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Synthesis, molecular docking, and in vitro tests of the Mannich base derivatives of Benzimidazolvanilin as an anti-inflammatory

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Abstract: Benzimidazole and vanillin are compounds which can act as anti-inflammatory. However, the potential of these two compounds is still low. The structure of benzimidazole and vanillin was combined with Mannich base substitution in order to increase potency and selectivity as an anti-inflammatory. In this study, benzimidazolvanillin (1) and its Mannich base derivatives were synthesized. The synthesized compounds (2a-d) show anti-inflammatory activity based on in vitro tests by using protein denaturation inhibition test. Furthermore, the IC50 obtained ranged from 210 -243 µM. This potency is lower than diclofenac (IC50: 2,99µM). In molecular docking, the results of the inhibition constant ratio between COX-1 / COX-1 as well as the interaction of the ligand and target protein show that benzimimidazolvanillin and its Mannich-base derivatives could be anti-inflammatory drug candidates that should be investigated further.

Keywords: Benzimidazolvanillin; Mannich base; anti-inflammatory; molecular docking

Introduction

Due to their efficacy as an analgesic and antipyretic, NSAIDs (Non-steroidal anti-inflammatory drugs) are popular drugs that are often used (Bindu, Mazumder, & Bandyopadhyay, 2020). However, NSAIDs have been widely reported to have side effects, such as intestinal problems, kidney ischemia, and cardiovascular complications (Puppala & Reddy, 2020).

Benzimidazole is a heterocyclic aromatic compound that consists of a benzene ring and an imidazole. Furthermore, this structure is a pharmacophore for various types of existing drugs; such as antibacterial, antiviral, antiparasitic, anticancer, antidiabetic, anti-inflammatory, and proton pump inhibitors (Vasuki B et al., 2021). The results of the Structure and Activity Relationship (SAR) analysis study, it shows that substituent at positions C2, C5, C6, and N1 of the benzimidazole core (Figure 1) have an effect on anti-inflammatory activity (Veerasamy, Roy, Karunakaran, & Rajak, 2021).

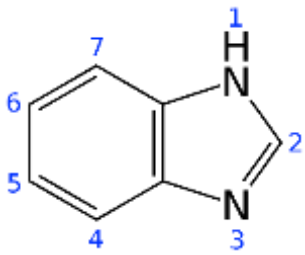

Figure 1. Structure of benzimidazole

Vanillin is a phenolic aldehyde compound that has been widely used as an additive in food, cosmetics, and pharmaceuticals. Both vanillin and its derivatives, such as vanilla acid, and vanilla alcohol are reported to have good affinity with TRPA-1 and TRPV-1 receptors which are associated with mediating a number of inflammatory mechanisms such as suppressing

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tachykinin production, decreasing macrophages, mast cell activation, reducing pro-inflammatory cytokines and reducing COX and NO synthase (Boiko, Nesterkina, Shandra, & Kravchenko, 2019).

Mannich bases are beta-amino ketones, which are essential building blocks for the synthesis of a wide range of substances, including medicines and natural chemicals. The actions of Mannich base heterocyclic compounds are diverse and include anti-inflammatory, anti-cancer, antibacterial, antifungal, anticonvulsant, anti-tuberculosis, and analgesic properties. They are also highly reactive (Geethapriya & Elumalaiim, 2021). Furthermore, Mannich base reactions allow the combining of amine fragments into various chemical structures, which allows for increasing the affinity of heterocyclic molecules towards their corresponding molecular targets (Janowska, Andrzejczuk, Gawryś, & Wujec, 2023). The application of Mannich bases to medicinal chemistry is important since Mannich bases can produce better biological activity, increase drug delivery into the body, and act as prodrugs so that it can release active substances under controlled conditions through deaminomethylation or deamination (Roman, 2015).

Method

The instruments used in this study were FTIR (Shimadzu IRXROSS QATR), magnetic stirrer (IKA C-MAG HS7), TLC vessel (CAMAG), melting point analog SMP11 (Stuart Scientific), analytical balance (Shimadzu), and glassware.

Meanwhile, the materials used were o-phenylenediamine (OPD), vanillin, pyrrolidine, morpholine, dimethylamine, diethylamine, formaldehyde, ethanol, and other chemical reagents from Merck.

Molecular docking hardware was Lenovo IdeaPad 5 Pro 14ACN6 with AMD Ryzen 7 5800U processor (CPU) with Radeon Graphics 1.90 GHz, 16 GB RAM memory, 512 GB SSD with Microsoft Windows 11 Home Version 22H2 operating system. Moreover, the software used was Autodock Tools 1.5.6, MarvinSketch 6.6.0, Pymol 2.5.5, Biovia Discovery Studio V21.1.0.20298, COX-1 target macromolecule (PDB ID: 1EQH) (Rimon et al., 2010) and COX -2 (PDB ID : 5IKQ) (Orlando & Malkowski, 2016) downloaded from the Protein Data Bank page http://www.rcsb.org/pdb.

Molecular docking of COX-1 and COX-2 receptors was conducted by using the target macromolecules 1EQH and 5IKQ which were downloaded from the RSCB PDB website. It is followed by separating the bound native ligands from each molecule. The chemical structure to be tested was drawn with MarvinSketch and it was converted to three-dimensional form in *.pdb format. Sodium diclofenac and celexocib as positive control was downloaded from go.drugbank.com/drugs. Molecular docking optimization was conducted by redocking native ligand molecules and analyzing their three-dimensional conformational interactions. The coordinates and size of the grid box were determined by the position of the native ligand which should cover the binding pocket of the target protein. Validation of the docking method was determined from the RMSD value resulting from the 3D conformational alignment of the binding site of the target protein. The docking method used is said to be valid if it has an RMSD value ≤ 2 Å. Each test ligand and positive control were docked and analyzed for affinity energy (ΔG, kcal.mol¬-1), binding pose at the active site, and type of binding interaction with amino acid residues (Shivanika et al., 2022).

