powder (36%).

Synthesis of 3,5-dimethyl-N-(4-(2,5-dioxo-2H-pirol-1(5H)-yl)phenyl)benzamide (M11)
Cyanuric fluoride (0.85 mL, 10.1 mmol) was added dropwise to a solution of 3,5-dimethylbenzoic acid (1.52 g, 10.1 mmol) and dry pyridine (0.82 mL, 10.1 mmol) in dry CH_2Cl_2 (40 mL) at 0 °C under a N_2 atmosphere. The reaction mixture was stirred at 0 °C for 20 min and then at ambient temperature for a further hour. The reaction mixture was diluted with CH_2Cl_2. The organic layer was washed with saturated NaCl solution, dried ($MgSO_4$) and the solvent was removed by evaporation under reduced pressure to afford a yellow solid (100%). The solid (1.52 g, 10.0 mmol) was then dissolved in dry CH_2Cl_2 (40 mL) was added dropwise (over 4 h) to a solution of 1,4phenylenediamine (3.26 g, 30.1 mmol) in dry CH_2Cl_2 (60 mL), under a N_2 atmosphere. The reaction mixture was stirred at ambient temperature overnight, and then diluted with CH_2Cl_2. The organic layer was washed successively with saturated $NaHCO_3$ solution and saturated NaCl solution, dried ($MgSO_4$) and concentrated by evaporation under reduced pressure. The residue was purified by column chromatography (SiO_2, gradient from 100:0 to 95:5 CH_2Cl_2/MeOH) to give a yellow solid (80%). The solid (0.95g, 3.95 mmol) obtained was dissolved in acetic acid (70 mL) to react with maleic anhydride (388 mg, 3.96 mmol). The reaction mixture was stirred at ambient temperature under a N_2 atmosphere for 6 h, and then heated to reflux for 16 h. The solvent was removed by evaporation under reduced pressure. The residue was purified by column chromatography (SiO2, gradient from 100:0 to 98:2 $CHCl_3$/MeOH) to give a yellow solid (66%).

Antimicrobial screening
All samples were tested using disc diffusion method against *Escherichia coli* (Gram negative), *Bacillus subtilis* (Gram positive) and *Saccharomyces cerevisiae*. The samples were prepared in two concentrations, 1.0 µg/µL (10 µg/disc) and 2.0 µg/µL (20 µg/disc) by dissolving the solid samples in appropriate solvent. The positive control for the bacteria was streptomycin at 10 µg/disc while nystatin at 20 µg/disc was used as positive control for the yeast. Nutrient agar and potato dextrose agar were prepared by dissolving the premixed powder in water following with heating and auto-clave. The dissolved solution was then poured on petri dishes, allowed to dry and ready to be used. The bacteria cultures were then spread on the surface of the Nutrient Agar (NA) and yeast cultures on Potato Dextrose Agar (PDA). Subsequently, the discs were impregnated with (10 µg/disc) and (20 µg/disc) test samples and then placed on the surface of the agar media. Experimental plates of bacteria cultures were incubated at 37 °C for 18 to 21 hours whereas the experimental plates of yeast cultures were incubated for 48 hours at 30 °C. The antimicrobial activity was the determined by measuring the diameter of inhibition zone (IZ) and compared with positive control.

Results and Discussion

Characterization study: N-(2-methylphenyl)maleimide (M1)
M.p. = 150.6-150.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $^\delta$H 7.34 (1H, d, *J* = 6.20 Hz, Ar*H*), 7.33 (1H, d, *J* = 6.24 Hz, Ar*H*) , 7.29 (1H, d, *J* = 7.72 Hz, Ar*H*), 7.10 (1H, d, *J* = 7.68 Hz, Ar*H*), 6.85 (2H, s, Ar*H*), 2.15 (3H, s, CH_3); ^{13}C NMR (100 MHz; $CDCl_3$) $^\delta$C 169.7 (*C*=O), 136.6 (*C*=*C*), 134.5 (q, 131.3 (Ar*C*), 130.0 (Ar*C*), 129.5 (Ar*C*), 128.8 (Ar*C*), 126.9 (q, 17.9 (*C*H_3); MS (EI) m/z 187.2 (M^+, 100 %); IR ν_{max} (KBr) cm-1: 2929.45 cm^{-1} (sp^3 CH), 1702.31 cm^{-1} (C=O), 1029.23 cm^{-1} (C-N).

