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BENZIMIDAZOLE DERIVATIVES AS POTENTIAL NEURAMINIDASE INHIBITORS: CONVENTIONAL AND MICROWAVE SYNTHESIS, *In Vitro* AND MOLECULAR DOCKING ANALYSIS

(Terbitan Benzimidazola yang Berpotensi sebagai Perencat Neuraminidase: Sintesis secara Konvensional dan Gelombang Mikro, Analisis *In Vitro* dan Pengedokan Molekul)

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Abstract

As a continuous effort to discover potential neuraminidase (NA) inhibitors, two series of benzimidazole derivatives consisting of esters, **5(a–g)** and carboxylic acid moieties, **6(a–g)** were synthesised under conventional and microwave conditions. The efficiency of both methods was compared, and their ability to inhibit the action of NA enzyme was examined *in silico* and *in vitro*. The microwave synthesis of the target compounds was more efficient and convenient than the conventional method as the former accelerated the reaction from hours to minutes, giving comparable yields. All compounds obtained were confirmed by the ^{1}H, ^{13}C NMR, and mass spectroscopic data. Out of six compounds tested in the carboxylic acid series, only **6f** showed inhibitory action towards NA with 15.2%. The binding interactions of **6(a–g)** were investigated further by molecular docking on the NA active site (PDB ID: 3TI6). **6f** was found to interact in the 430-loop cavity mainly by hydrophobic interactions. **6f** interacted at different active sites compared to DANA and oseltamivir. Although the compounds showed low inhibitory action, with strategic structural improvements, the benzimidazoles have the potential to be developed as NA inhibitors.

Keywords: benzimidazole, MAOS, Neuraminidase inhibitor, Molecular docking, MUNANA

Abstrak

Sebagai usaha berterusan untuk menemukan perencat neuraminidase (NA) yang berpotensi, dua siri terbitan benzimidazola yang terdiri daripada ester, **5(a–g)** dan moieti asid karboksilik, **6(a–g)** telah disintesis dalam keadaan konvensional dan gelombang mikro. Kecekapan kedua-dua kaedah telah dibandingkan dan keupayaan mereka untuk merencat enzim NA telah diselidik secara *in silico* dan *in vitro*. Sintesis sebatian sasaran melalui gelombang mikro adalah lebih cekap dan mudah berbanding kaedah konvensional kerana proses tindak balas dipercepat dari beberapa jam ke minit, memberikan hasil yang setanding. Semua sebatian yang diperoleh adalah bertepatan dengan data ^{1}H, ^{13}C NMR, dan spektroskopi jisim. Daripada enam sebatian yang diuji dalam siri

asid karboksilik, hanya **6f** menunjukkan tindakan perencatan terhadap NA sebanyak 15.2%. Interaksi pengikatan **6(a–g)** telah dikaji dengan lebih lanjut melalui pengedokan molekul terhadap tapak aktif NA (PDB ID: 3TI6). **6f** didapati berinteraksi dalam rongga 430 gelung terutamanya oleh interaksi hidrofobik. **6f** berinteraksi di tapak aktif yang berbeza berbanding DANA dan oseltamivir. Walaupun sebatian menunjukkan tindakan perencatan yang rendah, dengan penambahbaikan struktur strategik, benzimidazola mempunyai potensi untuk dibangunkan sebagai perencat NA.

Kata kunci: Benzimidazola, MAOS, Perencat neuraminidase, Pengedokan molekul, MUNANA

Introduction

Three main types of human influenza virus (A, B, and C) were reported with both types A and B virulent to humans [1]. The H1N1 influenza attack in 2009, derived from a triple-reassortant influenza virus comprising genes from avian, swine, and human origin, had caused the first pandemic outbreak of this century, raising the influenza pandemic alert level to phase 6 [2-4] until August 2010, after which the World Health Organization declared the end of the H1N1 pandemic. Vaccination is one of the most effective ways of protecting the body against viral infection. However, several drawbacks appeared in applying vaccines, such as the requirements of a new vaccine based on predicted strains that are circulating during flu season [5] and the lack of time to produce a mass number of vaccines, especially when the outbreak strikes [6]. Therefore, an anti-influenza drug is preferably applied as a forefront target in the early treatment of influenza attack.

The M2 ion protein channel and neuraminidase (NA) are two primary targets in the development of antiviral agents for influenza infection treatment or prevention [7, 8]. The M2 ion channel inhibitors (e.g., amantadine and rimantadine (adamantanes)) act by preventing the viral to uncover inside the cell. Unfortunately, these drugs are only useful for influenza virus having the M2 protein, such as type A, but ineffective for those without, such as influenza B [9]. In addition, the drugs are also associated with several adverse effects [10] and demonstrated resistance in most strains, making them less effective for treating the disease [11, 12].

On the other hand, NA works by cleaving the terminal sialic acid from the virion progeny and the host-cell surface. In doing so, it then assists the virus's movement through the respiratory tract [13, 14]. The NA active sites are surrounded by the conserved residues of amino acids of both influenza A and B strains. Thus, these sites become the ideal target for the design of NA inhibitors. Along with the advanced development in structure-based drug design and the availability of NA crystal structure, several NA inhibitors, such as zanamivir, oseltamivir, peramivir, and laninamivir, were developed to treat influenza [15]. Zanamivir (Relenza®) is a polar compound that needs to be administered by inhalation, which makes it inconvenient especially for children and elderly people. Oseltamivir carboxylate (Tamilflu®), while available in the form of tablets, can cause nausea and vomiting. Peramivir (Rapiacta®, Peramiflu®) received a fast-track designation from the US Food and Drug Administration to be used as a drug and was approved later for intravenous administration in December 2014. However, the reported side effects of this drug include gastrointestinal problems and abnormal neutrophil counts [16]. Laninamivir, recently authorised in Japan, was found to effectively inhibit H5N1 influenza virus and oseltamivir resistance mutants by single inhalation. It was also recently reported to be as efficient as zanamivir towards children affected with the influenza virus [17], although laninamivir is much more convenient as it only requires single inhalation for the treatment [4].

Benzimidazole is a heteroaromatic compound possessing diverse therapeutic and biological properties. Its derivatives were found to act as proton pump inhibitors (e.g., omeprazole), anthelmintics (e.g., mebendazole), and inodilators (e.g., pimobendan) [17]. Compounds possessing benzimidazole scaffold were also reported to exhibit antimicrobial and antifungal activities towards *S. aureus*, *E. coli, Xanthomonas sp*., and *Salmonella sp*., [18-21], antihistamine [22-25], anticancer [24, 26], and antifungal activities [26, 27]. These interesting properties warrant benzimidazole to be considered a privileged structure as the structure can be manipulated or modified to suit many different biological targets [28, 29].