The synthesis of benzimidazole vanillin (compound 1) was conducted by reacting ortho-phenylenediamine (5mmol) and vanillin (5mmol) in 100 mL ethanol solvent. This reaction was conducted by using a reflux apparatus at 80°C with stirring for 22 hours. During the reaction, monitoring was conducted every 2 hours by using TLC with the mobile phase chloroform: ethyl acetate: methanol (9:6:1). After the reaction was complete, the solution mixture was mixed with water until a precipitate formed. After that, evaporation was conducted at a temperature of 40°C in order to remove the ethanol solvent. After the ethanol evaporated, the precipitate formed was then filtered with filter paper by using a Buchner pump in order to separate the precipitate of compound 1 from water. The precipitate formed was washed with warm water in order to remove impurities and dried at a temperature of 35-40°C (Pessoa-Mahana, Espinosa-Bustos, Mella-Raipán, Canales-Pacheco, & Pessoa-Mahana, 2009).

Synthesis of Mannich base derivatives of benzimidazole vanilin (compounds 2a-d) was conducted by reacting 1 mmol of benzimidazole vanilin (product 1st step) with formaldehyde and secondary amine excess in ethanol solvent. The reaction was conducted at room temperature for 1 hour with stirring, followed by reacting with reflux at a temperature of 60-70 ^{o}C. The reaction was monitored by using TLC every 30 minutes until the reaction was complete. Following the completion of the reaction, the reaction mixture was evaporated in a vacuum oven at 30 ^{o} C, to eliminate excess formaldehyde, secondary amines, and solvent. It was followed by purification by column chromatography with the mobile phase ethyl acetate: methanol (1:4) in order to obtain pure compounds 2a-d (Marinescu et al., 2020).

Anti-inflammatory evaluation was conducted by using a protein denaturation inhibition test which was conducted with several modifications by using diclofenac as a standard. A total of 500µL of standard

solutions and test solutions in various concentrations were mixed in methanol with 4500µL of BSA solution (0.5%, w/v, pH 6.3) in TBS buffer solution. The solution combination was agitated and incubated at 37 °C for 15 minutes, then heated at 70°C for 10 minutes before being cooled to room temperature (25°C). The absorbance was then measured at 660 nm with a UV spectrophotometer. Each test chemical and standard were tested three times (in triplicate). 500 µL methanol was mixed with 4500 µL BSA (0.5%, w/v, pH 6.3) to make the negative control solution. The following equation was used to compute the percentage of protein denaturation inhibition:

$$\text{Inhibition (\%)} = \frac{\text{Abs. control} - \text{Abs. testing solution}}{\text{Abs. control}} \text{ x } 100\% \quad (1)$$

The IC50 value was calculated using a linear regression analysis of the association between the test compound's concentration and the percent inhibition. (Rahmawati, Hariyanti, Saputri, & Hayun, 2020).

Result and Discussion

Preparation of the macromolecular structure used will determine the prediction of the ligand-receptor interaction that will occur. At this stage, the 3D structure of each target protein is searched through the RCSB PDB website page. There are two target molecules (COX 1 and COX-2) was qualified, that have a three-dimensional structure as a result of x-ray diffraction because it can be applied to large macromolecular structures (>100 Kda) and is more precise and complex with the original ligand. The organism for the protein used is a human macromolecule, this is because this study is intended to develop drugs intended for humans.

Preparation of macromolecules, control ligands, and testing ligands were prepared using Autodock Tools. This preparation aims to remove water molecules and non-residue amino acid compounds. Next, the preparation of macromolecules continues by adding hydrogen atoms to adjust the pH atmosphere in the body and reappearing hydrogen atoms in the macromolecules so that the hydrogen bonds formed can be observed (Madhavi Sastry, Adzhigirey, Day, Annabhimoju, & Sherman, 2013). Apart from that, the Gasteiger charge is also added as a calculation of electrostatic interactions and desolvation energy (Forli et al., 2016).

The macromolecules that have been prepared are then re-docked with native ligands. This stage is carried out to validate the method, grid-box coordinates, and also the grid box size as the binding site for the test ligand. Redocking was carried out 100 times and then the RMSD value was obtained from the redocking results. RMSD is a value that determines the success of a docking method. If the value is <2 Å then the docking method is good. The grid box coordinates of each protein and the RMSD redocking results are in Table 1.

Table 1. Gridbox coordinates of 1EQH and 5IKQ macromolecules

Macromolecule	Coordinate			Dimension			RMSD
	X	Y	Z	X	Y	Z	
COX-1 (1EQH)	26.414	33.342	198.59	35	35	35	1,194
COX-2 (5IKQ)	22.431	51.926	17.405	40	40	40	0.416

Molecular docking uses computer-based techniques to predict complex interactions between ligands and receptors. Posing and scoring are the two primary steps in the docking process. (Torres, Sodero, Jofily, & Silva-Jr, 2019). The search algorithm will produce possible poses, which will then be ranked based on the score function (Agu et al., 2023). Binding free energy calculation shows the interaction of the ligand-receptor complex. ΔGbind = ΔG complex - ΔG protein + ΔG ligand) was the free binding energy equation used. A lower score indicates a higher binding energy (Owoloye et al., 2022). Stronger Van der Waals interactions arise from this increased binding, which is correlated with an increase in the protein-ligand interaction surface (Pantsar & Poso, 2018).