N-(3-methylphenyl)maleimide (M2)
^{1}H NMR (400 MHz, $CDCl_3$): $^{\delta}$H 7.34 (1H, t, *J* = 7.70 Hz, Ar*H*), 7.17 (1H, d, *J* = 7.72 Hz, Ar*H*), 7.11 (1H, d, *J* = 8.44 Hz, Ar*H*), 7.11 (1H, d, *J*= 8.44 Hz, Ar*H*), 6.80 (2H, s, Ar*H*), 2.37 (3H, s, CH_3); ^{13}C NMR (100 MHz; $CDCl_3$) $^{\delta}$C 169.7 (*C*=O), 139.3 (Ar*C*), 134.2 (q, 131.1 (Ar*C*), 129.0 (Ar*C*), 128.9 (Ar*C*), 126.9 (q, 123.4 (Ar*C*), 21.4 (*C*H_3); MS (EI) m/z 187.2 (M^+, 100 %); IR ν_{max} (KBr) cm-1: 2922.57 cm^{-1} (sp^3 CH), 1705.59 cm^{-1} (C=O), 1033.45 cm^{-1} (C-N).

***N*-(4-methylphenyl)maleimide (M3)**
M.p. = 150.5-150.7 °C; ^{1}H NMR (400 MHz, d-Acetone): $^{\delta}$H 7.27 (2H, d, *J* = 8.08 Hz, Ar*H*), 7.22 (2H, d, *J* = 8.44 Hz, Ar*H*), 7.00 (2H, s, Ar*H*), 2.35 (3H, s, CH_3); ^{13}C NMR (100 MHz; d-Acetone) $^{\delta}$C 169.8 (*C*=O), 137.4 (Ar*C*), 134.4 (q, 129.5 (Ar*C*), 129.3 (Ar*C*), 126.5 (q, 20.0 (*C*H_3); MS (EI) m/z 187.1 (M^+, 100 %); IR ν_{max} (KBr) cm-1: 3450.67 cm^{-1} (OH), 2967.11 cm^{-1} (sp^3 CH), 1702.41 cm^{-1} (C=O), 1041.54 cm^{-1} (C-N).

***N*-(4-florophenyl)maleimide (M4)**
M.p. = 151.8 –153.2 °C; ^{1}H NMR (400.1 MHz, d_6-Acetone): $^{\delta}$H 7.45-7.39 (2H, m; 2xAr*H*), 7.28-7.22 (2H, m, 2×Ar*H*), 7.03 (2H, s, Ar*H*); ^{13}C NMR (100.6 MHz, d_6-DMSO): $^{\delta}$C 169.7 (*C*=O), 161.6 (*C*F), 134.5 (Ar*C*H), 128.7 (Ar*C*H), 128.3 (Ar*C*-N), 115.6 (Ar*C*H).

***N*-(4-chlorophenyl)maleimide (M5)**
M.p. = 106.7 –108.5 °C; ^{1}H NMR (400.1 MHz, d_6-Acetone): $^{\delta}$H 7.53-7.51 (2H, m; 2xAr*H*), 7.43-7.41 (2H, m, 2×Ar*H*), 7.04 (2H, s, Ar*H*); ^{13}C NMR (100.6 MHz, d_6-DMSO): $^{\delta}$C 169.4 (*C*=O), 134.6 (Ar*C*H), 132.6 (Ar*C*-N), 131.1 (Ar*C*-Cl), 128.9 (Ar*C*H), 128.0 (Ar*C*H); ^{19}F NMR (376.5 MHz, d_6-DMSO) : $^{\delta}$F 115.6 (Ar*C*-F).

***N*-(4-bromophenyl)maleimide (M6)**
M.p. = 101.5 –103.9 °C; ^{1}H NMR (400.1 MHz, d_6-Acetone): $^{\delta}$H 7.68-7.65 (2H, m; 2xAr*H*), 7.38-7.35 (2H, m, 2×Ar*H*), 7.04 (2H, s, Ar*H*); ^{13}C NMR (100.6 MHz, d_6-DMSO): $^{\delta}$C 169.4 (*C*=O), 134.6 (Ar*C*H), 131.9 (Ar*C*H), 131.5 (Ar*C*-N), 128.3 (Ar*C*H), 120.6 (*C*-Br).