Gedye et al. [30] were the first to introduce the application of microwave in organic transformation and Giguere et al. [31] then opened up a new perspective on microwave reaction. Since then, many chemical syntheses utilising either domestic or single-mode microwave reactors have been reported [32-34]. Apart from shortening the reaction time and improving reproducibility, the percentage yield can be increased with low by-products formed [35, 36]. In contrast to the normal conventional method, the microwave method involves a conduction heating mechanism, where energy is transferred from the vessel wall to the adjacent solvent before it moves within the bulk solvent containing the reaction mixture. This process results in slow heating and thus requires a lot of energy to heat the glassware compared to the reaction mixture itself, making the temperature inside the target mixture often cooler than the outside [37].

The design of NA inhibitors mainly involves sialic acid, benzoic acid, and cyclopentane scaffolds. Only recently, benzimidazoles have also been investigated as NA inhibitors [37, 38]. Owing to benzimidazole's interesting structure and various therapeutic applications, we were interested to discover the potential of benzimidazole derivatives as NA inhibitors. In this work, the designed compounds were synthesised via microwave and conventional approaches. Both methods were compared, and the isolated compounds were evaluated by molecular docking and NA inhibition assay.

Materials and Methods

A single-mode microwave reactor (CEM™ Discover Labmate) was used for microwave experiments. ^{1}H, ^{13}C, and ^{19}F nuclear magnetic resonance (NMR) experiments were performed using an NMR spectrometer (Bruker AVANCE III 500 MHz). The chemical shifts are expressed in parts per million (ppm), and the splitting of signals are assigned as singlet (s), doublet (d), triplet (t), doublet of doublet (dd), and multiplet (m). Mass spectroscopic (MS) analyses were performed by an Agilent 1100 Series spectrometer. All chemicals purchased from suppliers were used without further purification.

Synthesis: Ethyl 4-fluoro-3-nitrobenzoate, 2

Conventional condition:

To a solution of 4-fluoro-3-nitro benzoic acid, **1** (2.00 g, 10.40 mmol) in ethanol (25 mL), 2.5 mL concentrated H_2SO_4 was added and refluxed at ~78 °C for 4–6 h. The progress of the reaction was monitored every hour by thin-layer chromatography (TLC). Upon completion, the solvent was removed under pressure. The mixture was dissolved in ethyl acetate (25 mL) and extracted with water containing 10% $NaHCO_3$. The organic layer was washed with water and dried in anhydrous Na_2SO_4. Filtered and concentrated crude product *in vacuo* was later purified using preparative thin-layer chromatography (PTLC) with ethyl acetate (EtOAc)/hexane (Hex) of 1:4 to give the ester product. The product obtained after concentrated *in vacuo* was 1.96 g (85%) of yellow solid.

Microwave condition:

Compound **2** was obtained after irradiation of a mixture containing 4-fluoro-3-nitrobenzoic acid, **1** (0.25 g, 1.35 mmol), 4% H_2SO_4 equiv. to the starting material, and 1.0 mL of ethanol. Similar work-up was carried out as in conventional procedure. The yellow solid obtained after concentrated *in vacuo* gave 0.21 g (74%).

R_f = 0.69 (EtOAc/Hex, 1:4). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.43 (t, J = 5.0 Hz, $OCH_2C\underline{H_3}$), 4.44 (q, J = 5.0, 10.0 Hz, $OC\underline{H_2}CH_3$), 7.39 (t, J = 10.0 Hz, benzene-$\underline{H_5}$), 8.31–8.35 (m, benzene-$\underline{H_6}$), 8.74 (dd, J = 5.0, 7.0 Hz, benzene-$\underline{H_2}$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.26, 62.13, 118.74, 127.61, 127.79, 136.49, 136.57, 158.03, 163.64; ^{19}F NMR (500 MHz, $CDCl_3$): -110.75; ESI-MS m/z calcd. for $[C_9H_8FNO_4]^+$ 213.2; found $[M + Na]^+$ 236.0.

Ethyl 4-(2-hydroxy-ethylamino)-3-nitro-benzoate, 3

Microwave condition:

4-fluoro-3-nitrobenzoic acid ethyl ester, **2** (0.25 g, 1.17 mmol) in 1 mL CH_2Cl_2 was mixed with N,N-diisopropylethylamine (DIPEA) (0.38 mL, 1.76 mmol), and ethanolamine (0.085 mL, 1.4 mmol) was added dropwise to the solution. The mixture was then irradiated in a CEM™ microwave at a temperature of 120 °C for 2 min (hold time). Upon completion, the mixture was filtered by suction and washed with hexane

to afford a yellow solid. The product was dried in a desiccator without further purification to give **3** (0.30 g, 94%).

Conventional condition:
4-fluoro-3-nitrobenzoic acid ethyl ester, **2** (1.00 g, 4.69 mmol) in 25 mL CH_2Cl_2 was mixed with DIPEA (1.51 mL, 7.0 mmol), and ethanolamine (0.33 mL, 5.63 mmol) was added dropwise to the solution. With the drying tube filled with anhydrous $CaCl_2$ attached to the round bottom flask, the mixture was stirred overnight at room temperature until a yellow precipitate formed. The precipitate was filtered by suction, washed with hexane, and dried in a desiccator without further purification to give **3** (1.18 g, 99%).

R_f = 0.60 (EtOAc/Hex, 4:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.40 (t, 3H, *J* = 5.0, 10.0 Hz, $OCH_2C\underline{H_3}$), 3.57 (q, *J* = 5.0, 10.0 Hz, $C\underline{H_2}CH_2OH$), 3.99 (q, *J* = 5.0, 10.0 Hz, $CH_2C\underline{H_2}OH$), 4.37 (q, *J* = 5.0, 10.0 Hz, $OC\underline{H_2}CH_3$), 6.92 (d, *J* = 10 Hz, benzene-$\underline{H_5}$), 8.31–8.34 (m, benzene-$\underline{H_6}$, 8.89 (d, *J* = 0.0 Hz, benzene-$\underline{H_2}$); ^{13}C NMR (125 MHz, $CDCl_3$): 14.39, 44.98, 60.72, 61.09, 113.48, 117.84, 129.47, 131.58, 136.43, 147.75, 165.16; ESI-MS m/z calcd. for $[C_{11}H_{14}N_2O_5]^+$ 254.2; found $[M + H]^+$ 255.1.

