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Research Article

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Synthesis, characterization and antibacterial activity of Hystatin 2 derivatives

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ABSTRACT

*Hystatin is a group of compounds resulted from the derivatization of aaptamine. A series of hystatin 2 derivatives (**3, 5-15**) have been synthesized and characterized using modern spectroscopic techniques, namely IR, ^{1}H and ^{13}C NMR, UV-Vis and Mass Spectrometer. The synthesized compounds were evaluated for their antibacterial activity against selected bacterial strains of both Gram positive and Gram negative groups, namely Bacillus cereus, Staphylococcus aureus, Micrococcus sp, Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae using standard microbiological protocol of disc-diffusion method. The antibacterial activities of the compounds were assessed by the presence and absence of inhibition zones and minimum inhibition concentration (MIC) values. In general, most of the derivatives showed significant antibacterial activity towards Gram positive bacteria. It was observed that compound **6** exhibited bacterial inhibition activity against all tested bacteria. Nonetheless, compounds **5**, **6** and **14** showed no antibacterial activities towards both Gram positive and Gram negative bacteria.*

Keywords: hystatin 2 derivatives; antibacterial activity; disc-diffusion method; minimum inhibition concentration.

INTRODUCTION

Parasite bacteria (Gram positive and Gram negative) continue to surround and kill millions of people in the world [1, 2]. Infectious diseases caused by these bacteria are a major cause of death, especially in developing countries [2]. Sixty million people are infected and about 15 million are death every year due to cause by *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella typhimurium* and *Escherichia coli*, which correspond to about one third of the death [1, 2]. These bacteria cause food poisoning, rheumatic, salmonellosis and diarrhea. Moreover, drug resistance to these diseases can attributed to the use of drugs (amoxicillin, norfloxacin, ciprofloxacin chloramphenicol) for treatment and to the adaptation of the bacteria parasite by developing alternate pathway for survival.

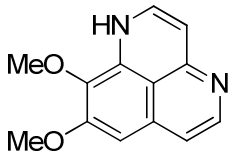
The rising incidence of infections caused by these multi-drug resistance Gram-positive and Gram-negative pathogens to available antimicrobial agents is a leading worldwide problem. Therefore, the development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from another source, including natural sources from any terrestrial or marine sources [3].

Marine invertebrates such as sponges are prominent sources of a wide variety of natural alkaloid. The aaptamines are an interesting group of biologically active marine alkaloids sharing a rare 1*H*-benzo[de]-1,6-naphthyridine skeleton. The importance of heterocyclic compounds has long been recognized in the synthetic organic chemistry. It is well known that a number of heterocyclic compounds containing nitrogen and sulphur exhibited a wide variety of biological activities such as antibacterial drugs and antifungal drugs [4]. Since 1982 when Nakamura's group [5] first discovered aaptamine (**1**) from marine sponge *Aaptosaaptos*, various aaptamine derivatives have been isolated from marine sponges belonging to the genera *Aaptos*, *Suberites*, *Luffariella*, *Hymeniacidon*, *Suberea* and

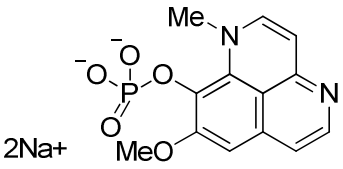
Xestospongia[6]. Aaptamine has potent cytotoxicity that may be explained by its ability to intercalate DNA [7]. Because of its various biological activities, such as α-adrenoceptor blocking, anti-HIV-1 [8], antimicrobial [9], antifungal, antiviral, antitumor [3] and sortase A inhibitory [10], this alkaloid has become an interesting focus for synthesis, structure-activity relationship and bioactivity studies.

Hystatin is a group of compound derivatized from aaptamine. The group of Pettit [11, 12] has synthesized Hystatin 1 (**2**), as well as N_1,N_4-bisbenzyl aaptamine derivatives, Hystatin 2 and Hystatin 3 (Figure 1) from unstable isoaaptamine [6]. Hystatin 2 (**3**) and hystatin 3 (**4**) exhibited significant inhibition activity against the murine P388 lymphocytic leukemia and human cancer cell line, and also demonstrated the most promising antibacterial profiles [12]. Herein we wish to report the synthesis, characterization and antibacterial activity of Hystatin 2 (**3**) [12] and a series of its derivatives (**5-15)**.

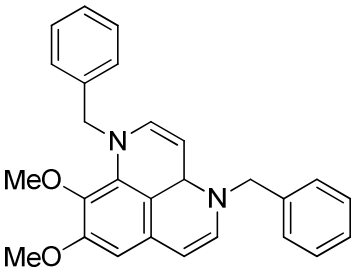
Figure 1. Structures of Aaptamine, Hystatin 1, Hystatin 2 and Hystatin 3



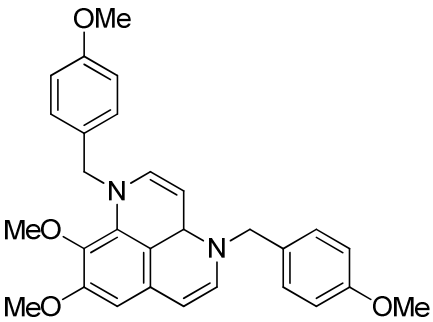
Aaptamine (**1**)