1-(3,5-bis(trifloromethyl)phenyl)-3-(4-(3-(2,5-dioxo-2H-pirol-1(5H)-yl)phenetyl)phenyl)urea (M8)
M.p. = 205.3 – 208.3 °C; ^{1}H NMR (300.1 MHz, d_6-DMSO) : $^{\delta}$H 9.36 (1H, s; N*H*), 8.89 (1H, s, N*H*), 8.13 (2H, s; 2×Ar*H*), 7.61 (1H, s; Ar*H*), 7.39 (2H, d, $^{3}J_{HH}$ 8.4 Hz; 2×Ar*H*), 7.33 (2H, d, $^{3}J_{HH}$ 8.4 Hz; 2×Ar*H*), 7.22 (2H, d, $^{3}J_{HH}$ 8.4 Hz; 2×Ar*H*), 7.18 (2H, d, $^{3}J_{HH}$ 8.4 Hz; 2×Ar*H*), 7.16 (2H, s; 2×C*H*), 2.94 – 2.82 (4H, m; 2×CH_2); ^{13}C NMR (75.5 MHz, d_6-DMSO): $^{\delta}$C 170.0 (*C*=O), 152.4 (*C*=O), 142.0 (Ar*C*), 141.3 (Ar*C*), 136.8 (Ar*C*), 135.5 (Ar*C*), 134.6 (Ar*C*H), 130.7 (q, $^{2}J_{CF}$ 32.7 Hz, Ar*C*), 129.3 (Ar*C*), 128.8 (Ar*C*H), 128.7 (Ar*C*H), 126.6 (*C*H), 123.3 (q, $^{1}J_{CF}$ 272.3 Hz, CF_3), 119.0 (Ar*C*H), 117.8 (m; Ar*C*H), 114.2 (m, Ar*C*H), 36.7 (*C*H_2), 36.3 (*C*H_2) ; ^{19}F NMR (376.5 MHz, d_6-DMSO) : $^{\delta}$F −62.2 (ArC*F*$_3$); MS (ES^-) *m/z* 546 ([M−H]$^-$, 100); HRMS (ES^-) *m/z* calculated for $C_{27}H_{18}N_3O_3F_6$ [M−H]$^-$ 546.1252, found 546.1257.

1-(4-(4-(2,5-dioxo-2H-pirol-1(5H)-yl)phenetyl)phenyl)-3-(3,5-dimethylphenyl)urea (M9)
M.p. = 229.3 –231.4 °C; ^{1}H NMR (400.1 MHz, $CDCl_3$): $^{\delta}$H 7.81 (1H, s; N*H*), 7.57 – 7.54 (2H, m; 2×Ar*H*), 7.46 (2H, s; 2×Ar*H*), 7.29 – 7.27 (2H, m; 2×Ar*H*), 7.25 – 7.22 (2H, m; 2×Ar*H*), 7.19 – 7.17 (3H, m; 3×Ar*H*), 6.84 (2H, s; 2×C*H*), 2.97 – 2.89 (4H, m; 2×CH_2),2.38 (6H, s; 2×CH_3); ^{13}C NMR (100.6 MHz, $CDCl_3$): $^{\delta}$C 169.8 (*C*=O), 166.1 (*C*=O), 141.8 (Ar*C*), 138.6 (Ar*C*), 137.7 (Ar*C*), 136.2 (Ar*C*), 135.2 (Ar*C*), 134.3 (*C*H), 133.5 (Ar*C*H), 129.4 (Ar*C*H), 129.2 (Ar*C*), 129.1 (Ar*C*H), 126.1 (Ar*C*H), 124.9 (Ar*C*H), 120.4 (Ar*C*H), 37.7 (*C*H_2), 37.3 (*C*H_2), 21.4 (*C*H_3); MS (ES^+) *m/z* 447 ([M+Na]$^+$, 75), 479 (100); HRMS (ES^+) *m/z* calculated for $C_{27}H_{24}N_2O_3Na$ [M+Na]$^+$ 447.1685, found 447.1674.

2-(4-(2,5-dioxo-2H-pirol-1(5H)-yl)phenyll) acetic acid (M10)
M.p. = 160.0 –162.0 °C; ^{1}H NMR (400.1 MHz, d_6-DMSO): $^{\delta}$H 12.07 (1H, s, CO_2*H*), 7.36 (2H, d, $^{3}J_{HH}$ = 8.5 Hz, 2×Ar*H*), 7.26 (2H, d, $^{3}J_{HH}$ = 8.5 Hz 2×Ar*H*), 7.18 (2H, s, 2×C*H*), 3.63 (2H, s, CH_2); ^{13}C NMR (100.6 MHz, d_6-DMSO): $^{\delta}$C 172.5 (CO_2H), 169.3 (*C*=O), 134.7 (Ar*C*-N), 134.6 (*C*H), 130.0 (Ar*C*), 129.9 (Ar*C*H), 126.6 (Ar*C*H),

40.1 (*C*H_2); MS (EI^+) *m/z* 254 ($[M+Na]^+$, 100); HRMS (EI^+) *m/z* calculated for $C_{12}H_9NO_4$ $[M+Na]^+$ 254.0429, found 254.0434.