Ethyl 3-amino-4-(2-hydroxy ethylamino) benzoate, 4
Microwave condition:
A mixture of 4-(2-hydroxy-ethylamino)-3-nitrobenzoic acid ethyl ester, **3** (0.60 g, 2.63 mmol), NH_4HCO_2 (0.52 g, 8.25 mmol), and 10% Pd/C (0.30 g) in 2 mL of ethanol was irradiated in a microwave at 100 °C for 2 min (hold time). The pressure profile was monitored using the Synergy^{TM} programme so that the pressure did not exceed 20 atm. Upon completion, the catalyst was filtered through Celite and washed with ethanol, while the filtrate was concentrated *in vacuo* to afford a brown solid. The product **4** (0.50 g, 94%) was used for subsequent reaction without further purification.

Conventional condition:
A mixture of 4-(2-hydroxy-ethylamino)-3-nitrobenzoic acid ethyl ester, **3** (0.35 g, 1.38 mmol), NH_4HCO_2 (0.30 g, 4.82 mmol), and 10 % Pd/C (0.18 g) in 5 mL of ethanol was refluxed at 79–80 °C for 1–2 h until all reactants were consumed, as monitored by TLC. Upon completion, the catalyst was filtered through Celite by suction and washed with ethanol, while the filtrate was concentrated *in vacuo* to afford a brown solid **4** (0.31 g, 99%). The compound was used for the subsequent reaction without further purification.

R_f = 0.69 (EtOAc/Hex, 1:4). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.43 (t, *J* = 5.0 Hz, $OCH_2C\underline{H_3}$), 4.44 (q, *J* = 5.0, 10.0 Hz, $OC\underline{H_2}CH_3$), 7.39 (t, *J* = 10.0 Hz, benzene-$\underline{H_5}$), 8.31–8.35 (m, benzene-$\underline{H_6}$), 8.74 (dd, *J* = 5.0, 7.0 Hz, benzene-$\underline{H_2}$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.26, 62.13, 118.74, 127.61, 127.79, 136.49, 136.57, 158.03, 163.64; ^{19}F NMR (500 MHz, $CDCl_3$): -110.75; ESI-MS m/z calcd. for $[C_9H_8FNO_4]^+$ 213.2; found $[M + Na]^+$ 236.0.

General procedure for the preparation of compounds 5(a–g)
Microwave method:
A solution of 3-amino-4-(2-hydroxy-ethanolamine)-benzoic acid ethyl ester (**4**) (1 equiv.) in CH_3CN (1 mL) was mixed with K10-montmorillonite (0.02–0.26 g) and the corresponding aldehyde (2.1 equiv. with respect to the starting material). The mixture was irradiated in the CEM^{TM} microwave at 80 °C, 150 W, 5 bar, and held for 5 min. Then, another aliquot of aldehyde (2.1 equiv.) was added, and the reaction mixture was irradiated again at the same condition as before. The reaction was monitored by TLC and was left for a few days before doing the work-up. K10-montmorillonite was removed by filtration, washed with DCM, and evaporated *in vacuo* to afford a brown precipitate. The crude product was later purified using PTLC (EtOAc/Hex, 4:1).

Conventional method:
A solution of 3-amino-4-(2-hydroxy-ethanolamine)-benzoic acid ethyl ester (**4**) (1 equiv.) in CH_3CN (5–10 mL) was mixed with K10-montmorillonite (0.30–0.50 g) and the corresponding aldehyde (2.1 equiv. with respect to the starting material). The reaction was refluxed at 78–80 °C for 4–5 h, and the progress was monitored by TLC every hour. Upon completion, K10-montmorillonite and CH_3CN were removed by filtration and under pressure, respectively, before the crude product was purified using PTLC (EtOAc/Hex, 4:1).

The product obtained in the conventional method was compared with the product obtained from microwave synthesis based on the R_f value.

5a

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.31 (t, *J* = 10.0, 15.0 Hz, $CH_2C\underline{H_3}$), 1.44 (t, *J* = 10.0, 15.0 Hz, $OCH_2C\underline{H_3}$), 1.76 (br, s, $\underline{H_2}O$), 2.89 (q, *J* = 10.0, 15.0 Hz, $C\underline{H_2}CH_3$), 4.09 (t, *J* = 5.0, 10.0 Hz, $C\underline{H_2}CH_2OH$), 4.24 (t, *J* = 5.0, 10.0 Hz, $CH_2C\underline{H_2}OH$), 4.39 (q, *J* = 10.0, 15.0 Hz, $OC\underline{H_2}CH_3$), 5.09 (br, s, OH), 7.21 (d, *J* = 15.0 Hz, benzene-$\underline{H_7}$), 7.77 (d, *J* = 15.0 Hz, benzene-$\underline{H_6}$), 7.87 (s, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.64, 14.43, 20.95, 46.53, 60.43, 60.74, 108.70, 120.71, 123.65, 124.31, 137.77, 141.22, 158.68, 166.86; ESI-MS m/z calcd. For $[C_{11}H_{16}N_2O_3]^+$ 262.3; found $[M + H]^+$ 263.1.

5b

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.34 (d, *J* = 10.0 Hz, $H(C\underline{H_3})_2$), 1.40 (t, *J* = 10.0, 15.0 Hz, $OCH_2C\underline{H_3}$), 3.28 (septet, *J* = 10.0, 15.0 Hz, $\underline{H}(CH_3)_2$), 3.75 (br, s, $O\underline{H}$), 3.99 (t, *J* = 10.0 Hz, $C\underline{H_2}CH_2OH$), 4.31 (t, *J* = 10.0 Hz, $CH_2C\underline{H_2}OH$), 4.36 (q, *J* = 10.0, 15.0 Hz, $OC\underline{H_2}CH_3$), 7.30 (d, *J* = 15.0 Hz, benzene-$\underline{H_7}$), 7.86 (d, *J* = 15.0 Hz, benzene-$\underline{H_6}$), 8.35 (sharp, s, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.36, 21.51, 26.34, 45.87, 60.75, 60.82, 109.14, 121.22, 123.73, 124.38, 138.18, 141.90, 162.42, 167.29; ESI-MS m/z calcd. for $[C_{15}H_{20}N_2O_3]^+$ 276.3; found $[M + H]^+$ 277.1.