Hystatin 1 (**2**)



Hystatin 2 (**3**)



Hystatin 3 (**4**)

EXPERIMENTAL SECTION

2.1. General Experimental Procedures

All reactions were performed under a nitrogen atmosphere. Principal reagents obtained from Sigma-Aldrich Co., Merck, and R&M chemicals were used without additional purification. Aaptamine (**1**) was isolated from *Aaptosaaptos* according to the literature [13, 14]. The reaction was monitored by thin layer chromatography with silica gel 60 F254 plastic-backed sheets 0.25 mm thick (Merck KGaA) and visualized with long wave UV (365 nm). Flash column chromatography was performed with silica gel 60 (particle size 0.063-0.200 mm, 70-230 mesh ASTM). Infrared spectra were recorded on Perkin Elmer 100 FTIR spectrometer. ^{1}H and ^{13}C NMR spectra were acquired on the Bruker Spectrospin-400 (400 MHz) and Jeol (500 MHz) spectrometer, using CD_3OD as the solvent. Coupling constants (*J*) are reported in Hertz (Hz) and multiplicities are indicated as s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), m (multiplet). All compounds were screened against the bacteria *Bacillus cereus*, *Staphylococcus aureus*, *Micrococcus* sp, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* according to disc diffusion standard protocol.

2.1.1. Preparation of Hystatin 2 (3) and Compounds 5-6 [12]

Aaptamine (1.58 mmol) was dissolved in anhydrous dimethylformamide (36 ml). Potassium carbonate (7.91 mmol) and benzyl halide (7.91 mmol) were added at room temperature. After stirring for 48 H at the same temperature, the solution was filtered and the solvent was removed in vacuo to leave brown oil. The residue was purified by column chromatography (silica gel, $CHCl_3$-CH_3OH). Analytically pure samples were obtained by crystallization from ethyl acetate-methanol using slow evaporation of the solvent.

N_1,N_4-Bisbenzylaaptamine (Hystatin 2) **(3)**[12]. Yield: 74%; IR (KBr) ν_{max}2980, 2940, 1648, 1569, 1304, 1207, 1060 cm^{-1}; UV (MeOH) λmax (log ε) 204 (4.67), 244 (4.39), 263 (4.39), 273 (4.33), 307 (3.62), 415 (3.92) nm ;^{1}H NMR (400MHz) δ 3.60 (s, 3H, OCH_3), 4.05 (s, 3H, OCH_3), 5.40 (s, 2H, NCH_2), 5.74 (s, 2H, NCH_2), 6.53 (d, *J*=7.6 Hz, 1H, H-3), 7.13 (d, *J*=7.6 Hz, 1H, H-6), 7.20 (d, *J*=7.2Hz, 2H, CH_{ar}), 7.30-7.46 (m, 9H, CH_{ar}), 7.62 (d, *J*=8.8 Hz, 1H, H-5), 7.97 (d, *J*=7.6 Hz, 1H, H-2); ^{13}C NMR (100MHz, CD_3OD) δ 55.8, 56.4, 60.084, 60.9, 97.7, 102.9, 114.6, 119.1, 125.8, 126.6, 127.5, 128.2, 128.6, 129.0, 132.9, 133.5, 133.8, 134.3, 135.1, 136.5, 149.1, 149.6, 159.0; DIMS *m*/*z* $[M]^+$ found 409.25 (calcd $C_{27}H_{25}N_2O_2$, 409.50).

N_1,N_4-Bis[(3,4,5-trimethoxy)benzyl]aaptamine **(5)**[12].Yield: 38%; IR (KBr) ν_{max}2920, 2850, 1647, 1592, 1332, 1241, 1125 cm^{-1}; UV (MeOH) λmax (log ε) 217 (5.61), 240 (5.41), 272 (5.06), 355 (4.37), 393 (4.30) nm; ^{1}H NMR (400MHz) δ 3.76 (s, 6H, OCH_3), 3.79 (s, 9H, OCH_3), 3.89 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 4.07 (s, 3H, OCH_3), 5.30 (s, 2H, NCH_2), 5.67 (s, 2H, NCH_2), 6.52 (s, 1H, H-6), 6.61-6.91 (m, 4H, CH_{ar}), 7.12 (d, *J*=7.2 Hz, 1H, H-3), 7.30 (s, 1H, H-7), 7.62 (d, *J*=7.2 Hz, 1H, H-5), 8.02 (d, *J*=8.0 Hz, 1H, H-2); ^{13}C NMR (100MHz, CD_3OD) δ 29.3, 41.9, 55.2, 55.3, 55.5, 55.8, 56.6, 59.7, 59.8, 60.9, 97.8, 103.0, 103.9, 104.3, 110.3, 114.5, 118.9, 129.6, 132.1, 132.9, 133.3, 134.2, 135.1, 148.8, 149.4, 153.5, 153.6, 153.9, 159.0.