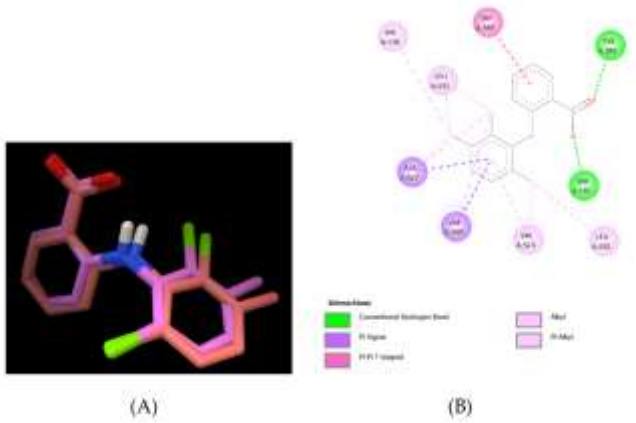


Figure 2. Superposition of Meclofenamic Acid in redocking with 5IKQ (A); ligand-receptor interaction Meclofenamic Acid with 5IKQ (B)

In redocking the native ligand (meclofenamic acid) on COX-2 and the native ligand (celecoxib) on COX-1, both produced an RMSD of < 2 Å. According to Gadhwal et al. Three binding sites are involved in COX-2 inhibition: (1) the hydrophilic amino acid residues Arg120, Tyr355, and Glu524 which are located at the entrance of the binding site; (2) the pocket at amino acid residues Tyr385, Leu 352, Trp387, Tyr248, and Phe518; (3) side pockets containing His90, Val523 and Arg513

(Gadhwal et al., 2011). This molecular docking uses sodium diclofenac as a standard ligand, which is one of the widely used NSAID drugs and celexocib, the COX-2 -selective inhibitor. The receptor-ligand interaction between diclofenac and the 5IKQ macromolecule involves the amino acid residues Ser530 and Tyr385 with hydrogen interactions and Val523, Ala527, Val349, Leu352, Phe518 with hydrophobic interactions. While celexocib inhibits COX-2, hydrogen interactions occur at Gln192, Ser353, and Phe518 as well as hydrophobic interactions with Leu531, Ala527, Val349, Val523, Leu352, Tyr385, Trp387, Leu384, and Met522. In the test ligand, compound 2b is the compound that has the lowest affinity energy and has the most amino acid residues in common with the amino acid binding site compared to the other test compounds. Even though compound 2b does not have hydrogen interactions with the amino acids contained in the binding site, it has hydrophobic interactions with Val523, Leu352, Tyr385, Trp387, and Tyr355, which are all residues of amino acids that are a component of the three primary binding sites for the inhibition of COX-2 (Figure 3). According to Deb et al., Van der Waals forces stabilize the majority of protein-ligand interactions in the largely hydrophobic COX-2 active site. (Deb, Mailabaram, Al-Jaidi, & Saadh, 2017). This allows compound 2b to have the lowest affinity energy compared to other test compounds.

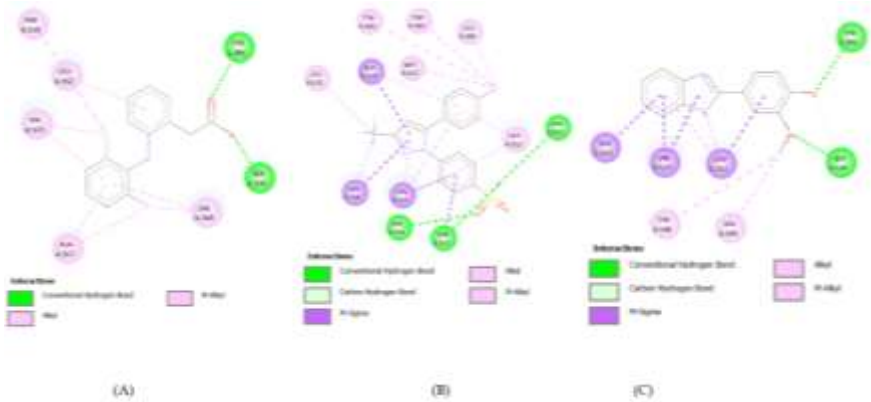


Figure 4. Ligand-receptor interaction of diclofenac (A); Celexocib (B) and Benzimidazolvanilin (C) with 5IKQ (COX-2)

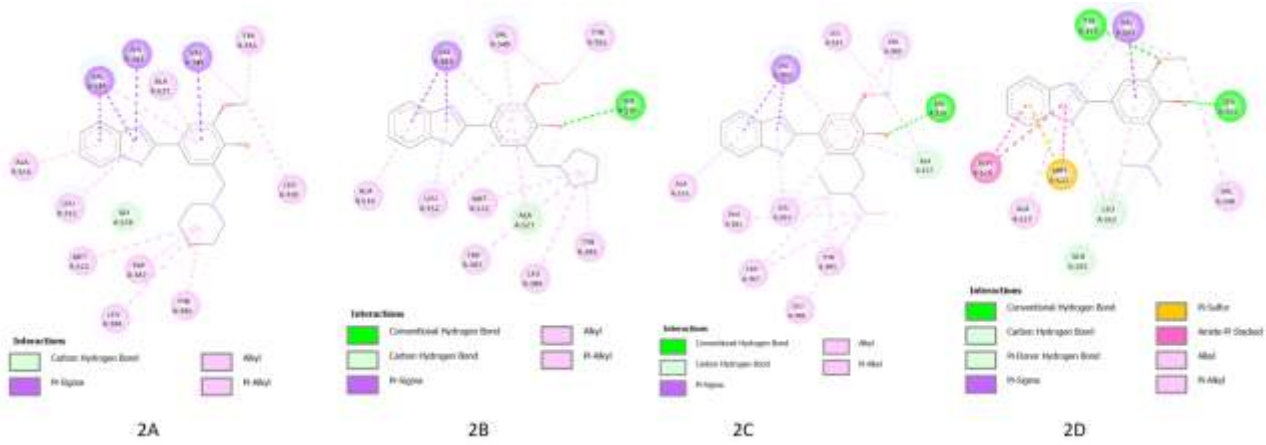


Figure 5. Ligand-receptors interaction of Mannich-base deravatives of Benzimidazolvanilin 2a-d with 5IKQ (COX-2)

Table 2. The affinity energy and ligand-receptor interactions that result from 5IKQ (COX-2) molecular docking