5c

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.98 (t, *J* = 10.0 Hz, $CH_2CH_2C\underline{H_3}$), 1.44 (t, *J* = 10.0, 15.0 Hz, $OCH_2C\underline{H_3}$), 1.74 (sextet, *J* = 10.0, 15.0 Hz, $CH_2C\underline{H_2}CH_3$), 2.85 (t, *J* = 10.0, 15.0 Hz, $C\underline{H_2}CH_2CH_3$), 4.09 (t, *J* = 5.0, 10.0 Hz, $C\underline{H_2}CH_2OH$), 4.25 (t, *J* = 5.0, 10.0 Hz, $CH_2C\underline{H_2}OH$), 4.39 (q, *J* = 10.0, 15.0 Hz , $OC\underline{H_2}CH_3$), 5.07 (br, s, $O\underline{H}$), 7.22 (d, *J* = 15.0 Hz, benzene-$\underline{H_7}$), 7.78 (dd, *J* = 0.0, 15.0 Hz, benzene-$\underline{H_6}$), 7.85 (s, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.89, 14.43, 21.00, 29.47, 46.59, 60.47, 60.73, 108.72, 120.72, 123.61, 124.34, 137.65, 141.26, 157.59, 166.83; ESI-MS m/z calcd. for $[C_{15}H_{20}N_2O_3]^+$ 276.3; found $[M + H]^+$ 277.1.

5d

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.93 (t, *J* = 10.0 Hz, $CH_2CH_2CH_2C\underline{H_3}$), 1.39 (septet, *J* = 10.0, 15.0 Hz, $CH_2CH_2C\underline{H_2}CH_3$), 1.43 (t, *J* = 7.5Hz, $OCH_2C\underline{H_3}$), 1.71 (quintet, *J* = 8.0 Hz, $CH_2C\underline{H_2}CH_2CH_3$), 2.86 (t, *J* = 8.0 Hz, $C\underline{H_2}CH_2CH_2CH_3$), 4.09 (t, *J* = 5.0 Hz, $C\underline{H_2}CH_2OH$), 4.25 (t, *J* = 5.0 Hz, $CH_2C\underline{H_2}OH$), 4.40 (q, *J* = 7.5 Hz, $OC\underline{H_2}CH_3$), 5.11 (br, s, OH), 7.21 (d, *J* = 8.5 Hz, benzene-$\underline{H_7}$), 7.77 (dd, *J* = 1.5, 8.5 Hz, benzene-$\underline{H_6}$), 7.85 (s, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.74, 14.43, 22.45, 27.28, 29.67, 46.65, 60.41, 60.73, 108.69, 120.63, 123.58 124.29, 137.60, 141.16, 157.79, 166.80; ESI-MS m/z calcd. for $[C_{16}H_{22}N_2O_3]^+$ 290.4; found $[M + H]^+$ 291.1.

5e

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.82 (t, *J* = 10.0, 15.0 Hz, $CH(CH_3)CH_2C\underline{H_3}$), 1.28 (d, *J* = 10.0 Hz, $CH(C\underline{H_3})CH_2CH_3$), 1.38 (t, *J* = 10.0 Hz, $OCH_2C\underline{H_3}$), 1.56–1.91 (m, $CH(CH_3)C\underline{H_2}CH_3$), 3.03 (sextet, *J* = 10.0, 15.0 Hz, $C\underline{H}(CH_3)CH_2CH_3$), 3.95 (t, *J* = 10.0 Hz, $C\underline{H_2}CH_2OH$), 4.30–4.38 (m, $OC\underline{H_2}CH_3$ and $CH_2C\underline{H_2}OH$), 7.33 (d, *J* = 15.0 Hz, benzene-$\underline{H_7}$), 7.87 (dd, *J* = 5.0, 12.5 Hz, benzene-$\underline{H_6}$), 8.37 (s, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 12.04, 14.36, 19.58, 29.13, 33.28, 45.66, 60.79, 61.10, 109.05, 121.45, 123.69, 124.47, 138.12, 142.23, 161.72, 167.26; ESI-MS m/z calcd. for $[C_{16}H_{22}N_2O_3]^+$ 290.2; found $[M + H]^+$ 291.1.

5f

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.80 (t, *J* = 10.0, 15.0 Hz, $CH(CH_2C\underline{H_3})_2$), 1.38 (t, *J* = 10.0, 15.0 Hz, $OCH_2C\underline{H_3}$), 1.67–1.90 (m, $CH(C\underline{H_2}CH_3)_2$), 2.86 (quintet, *J* = 5.0, 10.0 Hz, $C\underline{H}(CH_2CH_3)_2$), 3.97 (t, *J* = 10.0 Hz, $C\underline{H_2}CH_2OH$), 4.32–4.39 (m, $OC\underline{H_2}CH_3$ and $CH_2C\underline{H_2}OH$), 7.37 (d, *J* = 15.0 Hz, benzene-$\underline{H_7}$), 7.90 (dd, *J* = 0.0, 15.0 Hz, benzene-$\underline{H_6}$), 8.42 (s, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.96, 14.35, 27.45, 40.46, 45.72, 52.09, 60.73, 60.85, 109.35, 121.07, 123.62, 124.40, 138.14, 142.05, 160.84, 167.35; ESI-MS m/z calcd. for $[C_{17}H_{24}N_2O_3]^+$ 304.4; found $[M + H]^+$ 305.2.

5g

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.43 (t, *J* = 10.0, 15.0 Hz, $OCH_2C\underline{H_3}$), 1.74 (br, s, H_2O), 2.11 (s, $SC\underline{H_3}$), 2.92

(t, J = 10.0, 15.0 Hz, $CH_2C\underline{H_2}SCH_3$), 3.17 (t, J = 10.0, 15.0 Hz, $C\underline{H_2}CH_2SCH_3$), 4.07 (t, J = 5.0, 10.0 Hz, $C\underline{H_2}CH_2OH$), 4.29 (t, J = 5.0, 10.0 Hz, $CH_2C\underline{H_2}OH$), 4.39 (q, J = 10.0, 15.0 Hz, $OC\underline{H_2}CH_3$), 4.57 (br, s, OH), 7.27 (d, J = 10.0 Hz, benzene-$\underline{H_7}$), 7.82 (dd, J = 5.0, 12.5 Hz, benzene-$\underline{H_6}$), 7.97 (d, J = 5.0 Hz, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.41, 15.82, 27.80, 31.90, 46.76, 60.20, 60.82, 108.97, 120.66, 123.82, 124.48, 137.47, 141.00, 155.87, 166.73; ESI-MS m/z calcd. for $[C_{15}H_{20}N_2O_3S]^+$ 309.1; found $[M + H]^+$ 309.1.