N_4-[(4-nitro)benzyl]aaptamine (**6**). Yield: 95.15%; IR (KBr) ν_{max}3078, 2943, 1651, 1599, 1344, 1249, 1092cm^{-1}; UV (MeOH) λmax (log ε) 217 (4.37), 241 (4.36), 261 (4.42), 361 (3.71), 393 (3.66) nm; ^{1}H NMR (400MHz) δ 3.92 (s, 3H, OCH_3), 4.04 (s, 3H, OCH_3), 5.37 (s, 2H, NCH_2), 6.25 (d, *J*=6.8 Hz, 1H, H-3), 6.88 (d, *J*=7.6 Hz, 1H, H-6), 7.11 (s, 1H, H-7), 7.36 (d, *J*=7.6 Hz, 1H, CH_{ar}), 7.53 (d, *J*=8.4 Hz, 2H, CH_{ar}), 7.88 (d, *J*=7.2 Hz, 1H, H-5), 8.25 (d, *J*=8.8 Hz, 1H, H-2); ^{13}C NMR (100MHz, CD_3OD) δ 56.2, 57.0, 61.1, 97.7, 103.0, 114.4, 119.1, 125.2, 129.0, 132.5, 135.5, 143.2, 149.2.

2.1.2. Preparation of Compounds 7-9 [12]

Compounds **6**, **10** and **11** (1 mmol) were dissolved in anhydrous DMF. Potassium carbonate (5 eq) and benzyl bromide (5 eq) were added and stirred for 48 h at room temperature. The solution was filtered and the solvent was removed in vacuo. The residue was purified by column chromatography (silica gel, $CHCl_3$-CH_3OH).

N_1-benzyl-N_4-[(4-nitro)benzyl]aaptamine **(7).** Yield: 59% ; IR (KBr) ν_{max}3032, 2925, 1648, 1567, 1384, 1206, 1100 cm^{-1};UV (MeOH) λmax (log ε) 203 (4.48), 263 (4.17), 273 (4.13), 283 (4.11), 417 (3.53) nm; ^{1}H NMR (400MHz) δ 3.57 (s, 3H, OCH_3), 4.03 (s, 3H, OCH_3), 5.54 (s, 2H, NCH_2), 5.74 (s, 2H, NCH_2), 6.45 (d, *J*=7.6 Hz, 1H, H-3), 7.13 (d, *J*=7.6 Hz, 1H, H-6), 7.31 (s, 1H, H-7), 7.54-7.55 (m, 4H, CH_{ar}), 7.58-7.59 (m, 5H, CH_{ar}), 7.98 (d, *J*=7.6 Hz, 1H, H-5), 8.28 (d, *J*=8.8 Hz, 1H,H-2) ; ^{13}C NMR (100MHz, CD_3OD) δ 30.7, 57.0, 57.2, 61.6, 62.4, 69.8, 98.9, 104.6, 116.2, 125.3, 127.2, 128.7, 129.0, 129.1, 130.0, 130.4, 132.0, 134.2, 134.3, 135.5, 137.8, 142.7, 150.8, 151.1, 160.5.

N_1-benzyl-N_4-[(4-trifluoromethyl)benzyl]aaptamine **(8).** Yield: 44%; IR (KBr) ν_{max}3032, 1648, 1567,1384, 1207, 1119 cm^{-1}; UV (MeOH) λmax (log ε) 262 (4.08), 281 (4.04), 309 (3.34), 365 (3.43), 416 (3.62) nm; ^{1}H NMR (400MHz) δ 3.57 (s, 3H, OCH_3), 4.03 (s, 3H, OCH_3). 5.49 (s, 2H, NCH_2), 5.73 (s, 2H, NCH_2), 6.45 (d, *J*=8.0 Hz, 1H, H-3), 7.12 (d, *J*=7.2 Hz, 1H, H-6), 7.19 (s, 1H, H-7), 7.30-7.50 (m, 4H, CH_{ar}), 7.54-7.58 (m, 5H, CH_{ar}), 7.72 (d, *J*=8.0 Hz, 2H, H-5), 7.96 (d, *J*=8.0 Hz, 1H, H-2); ^{13}C NMR (100MHz, CD_3OD) δ 30.1, 30.7, 32.1, 57.2, 61.6, 62.4, 69.8, 99.0, 104.6, 116.2, 120.5, 127.2, 127.3, 128.6, 128.8, 129.0, 130.0, 130.4, 132.0, 134.2, 134.3, 134.9, 135.6, 136.6, 137.9, 139.9, 150.8, 151.1, 160.5.

N_1-benzyl-N_4-[(4-methoxyl)benzyl]aaptamine **(9).** Yield: 75%; IR (KBr) ν_{max}3030, 2970, 1646, 1611, 1374, 1248, 1155 cm^{-1}; UV (MeOH) λmax (log ε) 205 (4.623), 216 (4.54), 244 (4.10), 262 (4.10), 282 (4.01), 307 (3.32), 364 (3.36), 417 (3.54) nm; ^{1}H NMR (400MHz) δ 3.56 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 4.02 (s, 3H, OCH_3), 5.29 (s, 2H, NCH_2), 5.72 (s, 2H, NCH_2), 6.57 (d, *J*=8 Hz, 1H, H-3), 6.95 (d, *J*=8 Hz, 1H, H-6), 7.09 (d, *J*=8 Hz, 1H, H-5), 7.32 (s, 1H, H-7), 7.51-7.62 (m, 9H, CH_{ar}), 7.96 (d, *J*=8.0 Hz, 1H, H-2); ^{13}C NMR (100MHz, CD_3OD) δ 55.8, 57.2, 57.4, 61.4, 62.3, 69.7, 99.1, 104.2, 115.7, 115.9, 120.5, 126.9, 127.2, 128.8, 128.9, 129.7, 129.9, 130.4, 132.0, 134.2, 134.8, 135.5, 136.4, 137.9, 150.4, 150.8, 160.3, 161.4.