Ligand	Affinity Energy (kcal/mol)	Interactions between ligand and amino acid residues	
		Hydrogen	Non-Hydrogen
Meclofenamic Acid (native ligand)	-11,41	Ser530, **Tyr385**	Val116, Leu531, Ala527, Val349, **Leu352**, **Tyr385, Trp387**
Diclofenac	-8,38	Ser530, **Tyr385**	Ala527, Val349, **Val523**, **Leu352**, **Phe518**

Ligand	Affinity Energy (kcal/mol)	Interactions between ligand and amino acid residues	
		Hydrogen	Non-Hydrogen
Celexocib	-9,56	Gln192, Ser353, **Phe518**	Leu531,Ala527, Val349, **Val523**, **Leu352**,**Tyr385**, **Trp387**,Leu384,Met522
1	-7,80	Ser530, **Tyr385**	Val349, **Val523, Leu352,** Ser353, Tyr348
2a	-8,55	Gly526	Ala527, Val349, Val523, **Leu352**, **Tyr385, Trp387,** Ala516, Ser352, **Tyr355,** Leu359, Leu384, Met522
2b	-9,09	Ala527, Ser530	Val349, **Val523, Leu352, Tyr385, Trp387,** Ala516, **Tyr355,** Leu384, Met522
2c	-8,17	Ala527, Ser530	Leu531, Val349, **Val523, Leu352, Tyr385, Trp387,** Ala516, Leu384, Phe381
2d	-8,12	**Leu 352, Tyr355**, Ser353	Ala527, Val349, **Val523,** Met522, Gly526

*Amino acid residues in bold indicate the same amino acid residues at the binding-site

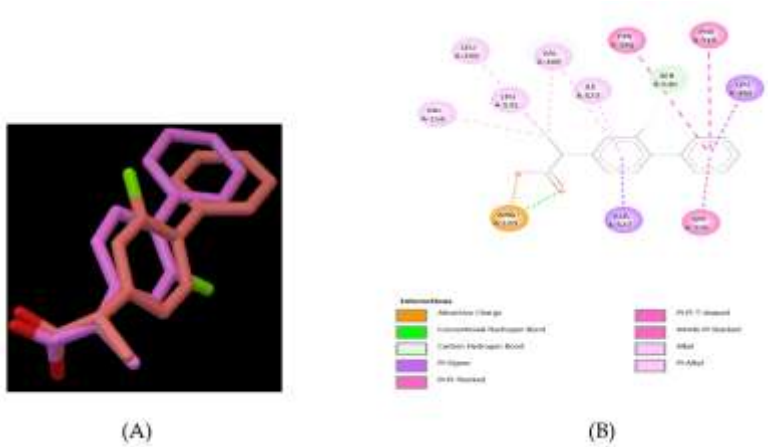


Figure 6. Superposition of Flurbiprofen in redocking with 1EQH (COX-1) (A) and its ligand-receptors interaction (B)

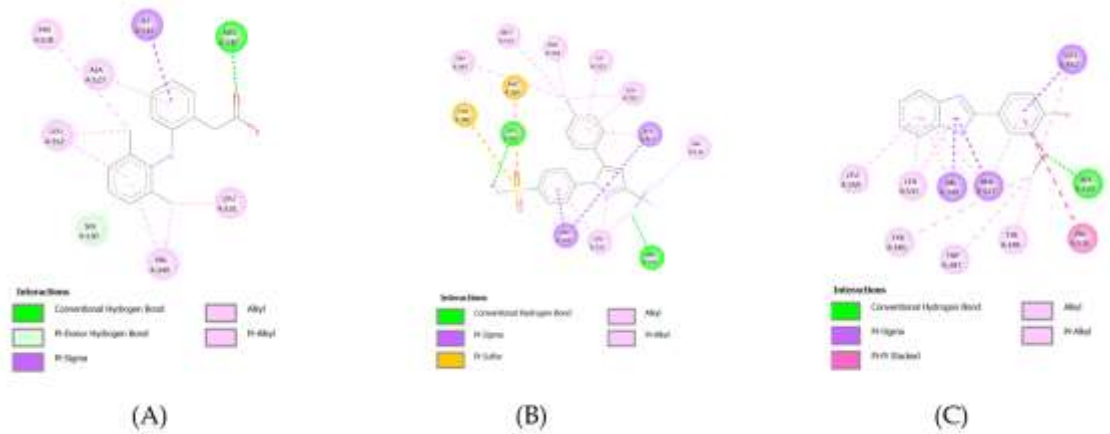


Figure 7. Ligand- receptors interactions of Diclofenac (A), Celexocib (B) and Benzimidazolvanilin (C) with 1EQH (COX-1)

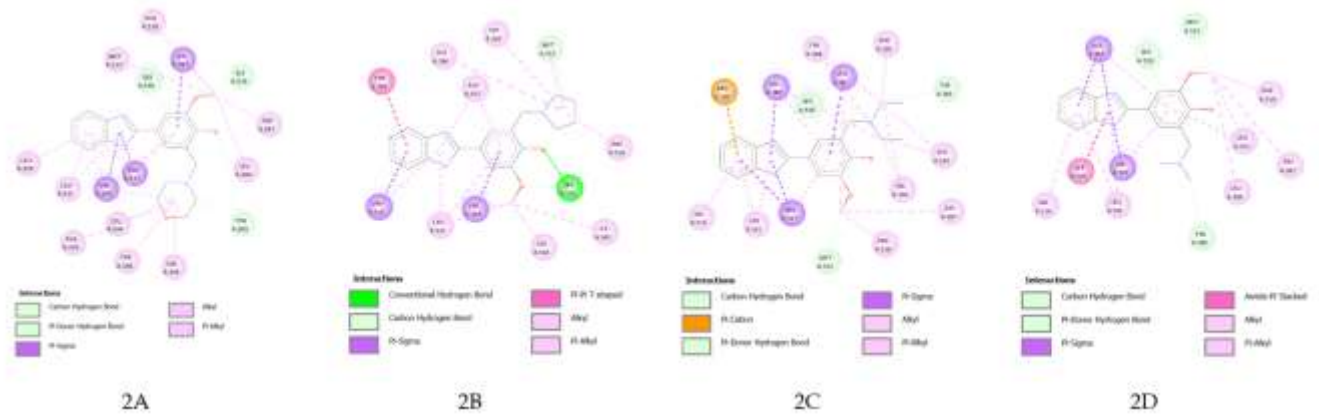


Figure 8. Ligand-receptors interaction of Mannich-base deravatives of Benzimidazolvanilin 2a-d with 1EQH (COX-1)