General procedure for the preparation of compounds 6(a–g)

Microwave condition:

The obtained ester product **5(a–g)** in THF:H_2O (2:1) was mixed with 4 N NaOH (0.5 mL). The mixture was irradiated in the microwave at 120 °C for 2–5 min. Upon completion, THF was removed under pressure, and the product was acidified using 2 M HCl to maintain the pH at 6–7. Removing excess water *in vacuo* gave a white precipitate, which later was dissolved in butanol to separate the carboxylic acid and salt. The filtrate was again evaporated under vacuum to obtain the crude product. Purification was later carried out using PTLC (EtOAc/Hex, 4:1) by dissolving the crude product with methanol.

Conventional condition:

The obtained ester product **5(a–g)** either in THF (2 mL) or in a ratio of THF:H_2O (2:1) was added with 4 N NaOH (1–5 mL). The mixture was refluxed for 2–18 h at 66–68 °C until all reactants were fully converted to the desired acid, as observed in the TLC. Upon completion, THF was removed under pressure and acidified using 2 M HCl to maintain the pH at 6–7. Removing excess water *in vacuo* gave a white precipitate, which later was dissolved in butanol to separate the carboxylic acid and salt. The filtrate was again evaporated under vacuum to obtain the crude product. Purification was done using PTLC (EtOAc/Hex, 4:1) by dissolving the crude product with methanol.

6a

R_f = 0.70 ($MeOH/CHCl_3$, 4:1). ^{1}H NMR (500 MHz, D_2O): δ 1.43 (t, J = 5.0, 10.0 Hz, $CH_2C\underline{H_3}$), 3.13 (q, J = 5.0, 10.0 Hz, $C\underline{H_2}CH_3$), 3.96 (t, J = 5.0 Hz, $C\underline{H_2}CH_2OH$), 4.45 (t, J = 5.0 Hz, $CH_2C\underline{H_2}OH$), 7.64 (d, J = 10.0 Hz, benzene-$\underline{H_7}$), 7.93 (d, J = 10.0 Hz, benzene-$\underline{H_6}$), 8.07 (s, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, D_2O): δ 9.88, 19.40, 46.68, 59.17, 111.32, 115.43, 125.67, 132.57, 133.25, 134.31, 157.68, 174.02; ESI-MS m/z calcd. for $[C_{12}H_{14}N_2O_3]^+$ 234.3; found $[M + H]^+$ 235.1.

6b

^{1}H NMR (500 MHz, D_2O): δ 1.36 (d, J = 5.0 Hz, $H(C\underline{H_3})_2$), 3.39 (septet, J = 5.0, 10.0 Hz, $\underline{H}(CH_3)_2$), 3.94 (t, J = 5.0, 10.0 Hz, $C\underline{H_2}CH_2OH$), 4.41 (t, J = 5.0, 10.0 Hz, $CH_2C\underline{H_2}OH$), 7.53 (d, J = 10.0 Hz, benzene-$\underline{H_7}$), 7.84 (dd, J = 0.0, 5.0, 10.0 Hz, benzene-$\underline{H_6}$), 8.10 (d, J = 5.0 Hz, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, D_2O): δ 20.74, 25.89, 45.52, 59.88, 109.99, 118.52, 123.69, 130.81, 136.73, 140.68, 163.71, 175.84; ESI-MS m/z calcd. for $[C_{13}H_{16}N_2O_3]^+$ 248.1; found $[M + H]^+$ 249.1.

6c

^{1}H NMR (500 MHz, D_2O): δ 1.03 (t, J = 5.0, 10.0 Hz, $CH_2CH_2C\underline{H_3}$), 1.91 (sextet, J = 5.0, 10.0 Hz, $CH_2C\underline{H_2}CH_3$), 3.20 (t, J = 5.0, 10.0 Hz, $C\underline{H_2}CH_2CH_3$), 3.99 (t, J = 5.0 Hz, $C\underline{H_2}CH_2OH$), 4.57 (t, J = 5.0 Hz, $CH_2C\underline{H_2}OH$), 7.81 (d, J = 10.0 Hz, benzene-$\underline{H_7}$), 8.08 (d, J = 10.0 Hz, benzene-$\underline{H_6}$), 8.25 (s, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, D_2O): δ 12.69, 19.62, 27.02, 47.32, 59.04, 112.78, 115.87, 126.89, 127.87, 129.74, 134.94, 156.90, 169.12; ESI-MS m/z calcd. for $[C_{13}H_{16}N_2O_3]^+$ 248.1; found $[M + H]^+$ 249.1.

6d

^{1}H NMR (500 MHz, D_2O): δ 0.97 (t, J = 5.0, 10.0 Hz, $CH_2CH_2CH_2C\underline{H_3}$), 1.46 (sextet, J = 5.0, 10.0 Hz, $CH_2CH_2C\underline{H_2}CH_3$), 1.85 (quintet, J = 5.0, 10.0 Hz, $CH_2C\underline{H_2}CH_2CH_3$), 2.99 (t, J = 5.0, 10.0 Hz, $C\underline{H_2}CH_2CH_2CH_3$), 3.98 (t, J = 5.0 Hz, $C\underline{H_2}CH_2OH$), 4.42 (t, J = 5.0 Hz, $CH_2C\underline{H_2}OH$), 7.57 (d, J = 10.0 Hz, benzene-$\underline{H_7}$), 7.88 (d, J = 10.0 Hz, benzene-$\underline{H_6}$), 8.14 (s, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, D_2O): δ 13.04, 21.83, 26.31, 28.98, 45.71, 59.92, 109.87, 123.65, 130.73, 140.70, 159.06, 175.84; ESI-MS m/z calcd. for $[C_{14}H_{18}N_2O_3]^+$ 262.3; found $[M + H]^+$ 263.1.