2.1.3. Preparation of Compounds 10-15 [7]

Aaptamine (0.95 mmol) was dissolved in dimethylformamide (22 ml) at 0 °C. To the solution, approximately 4 equivalents of potassium hydride (3.80mmol) were added and the mixture was stirred for 10 min, after which 2 equivalents of the appropriate alkyl halide (1.90 mmol) were added drop-wise. The reaction was stirred for an additional 1 hour and then allowed to warm to room temperature. While at room temperature, the reaction was monitored by TLC to insure complete conversion of starting material which occurred usually after 18-24 h. Workup consisted of aqueous extraction with chloroform, a brine wash and drying over sodium sulfate before evaporation under reduced pressure. The residue was purified by column chromatography (silica gel, $CHCl_3$-CH_3OH).

Analytically pure samples were obtained by crystallization from ethyl acetate-methanol using slow evaporation of the solvent.

N_4-[(4-methoxyl)benzyl]aaptamine **(10).** Yield: 50%; IR (KBr) ν_{max}2945, 2848, 1651, 1600, 1323, 1247, 1089 cm^{-1}; UV (MeOH) λmax (log ε) 201 (4.59), 218 (4.41), 239 (4.41), 259 (4.34), 270 (4.30), 306 (3.58), 357 (3.65), 395 (3.66) nm; ^{1}H NMR (400MHz) δ 3.80 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 4.08 (s, 3H, OCH_3), 5.21 (s, 2H, NCH_2), 6.45 (d, *J*=7.2 Hz, 1H, H-3), 6.95-6.98 (d, *J*=8.8 Hz, 3H, CH_{ar}), 7.16 (s, 1H, H-7), 7.27 (d, *J*=8.8 Hz, 1H, H-6), 7.44 (d, *J*=7.6 Hz, 2H, H-5), 7.87 (d, *J*=7.2 Hz, 1H, H-2); ^{13}C NMR (100MHz, CD_3OD) δ 54.5, 55.2, 59.3, 95.9, 101.0, 112.1, 117.5, 125.3, 125.4, 126.7, 130.7, 133.7, 138.7, 149.2; DIMS *m/z* $[M]^+$ found 348.35 (cacld $C_{21}H_{20}N_2O_3$, 348.40).

N_4-[(4-trifluoromethl)benzyl]aaptamine **(11).** Yield: 15%; IR (KBr) ν_{max} 3077, 2946, 1652, 1599, 1326, 1248, 1109 cm^{-1}; UV (MeOH) λmax (log ε) 202 (4.54), 240 (4.36), 259 (4.31), 269 (4.26), 278 (4.22), 305 (3.55), 356 (3.63), 397 (3.63) nm; ^{1}H NMR (400MHz) δ 3.83 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 5.25 (s, 2H, NCH_2), 6.18 (d, *J*=7.2 Hz, 1H, H-3), 6.81 (d, *J*=7.2 Hz, 1H, H-6), 7.03 (s, 1H, H-7), 7.27-7.39 (m, 3H, CH_{ar}), 7.61 (d, *J*=8.4 Hz, 1H, H-5), 7.78 (d, *J*=6.8 Hz, 1H, H-2); ^{13}C NMR (100MHz, CD_3OD) δ 55.8, 56.8, 57.1, 61.2, 98.1, 102.8, 114.8, 115.7, 127.2, 129.6, 132.9, 135.6, 161.3; DIMS *m/z* $[M]^+$ found 386.30 (cacld $C_{21}H_{17}F_3N_2O_2$, 386.37).

N_4-[(3,4,5-trimethoxyl)benzyl]aaptamine **(12).** Yield: 31%; IR (KBr) ν_{max}2943, 1651, 1598, 1322, 1247, 1126 cm^{-1}; UV (MeOH) λmax (log ε) 205 (4.72), 239 (4.42), 259 (4.35), 397 (4.62) nm; ^{1}H NMR (400MHz) δ 3.64 (s, 3H, OCH_3), 3.69 (s, 6H, OCH_3), 3.86 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 5.13 (s, 2H, NCH_2), 6.39 (d, *J*=7.6 Hz, 1H, H-3), 6.51 (s, 2H, CH_{ar}), 6.91 (d, *J*=7.6 Hz, 1H, H-6), 7.09 (s, 1H, H-7), 7.39 (d, *J*=7.6 Hz, 1H, H-5), 7.78 (d, *J*=7.2 Hz, 1H, H-2); ^{13}C NMR (100MHz, CD_3OD) δ 56.7, 57.1, 57.4, 61.3, 98.1, 103.0, 105.7, 115.2, 131.2, 135.6, 143.3, 155.3; DIMS *m/z* $[M]^+$ found 408.30 (cacld $C_{23}H_{24}N_2O_5$, 408.45).