3,5-dimethyl-N-(4-(2,5-dioxo-2H-pirol-1(5H)-yl)phenyl)benzamide (M11)
^{1}H NMR (300.1 MHz, $CDCl_3$): $^{\delta}$H = 7.86 (1H, s, NH), 7.79–7.74 (2H, m, ArH), 7.47 (2H, s, ArH), 7.37–7.32 (2H, m, ArH), 7.19 (1H, s, ArH), 6.85 (2H, s, ArH), 2.39 (6H, d, J = 0.5 Hz, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): $^{\delta}$C = 169.7 (*C*=O), 166.2 (*C*ONH), 138.8 (Ar*C*), 137.9 (Ar*C*), 134.8 (Ar*C*), 134.4 (Ar*C*H), 133.8 (Ar*C*H), 127.2 (Ar*C*), 126.9 (Ar*C*H), 124.9 (Ar*C*H), 120.6 (Ar*C*H), 21.4 (*C*H_3); MS (ES^+): m/z (%) = 321 ($[M+H]^+$, 100), 375 (70); HRMS (ES^+): m/z calc. for $[M+H]^+$ $C_{19}H_{17}N_2O_3$ 321.1239, found 321.1237.

The structure of the 11 maleimide compounds as shown in the following Table 2.

Table 2. Structure of eleven *N*-substituted maleimides

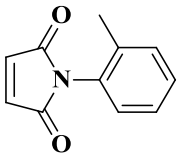
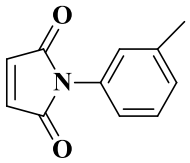
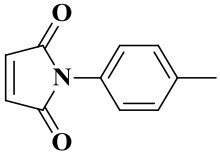
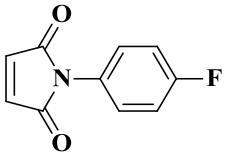
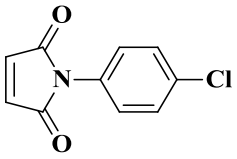
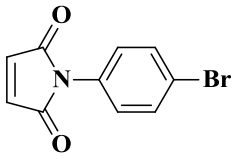
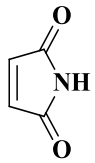
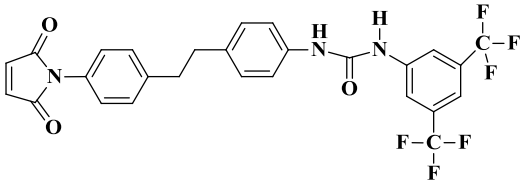
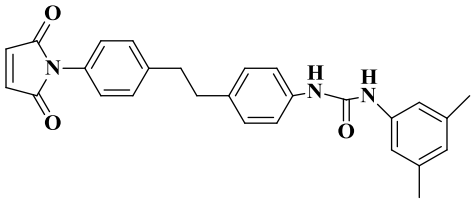
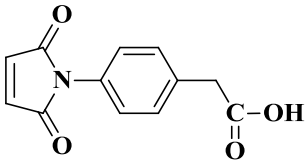
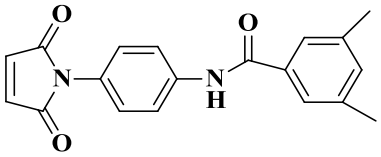
	Name of Compound	Structure	Yield (%)
M1	*N*-(2-methylphenyl)maleimide		35%
M2	*N*-(3- methylphenyl)maleimide		28%
M3	*N*-(4- methylphenyl)maleimide		84%
M4	*N*-(4-florophenyl)maleimide		40%
M5	*N*-(4-chlorophenyl)maleimide		45%
M6	*N*-(4-bromophenyl)maleimide		51%