6e

^{1}H NMR (500 MHz, D_2O): δ 0.92 (t, J = 5.0 Hz,

$CH(CH_3)CH_2C\underline{H_3}$), 1.47 (d, J = 5.0 Hz, $CH(C\underline{H_3})CH_2CH_3$), 1.84–1.96 (m, $CH(CH_3)C\underline{H_2}CH_3$), 3.52 (sextet, J = 5.0, 10.0 Hz, $C\underline{H}(CH_3)CH_2CH_3$), 4.01 (t, J = 5.0 Hz, $CH_2C\underline{H_2}OH$), 4.63 (t, $CH_2C\underline{H_2}OH$), 7.80 (d, J = 5.0 Hz, benzene-$\underline{H_7}$), 8.04 (dd, J = 10.0 Hz, benzene-$\underline{H_6}$), 8.17 (s, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, D_2O): δ 10.64, 17.72, 28.07, 32.39, 47.09, 59.02, 112.18, 114.81, 126.38, 130.71, 133.63, 133.78, 159.91, 173.48; ESI-MS m/z calcd. for $[C_{14}H_{18}N_2O_3]^+$ 262.3; found $[M + H]^+$ 263.1.

6f

^{1}H NMR (500 MHz, D_2O): δ 0.77 (t, J = 5.0, 10.0 Hz, $CH(CH_2C\underline{H_3})_2$), 1.71–1.84 (m, $CH(C\underline{H_2}CH_3)_2$), 3.03 (m, $C\underline{H}(CH_2CH_3)_2$), 3.92 (t, J = 5.0 Hz, $C\underline{H_2}CH_2OH$), 4.42 (t, J = 5.0 Hz, $CH_2C\underline{H_2}OH$), 7.54 (d, J = 10.0 Hz, benzene-$\underline{H_7}$), 7.82 (d, J = 10.0 Hz, benzene-$\underline{H_6}$), 8.09 (s, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, D_2O): δ 11.11, 27.37, 39.93, 45.50, 60.12, 110.08, 118.37, 123.58, 130.81, 136.72, 140.64, 161.89, 175.84; ESI-MS m/z calcd. for $[C_{15}H_{20}N_2O_3]^+$ 276.3; found $[M + H]^+$ 277.1.

6g

^{1}H NMR (500 MHz, D_2O): δ 2.10 (s, $SC\underline{H_3}$), 2.97 (t, J = 5.0, 10.0 Hz, $CH_2C\underline{H_2}SCH_3$), 3.13 (t, J = 5.0, 10.0 Hz, $C\underline{H_2}CH_2SCH_3$), 3.86 (t, J = 5.0 Hz, $C\underline{H_2}CH_2OH$), 4.21 (t, J = 5.0 Hz, $CH_2C\underline{H_2}OH$), 7.38 (d, J = 10.0 Hz, benzene-$\underline{H_7}$), 7.79 (dd, J = 10.0 Hz, benzene-$\underline{H_6}$), 8.07(s, benzene-$\underline{H_4}$); ^{13}C NMR (125 MHz, D_2O): δ 14.35, 26.60, 30.69, 45.70, 59.81, 109.81, 118.76, 123.80, 130.77, 136.66, 140.58, 156.24, 175.60; ESI-MS m/z calcd. for $[C_{13}H_{16}N_2O_3S]^+$ 280.3; found $[M + H]^+$ 281.1.

Molecular docking analysis

The structures were drawn and optimised into three-dimensional structures using Avogadro software [39]. Then, AutoDockTools (https://ccsb.scripps.edu/mgltools/) was employed to add non-polar hydrogens and merge them, define the atom types, and compute the Gasteiger charges. For the target protein, the NA protein structure (PDB ID: 3TI6) was retrieved from www.rcsb.org/pdb. Ligand, water, and ions were removed. Using AutoDockTools, polar hydrogen and Kollman charges were added before the file was saved in .pdbqt format. The size of grid points was set to 26, 30, and 40 with the grid centre to be 28.750, 14.639, and 19.556, for x-, y-, and z-coordinates, respectively. The spacing between points was set as default. Finally, Maestro 13.3 (Maestro, Schrödinger, LLC, New York, NY, 2021.) and PyMOL (PyMOL Molecular Graphics System, Version 2.0 Schodinger, LLC) were used to visualise the conformation of the best docking compounds.

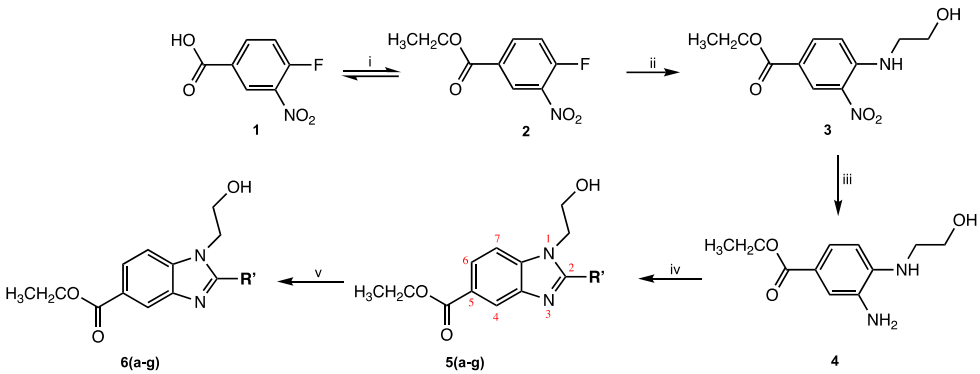
Enzyme inhibition study

The NA inhibition assay protocol was taken from Potier et al. [40] and Blick et al. [41]. The assay was performed in triplicate in a 96-well microplate. Firstly, 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (25 µL) was added to well 2–12. Then, the benzimidazole derivative (50 µL) was placed in the first well, and serial dilution was performed by taking 25 µL of the drug from the first well. To the plate, NA H1N1 (25 µL) virus was mixed with the dissolved benzimidazole in each well, sealed with aluminium foil, and then incubated at 37 °C for 30 min. 2'-(4-methylumbelliferyl)-alpha-D-N-acetylneuraminic acid (MUNANA) working solution was prepared 5 min before the end of the incubation period. After 30 min, MUNANA (50 µL) was added to each well, and two positive controls were prepared by adding (25 µL NA + 50 µL MUNANA) and (50 µL NA + 50 µL MUNANA), and a blank with 50 µL MES buffer into each well separately before they were incubated again at 37 °C for another 1 h. A stop solution (100 µL) was added to each well to terminate the reaction, and the microplate was immediately inserted into the microplate reader to measure the fluorescence of absorbance. The wavelengths set for the fluorescence detection were 355 nm and 460 nm for excitation and emission, respectively.