N_1-[(4-methoxyl)benzyl]aaptamine **(13).** Yield: 15%; IR (KBr) ν_{max}3079, 2930, 1646, 1603, 1339, 1249, 1105 cm^{-1}; UV (MeOH) λmax (log ε) 202 (4.63), 222 (4.42), 259 (4.31), 315 (3.56), 395 (3.80) nm; ^{1}H NMR (400MHz) δ 3.50 (s, 3H, OCH_3), 3.64 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 5.36 (s, 2H, NCH_2), 6.11 (d, *J*=7.6 Hz, 1H, H-3), 6.77 (d, *J*=8.8 Hz, 3H, CH_{ar}), 6.80 (s, 1H, H-7), 7.08 (d, *J*=8.8 Hz, 1H, H-6), 7.36 (d, *J*=3.6 Hz, 2H, H-5), 7.38 (d, *J*=4.4 Hz, 1H, H-2); ^{13}C NMR (100MHz, CD_3OD) δ 55.7, 56.7, 59.3, 61.9, 101.2, 103.7, 115.0, 115.2, 128.9, 130.6, 136.4, 159.6, 160.7; DIMS *m/z* $[M]^+$ found 348.30 (calcd $C_{21}H_{20}N_2O_3$, 348.40).

N_1-[(4-trifluoromethyl)benzyl]aaptamine **(14).** Yield: 26%; IR (KBr) ν_{max}2944, 2874, 1649, 1602, 1326, 1207, 1109 cm^{-1}; UV (MeOH) λmax (log ε) 203 (4.57), 244 (4.31), 258 (4.34), 277 (4.20), 314 (3.60), 394 (3.83) nm; ^{1}H NMR (400MHz) δ 3.60 (s, 3H, OCH_3), 4.02 (s, 3H, OCH_3), 5.76 (s, 2H, NCH_2), 6.45 (d, *J*=7.2 Hz, 1H, H-3), 7.02 (d, *J*=7.2 Hz, 1H, H-6), 7.22 (s, 1H, H-7), 7.40-7.45 (m, 3H, CH_{ar}), 7.69 (d, *J*=8.4 Hz, 1H, H-5), 7.87 (d, *J*=7.6 Hz, 1H, H-2); ^{13}C NMR (100MHz, CD_3OD) δ 55.7, 56.7, 59.3, 61.9, 101.2, 103.8, 115.0, 115.2, 129.0, 130.6, 135.1, 136.4, 146.8, 159.7, 160.8; DIMS *m/z* $[M]^+$ found 386.35 (cacld $C_{21}H_{17}F_3N_2O_2$, 386.37).

N_1-[(3,4,5-trimethoxyl)benzyl]aaptamine **(15).** Yield: 25%; IR (KBr) ν_{max}2940, 2840, 1646, 1600, 1335, 1239, 1124 cm^{-1}; UV (MeOH) λmax (log ε) 204 (4.80), 259 (4.34), 277 (4.21), 314 (3.59), 405 (3.83) nm; ^{1}H NMR (400MHz) δ 3.63 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 3.76 (s, 6H, OCH_3), 3.97 (s, 3H, OCH_3), 5.44 (s, 2H, NCH_2), 6.21 (d, *J*=7.6 Hz, 1H, H-3), 6.58 (s, 2H, CH_{ar}), 6.87 (s, 1H, H-7), 6.90 (d, *J*=6.4 Hz, 1H, H-6), 7.39 (d, *J*=7.6 Hz, 1H, H-5), 7.55 (d, *J*=6.4 Hz, 1H, H-2); ^{13}C NMR (100MHz, CD_3OD) δ 56.6, 56.7, 59.4, 61.1, 61.9, 100.6, 105.0, 115.1, 134.9, 135.0, 146.0, 154.8, 159.3; DIMS *m/z* $[M]^+$ found 408.25 (cacld $C_{23}H_{24}N_2O_5$, 408.45).

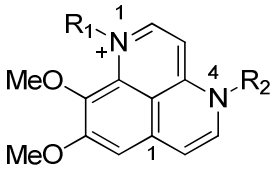
2.2. Antibacterial Study

The antibacterial activities of synthesized compounds against six bacteria were investigated by the standard protocol of disc-diffusion method. All tests were performed on Mueller-Hinton agar. The surface of agar was uniformly inoculated by bacterial suspension using sterile cotton swab. The bacterial suspensions were initially adjusted to equivalent turbidity of 0.5 McFarland standards. Sterile 6.0 mm diameter blank discs were used to load 20 µL of the compounds with various concentrations dissolved in methanol solvent. Ampicillin (10µg) and Streptomycin (10µg) were used as positive control and methanol (used as a solvent) loaded discs were the negative control. The compounds, methanol solvent and antibiotic discs were placed on Mueller-Hinton agar and incubated at 37 °C for 18-24 h. On the next day, the diameters of inhibition zone in mm were recorded [15].