Table 2 (cont'd). Structure of eleven *N*-substituted maleimides

	Name of Compound	Structure	Yield (%)
M7	Maleimide		*commercial available
M8	1-(3,5-bis(trifloromethyl)phenyl)-3-(4-(3-(2,5-dioxo-2H-pirol-1(5H)-yl)phenetyl)phenyl)urea		78%
M9	1-(4-(4-(2,5-dioxo-2H-pirol-1(5H) yl)phenetyl)phenyl)-3-(3,5-dimethylphenyl)urea		96%
M10	2-(4-(2,5-dioxo-2H-pirol-1(5H)-yl)phenyll) acetic acid		36%
M11	3,5-dimethyl-N-(4-(2,5-dioxo-2H-pirol-1(5H)-yl)phenyl)benzamide		66%

The *N*-substituted maleimides **M1** to **M11** were tested against two bacterial strains and a yeast. The biological screening activity of the studied compounds were determined by disc diffusion method. Data shown in Table 3 indicate that all compounds exhibited notable antibacterial and antifungal activity except **M8**, **M9** and **M11**.

According to Table 3, the reactivity of **M1**, **M2** and **M3** toward all three microbes tested were positive, showing inhibition zone ranging from 9 mm to 21 mm for sample concentration up to 20 µg/disc. Whereas, the inhibition zone for **M4**, **M5** and **M6** were around 10 mm, except for **M5** at 20 µg/disc showed 18 mm inhibition zone on the yeast, *Saccharomyces cerevisiae*. Surprisingly, **M4** which showed inhibition towards *Escherichia coli* at 10 µg/disc, became inactive when the concentration double up to 20 µg/disc. On the other hand, unsubstituted maleimide, **M7** is very reactive towards *Saccharomyces cerevisiae*. **M8** possess a trifluoromethane moiety which is often used to increase the biological stability of a drug. Nonetheless, our finding indicated that samples **M8** to **M11** are generally unreactive, except that **M10** showed a small inhibition zone of 9 mm towards *Saccharomyces cerevisiae*.

Generally, most of the samples were biologically active against the yeast *Saccharomyces cerevisiae* compared to *Escherichia coli* and *Bacillus subtilis*. It is also noted that the biological reactivity of the samples does not directly proportional to the dosage concentration. This might be due to the reason that maleimide compounds only show optimum biological reactivity at optimum dosage. Hence, any concentrations beyond the optimum range would have no effect against the microbes tested. Fascinatingly, maleimides substituted with methyl phenyl group are among the most reactive compounds tested. Their inhibition zone is comparable to that of the positive control.

Table 3. Biological activity of *N*-substituted maleimide towards three microbes

Compound	**Inhibition Zone (mm)**					
	Escherichia coli		***Bacillus subtilis***		***Saccharomyces cerevisiae***	
	10 µg/disc	**20 µg/disc**	**10 µg/disc**	**20 µg/disc**	**10 µg/disc**	**20 µg/disc**
M1	15	15	10	15	21	18
M2	11	13	11	12	15	19
M3	10	11	9	10	12	14
M4	10	-	10	11	10	10
M5	9	12	11	12	15	18
M6	8	10	10	10	12	10
M7	22	25	21	25	30	29
M8	-	-	-	-	-	-
M9	-	-	-	-	-	-
M10	-	-	-	-	-	9
M11	-	-	-	-	-	-
Negative control	-	-	-	-	-	-
Streptomycin 10 µg	16	NA	15	NA	NA	NA
Nystatin 20 µg	NA	NA	NA	NA	NA	-

Note: Experiments are done in triplicate to rule out experimental bias or random error.

In addition, unsubstituted maleimide was more reactive towards the microbes compared to the *N*-substituted maleimide since the maleimide core itself is the main active centre which possess good biological activites [15]. Thus, it is apparent that the introduction of bulky substituents at the *N* position of the maleimide ring had different influence on the biological activities. A possible explanation is that maleimide and derivatives could inactivate some enzymes in the microbes. With change in the polarity and chain length of *N*-phenyl substituents, their capacity to bind to eznyme differs and therefore also had different influence on biological activities.

Conclusion

In conclusion, the present work has demonstrated that unsubstituted maleimide was most reactive while simple *N*-phenyl substituted maleimide exhibit potential activity against the tested microbes. Nevertheless, bulky *N*-phenyl substituted maleimides were biologically inactive with rapid decreasing action towards the microbe when more complex moieties inserted. This might be explained by the nitrogen–carbon distances betweeen the two rings which play an important role in the antimicrobial activities of these compounds. Furthermore, steric hindrance might affect inhibition activity. For further investigation, more sensitive antimicrobe screening method such as dilution method should be used to evaluate the potential of maleimide derivatives against different types of microbes. Structural optimisation of these compounds are underway.

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