Results and Discussion

Chemistry

The multi-step reactions to prepare alkyl benzimidazoles **5(a–g)** in conventional and microwave approaches are outlined in Scheme 1. The spectroscopic data for this series confirmed the synthesised structures with the appearance of the aliphatic substituent signals in ^{1}H NMR and the presence of quaternary carbon in ^{13}C NMR that correlates with cyclisation occurring at C-2 around 159 ppm.



Label	R'
a	-CH_2CH_3
b	-$CH(CH_3)_2$
c	-$(CH_2)_2CH_3$
d	-$(CH_2)_3CH_3$
e	-$CH(CH_3)CH_2CH_3$
f	-$CH(CH_2CH_3)_2$
g	-$(CH_2)_2SCH_3$

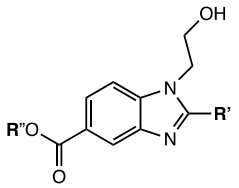
Scheme 1. Reagents and conditions: Conventional: (i) CH_3CH_2OH, H_2SO_4, 4–6 h, ~78 °C, 85%; (ii) $NH_2(CH_2)_2OH$, DIPEA, DCM, overnight, rt, 99%; (iii) Pd/C, NH_4HCO_2, ethanol, 1–2 h, 79–80 °C, 99%; (iv) K10-montmorillonite, MeCN, **R**'CHO, 4–5 h, 78–80 °C, 52–89%; (v) 4 N NaOH, THF, 2 M HCl, 4–6 h, 66–68 °C, 45–95%; Microwave: (i) CH_3CH_2OH, H_2SO_4, 15 min, 130 °C, 1–98%; (ii) $NH_2(CH_2)_2OH$, DIPEA, DCM, 2 min, 120 °C, 94%; (iii) Pd/C, NH_4HCO_2, ethanol, 2 min, 100 °C, 94%; (iv) K10-montmorillonite, MeCN, **R**'CHO, 5 min, 80 °C, 150 W, 5 bar, 29–66%; (v) 4 N NaOH, THF, 2 M HCl, 2–5 min, 120 °C, 59–90%. The numbering of the benzimidazoles is as indicated in **5(a–g)**

Due to the low solubility of the ester moieties in the buffer solution, the compounds were hydrolysed to carboxylic acids **6(a–g)** to increase their polarity. The disappearance of ester group signals in both ^1H and ^{13}C NMR spectra confirmed the formation of the products. Single crystals of **5c**, **5g**, and **6f** characterised by X-ray crystallography further confirmed their molecular structures [42-44]. The yield and duration for the preparation of the compounds from both conventional and microwave experiments are summarised in Table 1. Both techniques exhibited good to excellent conversions during esterification, S_NAr, reduction, and hydrolysis reactions, ranging from 59% to 94% for microwave and 45% to 99% for the conventional method. In contrast, during the cyclisation step, most of the compounds obtained gave low to moderate yields (23–69%), except **5f** (89%). However, there was a marked difference in terms of the duration needed to complete the reaction. In general, the reactions need 5–10 min under the microwave condition, although in some cases, the reactions can be performed in 2 min for the target compounds to yield almost similar results as under conventional conditions.

Neuraminidase inhibition assay

Benzimidazole carboxylic acid series **6(a–g)** were selected for bioassay analysis as the compounds showed good solubility in MES buffer. The NA inhibition assay was performed following the established protocols [39, 45]. One of the most potent NA inhibitors, 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (DANA), was used as a control for comparison. Once cleaved by the NA, MUNANA substrate will release the fluorescent methylumbelliferone and will be detected by the fluorometric method. The intensity of fluorescence relates directly to the amount of NA activity. The NA inhibition results for **6(a–g)** series tested at a single concentration of 250 µg/mL are shown in Figure 1. Compound **6f** gave the best inhibition at 15.2%, while the remaining compounds were shown to give inhibition of less than 10%. The data demonstrated that branched alkyl moieties at the C-2 position, such as in **6f** and **6b**, are slightly more tolerable for the inhibitory activity. In contrast, different alkyl chain substituents (up to four carbons) at the C-2 position are not favourable even with the presence of sulphur, as observed in compounds **6a**, **6c**, **6d**, and **6g**. Compound **6e**, although also possesses branched alkyl substituent at the C-2 position, showed lower inhibition results than **6f** and **6b**. Unfortunately, we cannot provide a detailed explanation for this observation other than the unsymmetrical branched alkyl that may affect the inhibition towards the enzyme.

Table 1. Reaction time and yield of products of benzimidazole derivatives under microwave and conventional conditions



5 & **6**

Compound			Time		[a]Yield (%)	
	R'	R"	Microwave (min)	Conventional (h)	Microwave	Conventional
2	-	-	15	2	74	85
3	-	-	2	24	94	99
4	-	-	2	1-2	94	99
5a		$-CH_2CH_3$	10	5	55	53
5b		$-CH_2CH_3$	10	4	51	69
5c		$-CH_2CH_3$	10	5	30	52
5d		$-CH_2CH_3$	10	5	40	23
5e		$-CH_2CH_3$	10	5	48	64
5f		$-CH_2CH_3$	10	5	66	89
5g		$-CH_2CH_3$	10	5	35	47
6a		H	5	3	76	45
6b		H	5	2	70	79
6c		H	5	3	83	77
6d		H	5	5	84	75
6e		H	5	18	59	95
6f		H	5	2	90	81
6g		H	5	8	86	74

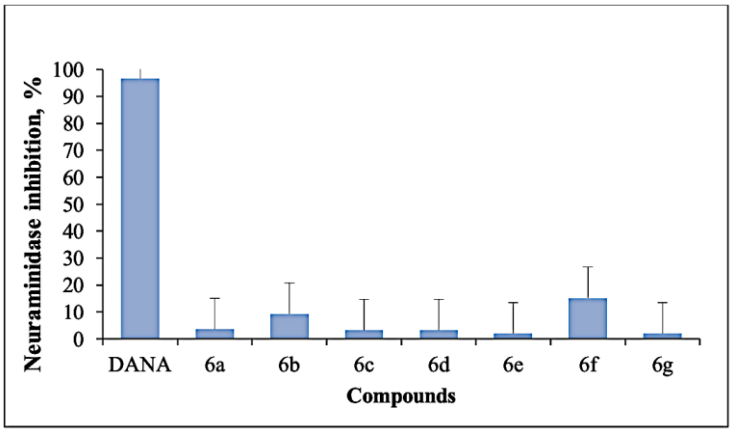


Figure 1. Percentage inhibition of **6(a–g)** analogues with DANA as a control at 250 µg/mL

Molecular docking

A molecular docking study was performed to gain a better understanding of how **6f** interacts in NA active sites at the molecular level. The NA crystal structure of 2009 H1N1 (PDB ID: 3TI6) with DANA as a reference was used in the study. The co-crystallised oseltamivir in the protein was removed and re-docked again into the NA active sites. Comparison between the co-crystallised and re-docked oseltamivir docking poses shows that the re-dock oseltamivir bound at the same location with the original ligand with slightly different conformation to give RMSD of 1.566 Å, suggesting the reliability of the docking algorithm in AutoDock VINA as the value is below the cut-off point 2.0 Å.