RESULTS AND DISCUSSION

Hystatin 2 (**3**) and a series of its derivatives (compounds **5**-**15**) were synthesized according to the literature, with some modifications [12, 7].

Figure 2. Analogues of hystatin 2



3, R_1=R_2=Bn
5, R_1=R_2=3,4,5-trimethoxy

6, R_1=H, R_2=4-nitro

7, R_1=Bn, R_2=4-nitro
8, R_1=Bn, R_2=4-trifluoromethyl
9, R_1=Bn, R_2=4-methoxy

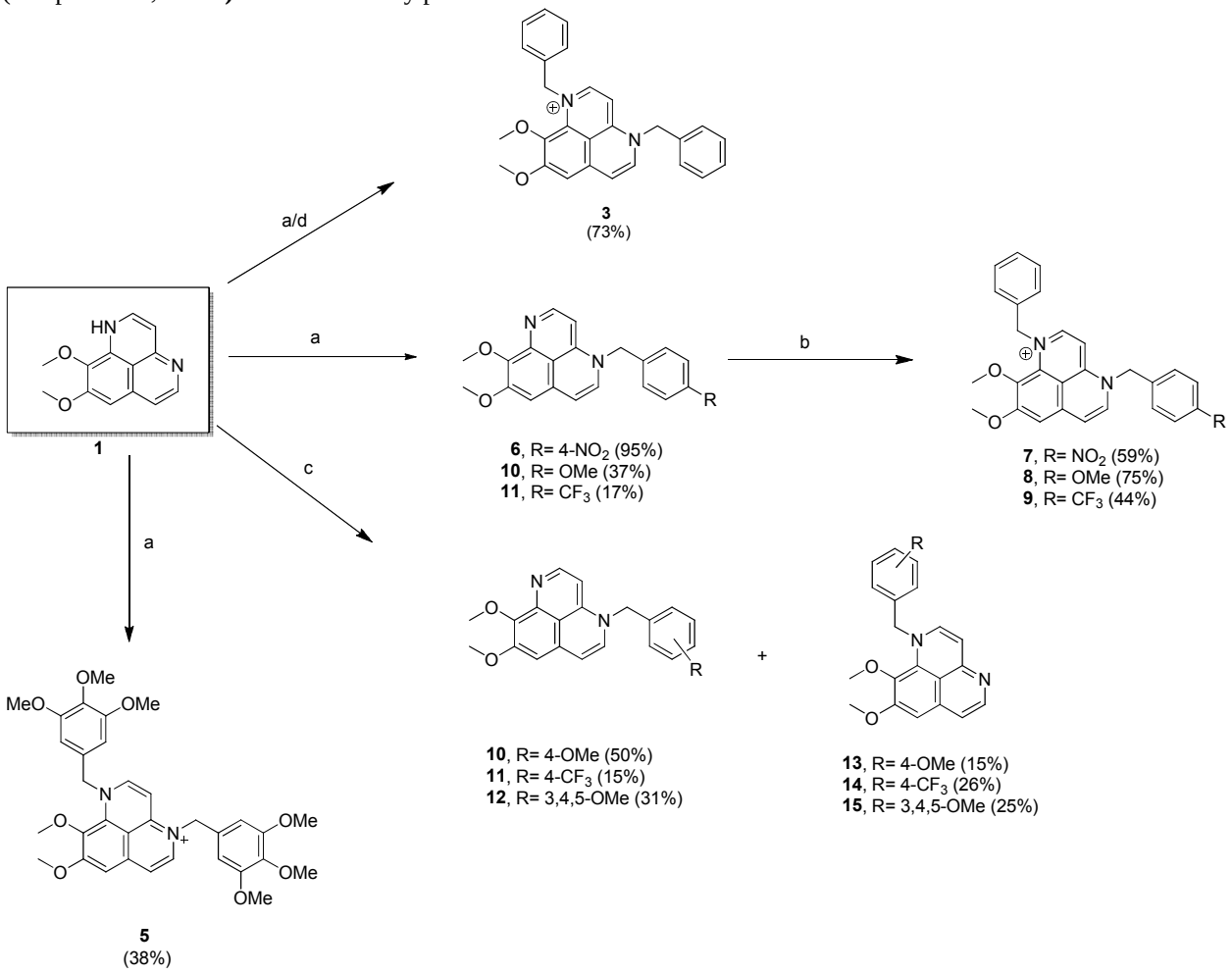
10, R_1=H, R_2=4-methoxy
11, R_1=H, R_2=4-trifluoromethyl
12, R_1=H, R_2=3,4,5-trimethoxy

13, R_1=4-methoxy, R_2=H
14, R_1=4-trifluoromethyl, R_2=H
15, R_1=3,4,5-trimethoxy, R_2=H

3.1. Chemistry

N_1, N_4 di-substituted derivatives (Hystatin 2 (**3)** and compound **5**) [12] were prepared by reacting of aaptamine (**1**) with benzyl halide and 3,4,5-trimethoxybenzyl halide, respectively, in the presence of potassium carbonate [12] (Scheme 1).