Table 3. The affinity energy and ligand-receptor interactions that result from 1EQH (COX-1) molecular docking

Ligand	Affinity Energy (kcal/mol)	Interactions between ligand and amino acid residues	
		Hydrogen	Non-Hydrogen
Flurbiprofen	-9,00	**Ser530**	Val116,Leu531, **Leu352, Ile523**, **Val349**, **Tyr385**, **Phe518**,**Gly526**, **Leu352**, **Ala527**, **Arg120**
Natrium Diklofenak	-7,85	**Arg120, Ser530**	Leu531, **Val349**, **Phe518**, **Leu352**, **Ala527**
Celexocib	-6,29	**Ser530, Arg120**	**Trp387**,**Met522**, **Phe518**, **Ile523**, **Leu352** ,**Ala527**, Val116, Leu531, **Val349, Tyr385**, Phe205
1	-7,87	**Ser530**	Leu531, **Leu352**, **Val349**, **Tyr385**, **Phe518**, Leu359, **Ala527**, **Trp387**, Tyr348
2a	-7,16	**Ser530, Gly526, Tyr385**	**Phe518**, **Met522**, **Leu352**, **Trp387, Leu384,** Val344, Tyr348, Leu534, Phe205, Leu531, Leu359, **Ala527, Val349**
2b	-7,25	**Ser530, Met522**	**Leu384, Ala527, Trp387, Phe518,** Ile345, Leu534, **Val349,** Leu531, Val116, **Tyr355**
2c	-7,43	**Met522, Ser530, Tyr385**	**Arg120, Val349,** Tyr348, **Leu352,** Phe205, Leu534, **Trp387, Phe518,** Val344, **Ala527**, Leu531, Val116
2d	-7,74	**Met522, Ser530, Tyr385**	**Ala527,** Val116, **Gly526**, **Val349**, Leu531, **Leu384, Trp387, Leu352, Phe518**

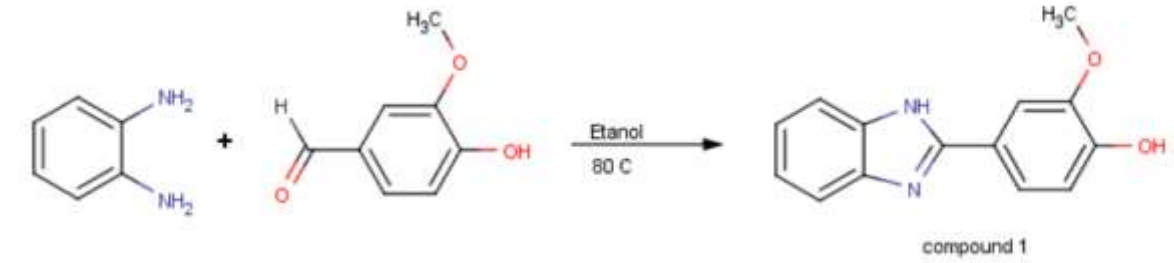
*Amino acid residues in bold indicate the same amino acid residues at the binding-site

The amino acid residues Ala527, Tyr355, Val349, Arg120, and Ser353 form the proximal binding pocket in COX-1. The amino acid residues Tyr385, Leu352, Leu384, Phe381, Phe518, Gly526, Met522, Trp387, and Ser530 form the central binding pocket; and the amino acid residues Ile434, Ile523, and His513 form the side pockets (Rouzer & Marnett, 2020). The amino acid sequences of COX-1 and COX-2 are similar to each other to the extent of 60–80%. The N (amino) and C (carboxyl) terminal amino acid sequences differ from one another. There are 17 amino acids at the COX-1 N-terminus that are absent from COX2. Conversely, COX-2's C-terminal contains eighteen amino acid sequences that are absent from COX-1. The 'side pockets' of COX-1 and COX-2 differ in their amino acid composition; specifically, COX-1 has Ile434, Ile523 and His513; while COX-2 has Val434, Arg513, and Val523. This difference in amino acid composition accounts for COX-2's larger binding pocket than COX-1's. (Faki & Er, 2021). The standard compound and the test compound were each observed for their ligand-receptor interactions (Table 2) and the inhibition constant ratio of COX-1/COX-2 was determined (Table 3). The compound is more selective against COX-2 when has a higher COX-1/COX-2 ratio

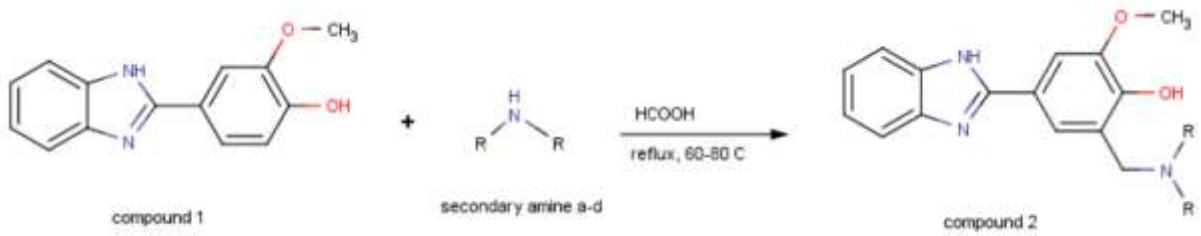
value. (Mengle-Gaw & Schwartz, 2002). Benzimidazolvanillin's selectivity index value is less than one, as can be seen from the data. On the other hand, the selectivity index (SI>1) seems to rise with the Mannich base derivative. While it has the highest selectivity index among the Mannich base derivatives, the pyrrolidine-substituted benzimidazolvanillin compound (2b) still has a lower selectivity index value than celexocib (SI=252.4). This indicates that benzimidazolvanillin's Mannich base derivative merits additional investigation as a selective COX-2 inhibitor.