The NA active site of the 2009 pandemic H1N1 is unique from other N1-NA subtypes as it only consists of a sialic acid cavity and a 430-loop cavity with the absence of a 150-loop [39, 45]. In this study, DANA, which was used as a reference in the NA assay and docking study, gave the best docking energy of -6.1 kJ/mol, a slightly lower value than **6f** (-6.5 kJ/mol). Interestingly, the best docking pose of **6f** was found to bind at the 430-loop region, in contrast to DANA, which was found to bind at the sialic acid active site (Figure 2a). Further analysis on the 2D-ligand interaction between **6f** and DANA with 3TI6 was evaluated using Maestro (Schrödinger Release 2022-3, 2021).

Figure 2b(i) shows that there are nine amino acid residues take place in the binding interactions of **6f** in the 430-cavity with one hydrogen bond interaction between the carboxylic acid moiety and SER404. The remaining interactions are mostly hydrophobic interactions with ASN347, ARG371, ASN 372, ILE427, TRP403, LYS432, PRO431, ILE149, and ARG118 residues. The 3-pentanyl moiety of **6f** was found to interact with the ILE149 residues, suggesting that this type of branch alkyl group forms favourable hydrophobic interactions compared to other substituents in the carboxylic acid series. Meanwhile, DANA, which prefers to sit in the sialic acid cavity, also has one hydrogen bond interaction with THR225, while the remaining residues mainly form hydrophobic interactions. Although hydrophobic interaction is not as strong as hydrogen bonds, it can affect the docking free energy between ligand and enzyme. However, the presence of carboxylic acid group, especially for NA inhibitors, is crucial as it forms a strong hydrogen bond between the polar and charged residues of arginine with the oxygen atoms, which could give partial stability to the overall binding [46].

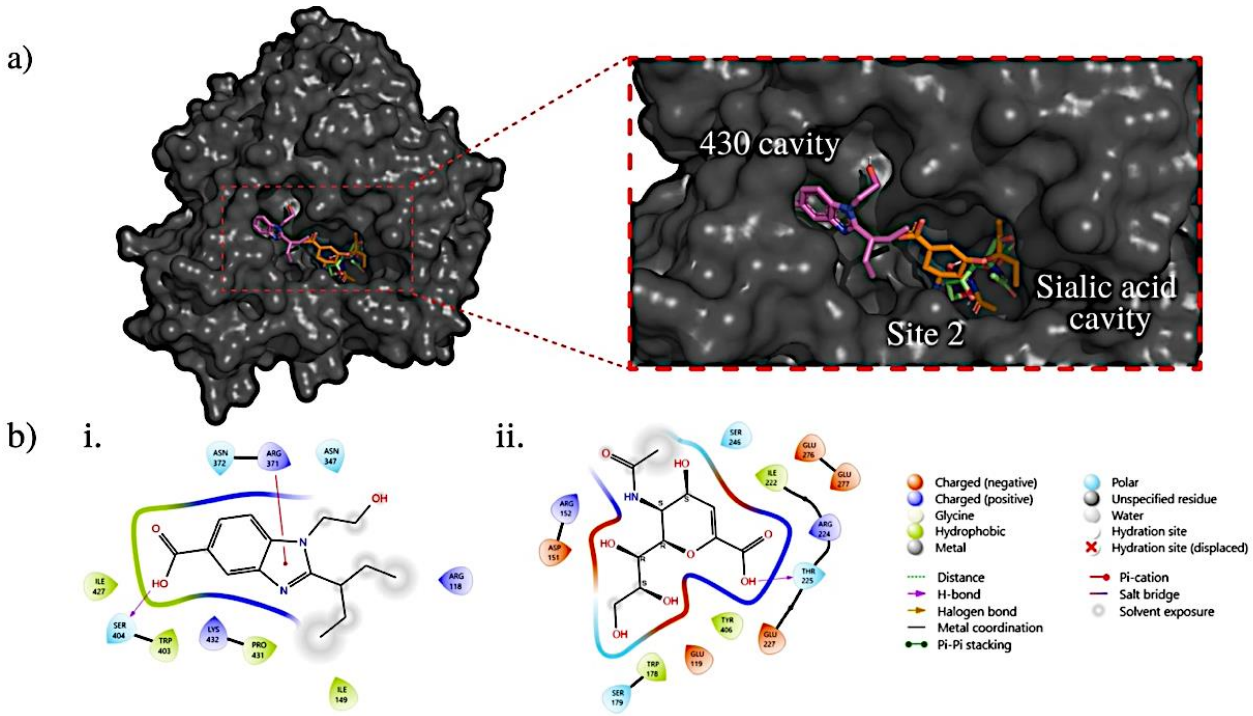


Figure 2. a) Best score docking poses of **6f** (yellow) in the 430-cavity in relation to the original ligand in the crystal structure (oseltamivir-orange) at site 2 and DANA (green) in the sialic acid region and b) the 2D-ligand interaction of i) **6f** and ii) DANA calculated in 4 Å distances from H1N1-NA (PDB ID: 3TI6) residue

Conclusion

In the present study, two series of benzimidazole analogues **5(a–g)** and **6(a–f)** were synthesised with good to excellent yields using both microwave and conventional methods. Microwave reaction was more effective than the conventional method as it can speed up the reaction time, although the yields obtained were generally slightly lower than those obtained by the conventional method. Compound **6f** was found to give the highest NA inhibition activity with 15.2% at 250 µg/mL, while others gave less than 10% inhibition. It is interesting to note that the molecular docking analysis revealed **6f** to bind at the 430-cavity of the NA active site, in contrast to DANA, which is at the sialic acid site, and oseltamivir at site 2. Despite the low inhibition data, the results show that benzimidazoles have the potential to be developed as NA inhibitors. Subsequent structural improvements of the benzimidazole scaffold could be designed towards more effective NA inhibitors.

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