Unfortunately, the desired N_1,N_4-disubstituted derivatives were not observed when the similar approach was applied to 4-nitro, 4-methoxy and 4-trifluoromethyl benzyl halides. Instead, only compounds with N_4-substituted (compounds **6**, **10**-**11)** were exclusively produced.



Scheme 1.General synthesis scheme of hystatin 2 derivatives

Condition and reagents: (a) $BnCH_2X$, K_2CO_3, DMF, rt, 24 h; (b) $BnCH_2Br$, K_2CO_3, DMF, rt, 24 h; (c) $BnCH_2Cl$, KH,DMF, 0 °C, 24-48 h ; (d) $BnCH_2Br$, KH, DMF, 0 °C, 24-48 h.

The ^{1}H NMR of compounds **3** and **5** showed two signals noted as singlet, at δ_H5.74 and 5.40ppm (for compound **3**) and δ_H 5.67 and 5.30 ppm (for compound **5**), corresponds to CH_2 of benzyl group that attached to N_1 and N_4 positions which is in good agreement with previous reported literature [12].

On the contrary, ^{1}H NMR of compounds **6** showed one signal noted as singlet at δ_H 5.37 ppm which correspond to CH_2 at N_4 position. The ^{13}C NMR of compounds **6** showed 20 carbon signals, which further confirmed the molecular structures of these compounds.

Analysis of the DIMS data of compound **10** (m/z 348.35 for [M^+]) suggested the molecular formula $C_{12}H_{20}N_2O_3$ and indicated a coupling product of **1** with 4-methoxybenzyl moiety attached at N_4. The ^{1}H NMR of compound **10** showed three singlet at δ_H3.79, 3.95 and 4.07 ppm assigned to three O-CH_3 presence in the structure which one O-CH_3 from 4-methoxybenzyl moiety and two of them from **1**. Another singlet atδ_H 5.21 ppm indicated a CH_2 of 4-methoxybenzyl that successfully bonded at N_4. The ^{13}C NMR data of **10** showed 21 carbon signals, which further confirmed the identity of compound **10**.

The DIMS data of **11** (m/z 386.30 for [M^+]) suggested a molecular formula $C_{21}H_{17}F_3N_2O_2$ and indicated a coupling product of **1** with 4-trifluoromethylbenzyl moiety bonded at N_4. ^{1}H NMR data of **11** showed two singlet signals at δ_H 3.83 and 3.95 ppm indicated O-CH_3 moiety at C-9 and C-8 respectively. Another singlet signal at δ_H 5.25 ppm showed a CH_2 of 4-trifluoromethylbenzyl that successfully bonded to N_4 of **1**. The ^{13}C NMR data of **11** showed 21 carbon signals, which confirmed a structure of **11**. In addition, further treatment of compounds **6**, **10** and **11** with benzyl halide and K_2CO_3 in DMF led to the formation of compounds **7**-**9** with N_1,N_4-disubstituted derivatives (Scheme 1).

Surprisingly, when the different method was applied by using potassium hydride as a base [7], compounds with N_4 substituted benzyl derivatives (**10**-**12)** were formed predominantly, along with N_1 substituted compounds (**13-15**) (Scheme 1).

The DIMS data of **12** (m/z 408.30 for [M^+]) suggested a molecular formula $C_{23}H_{24}N_2O_5$ and indicated a coupling product of **1** with 3,4,5-trimethoxybenzyl moiety bonded at N_4. ^{1}H NMR data of **12** showed five singlet at δ_H 3.63, 3.69, 3.69, 3.85 and 3.96 ppm indicated O-CH_3, which three of them from 3,4,5-trimethoxybenzyl moiety that bonded to N_4 and another two was originally from **1**. Another singlet signal at δ_H 5.12 ppm showed a CH_2 of 3,4,5-trimethoxybenzyl that successfully bonded to N_4 of **1**. The ^{13}C NMR data of **12** showed 23 carbon signals, which confirmed the structure of **12**.

The DIMS data of **13** (m/z 348.30 for [M^+]) suggested a similar molecular formula as **10**. The ^{1}H NMR data of **13** showed three singlets at δ_H3.50, 3.64 and 3.85 ppm indicated three O-CH_3 presence in the structure which one O-CH_3 from 4-methoxybenzyl moiety and two of it from **1** at C-9 and C-8. A singlet signal at δ_H 5.36 ppm showed a CH_2 of 4-methoxybenzyl moiety with slightly shifted to downfield due to the shielding effect, that indicated an expected coupling product of **10**. This confirmed that it was successfully bonded at N_1. The ^{13}C NMR data of **13** showed 21 carbon signals, which further confirmed the identity of compound **13**.

The DIMS data of **14** (m/z 386.35 for [M^+]) suggested a similar molecular formula as **11**. The ^{1}H NMR data of **14** showed two singlets at δ_H 3.59 and 4.01 ppm indicated O-Me moiety at C-9 and C-8 respectively. A singlet signal at δ_H5.75 ppm showed a CH_2 of 4-trifluoromethylbenzyl with slightly shifted to downfield, that indicated an expected coupling product of **11**. This confirmed that it was successfully bonded at N_1. The ^{13}C NMR data of **14** showed 21 carbon signals, which confirmed a structure of **14**.