Table 4. Selectivity Index (SI) of benzimidazole vanillin compounds and their Mannich base derivatives towards COX-2 and COX-1

Compound	Ki COX-2 (µM)	Ki COX-1 (µM)	Selectivity value (COX-1/ COX-2)	IC50 (µM)
Diklofenak	0,719	1,76	2,45	2,99
Celexocib	0,0977	24,66	252,4	-
1	1,92	1,72	0,90	270,55
2a	0,5413	5,61	10,36	231,06
2b	0,2182	4,87	22,32	210,43
2c	1,03	3,59	3,49	243,74
2d	1,12	2,14	1,91	229,55



(A)



(B)

Figure 2. Reaction scheme of Benzimidazolvanillin (1) and its Mannich base derivatives (2a-d)

Two steps were involved in the synthesis of the benzimidazole vanillin compound (1) and its Mannich base derivatives (2a-b). The condensation reaction between the aldehyde group of vanillin and o-phenylenediamine was the initial step. When carbonyl compounds and o-phenylenediamine condensed, a Schiff base intermediate was created. The primary amine of o-phenylenediamine acted as a nucleophilic, attacking the aldehyde group which was electrophilic in order to form an intermediate compound known as hemiaminal. Furthermore, hemiaminal compounds would form iminium ions which then rearranged to become imine without a proton on the N atom. At this step, 78.63% of the benzimidazole vanillin product was produced. Meanwhile, in step 2, a Mannich base reaction occurred between compound (1) with formaldehyde and secondary amines a-d (a is morpholine; b is pyrrolidine, c is diethylamine, d is dimethylamine) shown in Figure 2. In the Mannich base reaction, secondary amines that acted as nucleophiles would attack the aldehyde group of formaldehyde in order to form iminium ions accompanied by the release of water molecules. In the second step, a yield of 35-40% was produced. The resulting structure was then elucidated by using 13CNMR, 1HNMR, and FTIR instruments.

Table 4. Characterization and Elucidation Benzimidazolvanilin and its Mannich Base Derivatives

Compound	1	2a	2b	2c	2d
Characterization	Brownish-yellow solid M.P= 214-216°C	Light brown solid M.P =146-148°C	Brown solid M.P=110-112 C	Brownish-Yellow solid M.P= 44-46° C	Brown-Yellow solid M.P=50-52° C
FTIR	3317,62 (O-H bonding) 1602,90 (C=N) 1437,96 and 1497,75 (C=C aromatic) 1364,66 (C-H) 1265,32 (C-N) 1214,21 (Ar-O-C)	2850,79 (C-H aliphatic) 1602,85 (C=N) 1490,97 and 1435,04 (C=C aromatic) 1350,17 (C-H aromatic) 1273,02 (Ar-O-C) 1228,66 (C-N)	3118,90 and 2962,66 (C-H aliphatic) 1602,85 (C=N) 1431,18 and 1490,97 (C=C aromatic) 1342,46 (C-H aromatic) 1230,58 (C-N)	3151,69 (C-H aliphatic) 1602,85 (C=N) 1469,76 (C=C aromatic) 1269,16 and 1082,07 (Ar-O-C) 1201,65 (C-N)	2877,79 and 3091,89 (C-H aliphatic) 1595,13 (C=N) 1431,18 (C=C aromatic) 1269,16 and 1083,39 (Ar-O-C) 1230,58 (C-N)
12CNMR	55,10 (1C); 109,92 (1C); 114,30 (1C); 115,25 (1C); 119,79 (1C); 121,14 (1C); 128,16 (2C); 137,90 (1C); 138,40 (1C); 146,71 (1C); 148,10 (1C); 148,79 (1); 152,53 (1C)	50,61 (1C); 52,59 (2C); 55,07 (1C); 66,49 (2C); 109,33 (1C); 114,26 (1C); 118,18 (1C); 125,23 (1C); 127,32 (1C); 128,43 (2C); 134,65 (1C); 137,45 (1C); 138,48 (1C); 147,09 (2C); 149,43 (1C)	23,04 (2C); 52,95 (2C); 55,12 (1C); 56,64 (1C); 109,25 (1C); 119,96 (2C); 122,09 (1C); 127,52 (1C); 128,26 (2C); 134,76 (1C); 137,52 (1C); 140,35 (1C); 147,87 (1C); 148,73 (1C); 151,16 (1C)	13,73 (2C); 49,26 (2C); 52,71 (1C); 58,29 (1C); 111,09 (1C); 115,23 (1C); 120,53 (1C); 125,01 (1C); 127,52 (1C); 128,20 (2C); 134,85 (1C); 138,32 (2C); 147,23 (2C); 150,23 (1C)	43,12 (2C); 57,19 (1C); 60,23 (1C); 112,15 (1C); 115,23 (1C); 119,62 (1C); 125,63 (1C); 127,41 (1C); 128,21 (2C); 134,65 (1C); 138,23 (1C); 139,76 (1C); 148,42 (1C); 149,98 (1C); 152,52 (1C)
1HNMR	4,00 (3H, s) ; 6,94 (1H, d, 8,25 Hz); 7,18 (1H,q, 2,5/ 9,2 Hz); 7,22 (2H, tt); 7,56 (2H, dd, 10); 7,74 (1H, d, 2,5)	2,45 (4H, tt); 3,62 (4H,tt); 3,69 (3H,s); 4,12 (2H,s); 7,25 (2H,d, 8,1Hz); 7,3791 (2H,d,8Hz); 7,63 (1H,s); 7,72 (1H,s)	1,93 (4H,tt); 3,32 (4H,t); 3,97 (3H,s); 4,02 (2H,s); 7,24 (2H,q,3/9,1 Hz); 7,50 (1H,d); 7,57 (2H,s); 7,66 (1H,d,2 Hz)	1,18 (6H,t,7,2 Hz); 2,68 (4H,q,7 Hz); 3,67 (2H,s); 3,82 (3H,s); 7,2 (2H,d,4,5 Hz); 7,37 (1H,d,2 Hz); 7,41 (1H,s); 7,63 (1H,d, 1,45Hz); 7,86 (1H,s)	2.32 (6H,s); 3,65 (2H,s); 3,71 (3H,s); 7,2 (2H,d,5 Hz); 7,35 (1H,d, 2 Hz); 7,44 (1H,s); 7,56 (1H,d,2 Hz); 7,73 (1H,s)

Characterization and structure elucidation data are in table 4. In the FTIR spectrum of compound 1, the spectrum at wavenumber is 3317 (Ar-OH/phenolic), 1602 (C=N in benzimidazole), 1497 (C=C aromatic), 1265 (C-N), and 1214 (Ar-O-C).In the Mannich derivative base spectrum, a spectrum at 2800-3100 cm-1 is formed, indicating the aliphatic of the secondary amine, and there is also a shift in the wave number towards a lower wave number (1220-1230), indicating an increase in intermolecular forces caused by the Mannich base substituent. A spectrum of 13-50 ppm in CNMR and 1.1 - 2.45 ppm in NMR appears, indicating the presence of aliphatic carbon from Mannich base amine

The heat-induced protein denaturation method was used to test the anti-inflammatory activity. As a protein substrate, 0.5% bovine serum albumin (BSA) was used in this method. After the BSA was heated, the turbidity of the solution was measured for absorbance at a wavelength of 660nm. This wavelength is used to measure the optical density of protein aggregation. The anti-inflammatory test results show that the benzimidazole vanillin compound and its Mannich base derivative have lower anti-inflammatory activity than sodium diclofenac (IC50: 2,99 µM). For the Selectivity Index indicated by the COX1/COX2 inhibition constant ratio, the The selectivity index value of benzimidazolvanillin and its Mannich base derivatives is higher than that of diclofenac sodium but lower than that of celexocib. This indicates that further research is needed to determine whether benzimimidazolvanillin and its Mannich-base

derivatives are viable options for anti-inflammatory medications. The reason for this could be that the pharmacophore of COX-2-selective inhibitors has not been fulfilled by the structure of benzimidazolvaniline and its Mannich base derivatives in this investigation. (Michaux et al., 2006) reported that the pharmacophore of selective COX-2 inhibitors comprises two hydrophobic groups, one hydrogen bond acceptor, and one aromatic ring. In this particular compound, there is only one aromatic ring, which is the benzimidazole building block, along with one hydrophobic group derived from methoxybenzene and one hydrogen acceptor from tertiary amine.

Conclusion

The compound benzimidazole vanillin and its Mannich base derivatives have been successfully synthesized. Its potential as an anti-inflammatory is lower than sodium diclofenac with a range of IC_{50} 210-243 µM. However, related to the selectivity of inhibition, molecular docking towards COX-1 (1EQH) and COX-2 (5IKQ) suggests that benzimimidazolvanillin and its Mannich-base derivatives could be anti-inflammatory drug candidates that should be investigated further.

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Author Contributions
All aspects of the study, including procedures, reagents, and material acquisition, were designed by Inas and Hayun. Inas conducted the research, gathered data, and wrote and edited the manuscript with Hayun. Hayun and Arry oversaw the research process, gathered data, and made writing suggestions. All contributing authors approved the final manuscript, and permission was granted for the final draft to be published.

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The authors state unequivocally that no conflict of interest exists.

References

Agu, P. C., Afiukwa, C. A., Orji, O. U., Ezeh, E. M., Ofoke, I. H., Ogbu, C. O., … Aja, P. M. (2023). Molecular docking as a tool for the discovery of molecular targets of nutraceuticals in diseases management. *Scientific Reports*, *13*(1). https://doi.org/10.1038/s41598-023-40160-2

Bindu, S., Mazumder, S., & Bandyopadhyay, U. (2020). Non-steroidal anti-inflammatory drugs (NSAIDs) and organ damage: A current perspective. *Biochemical Pharmacology*, *180*, 114147. https://doi.org/10.1016/j.bcp.2020.114147

Boiko, Y. A., Nesterkina, M. V., Shandra, A. A., & Kravchenko, I. A. (2019). Analgesic and Anti-Inflammatory Activity of Vanillin Derivatives. *Pharmaceutical Chemistry Journal*, *53*(7), 650–654. https://doi.org/10.1007/s11094-019-02056-2

Deb, P. K., Mailabaram, R. P., Al-Jaidi, B., & Saadh, M. J. (2017). Molecular Basis of Binding Interactions of NSAIDs and Computer-Aided Drug Design Approaches in the Pursuit of the Development of Cyclooxygenase-2 (COX-2) Selective Inhibitors. In *Nonsteroidal Anti-Inflammatory Drugs, 2*, 64. https://doi.org/10.5772/intechopen.68318

Faki, Y., & Er, A. (2021). Different chemical structures and physiological/pathological roles of cyclooxygenases. *Rambam Maimonides Medical Journal*, *12*(1). https://doi.org/10.5041/RMMJ.10426

Forli, S., Huey, R., Pique, M. E., Sanner, M. F., Goodsell, D. S., & Olson, A. J. (2016). Computational protein-ligand docking and virtual drug screening with the AutoDock suite. *Nature Protocols*, *11*(5), 905–919. https://doi.org/10.1038/nprot.2016.051

Gadhwal, M. K., Article, R., D'mello, P., Kumar Gadhwal, M., Joshi, U., & Shetgiri, P. (2011). Modeling of COX-2 inhibotory activity of flavonoids. In *Article in International Journal of Pharmacy and Pharmaceutical Sciences, 3*(4), 33-40. Retrieved from https://www.researchgate.net/publication/224054089