The DIMS data of **15** (m/z 408.25 for [M^+]) suggested a similar molecular formula of **12**. The ^{1}H NMR data of **15** showed five singlets at δ_H 3.62, 3.72, 3.76, 3.86, 3.86 and 3.97 ppm indicated O-Me, which three of them from 3,4,5-trimethoxybenzyl moiety that bonded to N_1 and another two was originally from **1** at C-9 and C-8. Another singlet at δ_H5.43 ppm showed a CH_2 of 3,4,5-trimethoxybenzyl with slightly shifted than **12** toward downfield that confirmed it was successfully bonded at N_1. The ^{13}C NMR data of **15** showed 23 carbon signals, which confirmed the structure of **15**.

3.2. Antibacterial Study

The antimicrobial activities of all the compounds against the six bacteria strains were assessed using standard protocol of disc-diffusion method by the presence or absence of inhibition zones and calculating the minimum inhibition concentration (MIC) values. MIC for compounds was tested within the range 1.00-0.25mg/ml. The MIC values and the diameter of inhibition zones of the all compounds tested for antibacterial activity are given in Table 1.

In general, aaptamine (**1**), hystatin 2 (**3**) and its derivatives (compounds **5-15**) exhibited significant activity against both Gram positive and Gram negative bacterial strain, where most of the derivatives showed activities for Gram positive. Nonetheless, derivatives **5**, **6** and **14** showed no activities against both Gram positive and Gram negative bacteria. The group of Pettit reported that the antibacterial of derivative **5** only had minor or almost no antibacterial activity against both Gram positive and Gram negative bacterial except *Micrococcusluteus*at 64 µg/ml [6, 12]. Interestingly, derivative **9** exhibited antibacterial activity against all tested bacteria. However, the lowest MIC (0.25mg/ml) for derivative **9** were observed against bacteria *Klebsiellapneumoniae* and *Staphylococcus aureus* only, while hystatin 2 (**3**) exhibited the most potential antibacterial activity at 0.25mg/ml against all bacterial strains except *Klebsiellapneumoniae*. Pettit *etal*. reported that hystatin 2 (**3**) inhibited *Micrococcusluteus*, *Escherichiacoli* and no inhibition for *Staphylococcusaureus*[12]. Where in this study, derivative **3** have an excellent antibacterial activity where it's inhibited all bacterial strains except *Klebsiellapneumoniae*. On the other hand, aaptamine (**1**) only showed selective activity against Gram negative bacteria at 1.00mg/ml. The result was consistent with previous studies done by Bowling *et al*., (2008) [7]. In contrast, compounds **7**, **12**, **13** and **15** only exhibited antibacterial activity exclusively against Gram positive bacteria. It is also observed that compound **8** exhibited activity at 0.25mg/ml against all Gram positive bacteria and *Escherichia coli* (Gram negative) at 1.0mg/ml. Whereas, compound **10** showed activities against *Escherichia coli* (0.5mg/ml),*Staphylococcus aureus*(1.0mg/ml) and *Micrococcus* sp (1.0mg/ml).

Table 1. Antibacterial activity for aaptamine (1), hystatin 2 (3) and its derivatives (compounds 5-15)

	Gram negative bacteria			Gram positive bacteria		
	MIC mg/ml (zone of inhibition)					
Compounds	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*B. cereus*	*S. aureus*	*Micrococcus* sp.
1	1.0 (10mm)	NA	1.0 (9mm)	NA	NA	NA
3	0.25 (11 mm)	0.25 (8 mm)	NA	0.25 (8 mm)	0.25 (25 mm)	0.25 (12 mm)
5	NA	NA	NA	NA	NA	NA
6	NA	NA	NA	NA	NA	NA
7	NA	NA	NA	NA	0.5 (9 mm)	0.25 (15 mm)
8	1.0 (7 mm)	NA	NA	0.25 (8 mm)	0.25 (8 mm)	0.25 (17 mm)
9	0.5 (7 mm)	0.5 (11 mm)	0.25 (8 mm)	0.5 (8 mm)	0.25 (11 mm)	0.5 (11 mm)
10	0.5 (9 mm)	NA	NA	NA	1.0 (9 mm)	1.0 (8 mm)
11	NA	NA	NA	1.0 (11 mm)	1.0 (12 mm)	0.25 (9 mm)
12	NA	NA	NA	1.0 (13 mm)	NA	NA
13	NA	NA	NA	1.0 (11 mm)	NA	0.5 (8 mm)
14	NA	NA	NA	NA	NA	NA
15	NA	NA	NA	NA	1.0 (9 mm)	0.5 (9 mm)
P	12	11	-	27	10	36
S	30	12	16	13	8	27

NA = no activity
P= Penicillin, S= Streptomycin

CONCLUSION

In conclusion, we have synthesized a series of di- and mono- benzylation of hystatin 2 derivatives and showed the potential of hystatin 2 (**3**) and its derivatives (**5-15)** against six bacteria. Most of hystatin 2 derivatives showed high potential as antibacterial agent and further evaluation of this class of compound against a wider range of bacteria strains would be useful.

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