Geethapriya, C., & Elumalaiim, K. (2021). Mannichbases: An Overview Of Heterocyclic Compound With Various Biological Activities. *International Journal of Pharmaceutical Sciences and Research*, *12*(12), 6151. https://doi.org/10.13040/IJPSR.0975-8232.12(12).6151-65

Janowska, S., Andrzejczuk, S., Gawryś, P., & Wujec, M. (2023). Synthesis and Antimicrobial Activity of New Mannich Bases with Piperazine Moiety. *Molecules*, *28*(14). https://doi.org/10.3390/molecules28145562

Madhavi Sastry, G., Adzhigirey, M., Day, T., Annabhimoju, R., & Sherman, W. (2013). Protein and ligand preparation: Parameters, protocols, and influence on virtual screening enrichments. *Journal of Computer-Aided Molecular Design*, *27*(3),

221–234. https://doi.org/10.1007/s10822-013-9644-8

Marinescu, M., Cinteză, L. O., Marton, G. I., Chifiriuc, M. C., Popa, M., Stănculescu, I., … Stavarache, C. E. (2020). Synthesis, density functional theory study and in vitro antimicrobial evaluation of new benzimidazole Mannich bases. *BMC Chemistry*, *14*(1). https://doi.org/10.1186/s13065-020-00697-z

Mengle-Gaw, L. J., & Schwartz, B. D. (2002). Cyclooxygenase-2 inhibitors: Promise or peril? *Mediators of Inflammation*, *11*, 275–286. https://doi.org/10.1080/09629350290000041

Michaux, C., de Leval, X., Julémont, F., Dogné, J. M., Pirotte, B., & Durant, F. (2006). Structure-based pharmacophore of COX-2 selective inhibitors and identification of original lead compounds from 3D database searching method. *European Journal of Medicinal Chemistry*, *41*(12), 1446–1455. https://doi.org/10.1016/j.ejmech.2006.07.017

Orlando, B. J., & Malkowski, M. G. (2016). Substrate-selective inhibition of cyclooxygeanse-2 by fenamic acid derivatives is dependent on peroxide tone. *Journal of Biological Chemistry*, *291*(29), 15069–15081. https://doi.org/10.1074/jbc.M116.725713

Owoloye, A. J., Ligali, F. C., Enejoh, O. A., Musa, A. Z., Aina, O., Idowu, E. T., & Oyebola, K. M. (2022). Molecular docking, simulation and binding free energy analysis of small molecules as Pf HT1 inhibitors. *PLoS ONE*, *17*(8), e0268269. https://doi.org/10.1371/journal.pone.0268269

Pantsar, T., & Poso, A. (2018). Binding affinity via docking: Fact and fiction. *Molecules*, *23*(8), 1899. https://doi.org/10.3390/molecules23081899

Pessoa-Mahana, D., Espinosa-Bustos, C., Mella-Raipán, J., Canales-Pacheco, J., & Pessoa-Mahana, H. (2009). Microwave-assisted synthesis and regioisomeric structural elucidation of novel benzimidazo[1,2d][1,4]benzodiazepinone derivatives. *General Papers ARKIVOC*, 131–140. Retrieved from https://repositorio.uchile.cl/handle/2250/120942

Puppala, N., & Reddy, G. A. (2020). Review on Effects of NSAID`S on Different Systems. *Asian Journal of Pharmaceutical Research and Development*, *8*(1), 100–109. https://doi.org/10.22270/ajprd.v8i1.621

Rahmawati, N., Hariyanti, H., Saputri, F. C., & Hayun, H. (2020). Synthesis and preliminary in vitro anti-inflammatory evaluation of mannich bases derivatives of 4′-methoxy-substituted of asymmetrical cyclovalone analogs. *Indonesian Journal of Pharmacy*, *31*(1), 35–41. https://doi.org/10.14499/indonesianjpharm31iss1pp35

Rimon, G., Sidhu, R. S., Lauver, D. A., Lee, J. Y., Sharma, N. P., Yuan, C., … Smith, W. L. (2010). Coxibs interfere with the action of aspirin by binding tightly to one monomer of cyclooxygenase-1. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(1), 28–33. https://doi.org/10.1073/pnas.0909765106

Roman, G. (2015). Mannich bases in medicinal chemistry and drug design. *European Journal of Medicinal Chemistry*, *89*, 743–816. https://doi.org/10.1016/j.ejmech.2014.10.076

Rouzer, C. A., & Marnett, L. J. (2020, August 12). Structural and Chemical Biology of the Interaction of Cyclooxygenase with Substrates and Non-Steroidal Anti-Inflammatory Drugs. *Chemical Reviews*, *120*, 7592–7641. https://doi.org/10.1021/acs.chemrev.0c00215

Shivanika, C., Deepak Kumar, S., Ragunathan, V., Tiwari, P., Sumitha, A., & Brindha Devi, P. (2022). Molecular docking, validation, dynamics simulations, and pharmacokinetic prediction of natural compounds against the SARS-CoV-2 main-protease. *Journal of Biomolecular Structure and Dynamics*, *40*(2), 585–611. https://doi.org/10.1080/07391102.2020.1815584

Torres, P. H. M., Sodero, A. C. R., Jofily, P., & Silva-Jr, F. P. (2019, September 2). Key topics in molecular docking for drug design. *International Journal of Molecular Sciences*, *20*. https://doi.org/10.3390/ijms20184574

Vasuki B, Mahadevan N, Vijayabaskaran M, Mohanapriya K, Kosilamani P, Balaji K, … Sambathkumar R. (2021). An insight into the medicinal perspectives of mannich bases of benzimidazole derivatives: A review. *International Journal of Research in Pharmaceutical Sciences*, *12*(3), 1895–1901. https://doi.org/10.26452/ijrps.v12i3.4790

Veerasamy, R., Roy, A., Karunakaran, R., & Rajak, H. (2021). Structure–activity relationship analysis of benzimidazoles as emerging anti-inflammatory agents: An overview. *Pharmaceuticals*, *14*. https://doi.org/10.3390/ph14070663