International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.07 pp 453-465, 2016

Identification of triazole bridged amino acids appended indoles as dual inhibitors of 5-LOX and COX-2 enzymes

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Abstract : A new set of compounds containing indole nucleus appended with carefully selected amino acids via a triazole linker are identified. These compounds are found to be effective against the enzymes responsible for inflammation. The in-vitro enzyme immunoassay studies on the test compounds indicated substancial activities which become amplified for the compounds with free carboxyl group. The kinetic experiments indicated favourable enzyme-drug interactions with 1:1 stoichiometry being the most favoured. The in vivo experiments on these compounds indicated the acceptable limits of toxicity compared to the control. All these findings were in close coordination with the docking investigations.
Keywords : 5LOX – 5lipoxygenase, COX-1/2 – cyclooxygense ½, MIC_{80} - minimum inhibitory concentration for 80% inhibition, ITC – isothermal titration calorimetry.

1. Introduction

Identification of various enzymes participating in the arachidonic acid metabolism and recognition of inflammatory metabolites led to systematic studies for the development of anti-inflammatory drugs.[1,2,3] Starting with the stimulatory role of phospholipases to release arachidonic acid from phospholipids, a COX-2 and 5-LOX over-expression causes an abnormal production of the respective metabolites. Excessive production of prostaglandins and leukotrienes[4] leads to chronic inflammation in humans.[5] The consequent ailments are expressed in the form of asthma, atherosclerosis, irritable bowel syndrome, arthritis and cancer.[6] Targeting the induced isoform of cyclooxygenase viz. COX-2, the anti-inflammatory medication has traversed a long journey.[7] Starting from aspirin to COXIBS several SAIDs′ and NSAIDs′ have been developed for targeting the enzymes of arachidonic acid pathway and hence for the treatment of inflammation. However there are certain limitations allied with these drugs, e.g., the prolonged use of COXIBS[8] leads to the inhibition of COX-2 in blood vessels which contributes to a decrease in the production of prostacyclin[9] which consequently facilitates the platelet aggregation and hence vasoconstriction. This may lead to a higher blood pressure thus increasing the risk of heart attack and stroke.[10] It was reported that 80,000 to 1,39,000 patients suffered from serious heart diseases due to the overuse of the drug *rofecoxib*, which caused the withdrawal of the drug by Merck.[11] Other COXIBS like valdecoxib and celecoxib have faced similar setbacks. Hence, it seems quite obvious that the rhythm of the development of the anti-inflammatory medication has not kept a pace with the current clinical demands because irrespective of their effectiveness, the safety usage is still a major challenge. Certainly, there is a need for the development of effective, target specific drugs having minimum side-effects.

Indole is one of the naturally occurring heterocycles which is a part of numerous biologically active substances (Figure 1), like amino acids (tryptophan), alkaloids (tryptamine), hormones (melatonin, auxins), neurotransmitters (serotonin) and peptide hormones (growth hormone releasing peptide) etc.

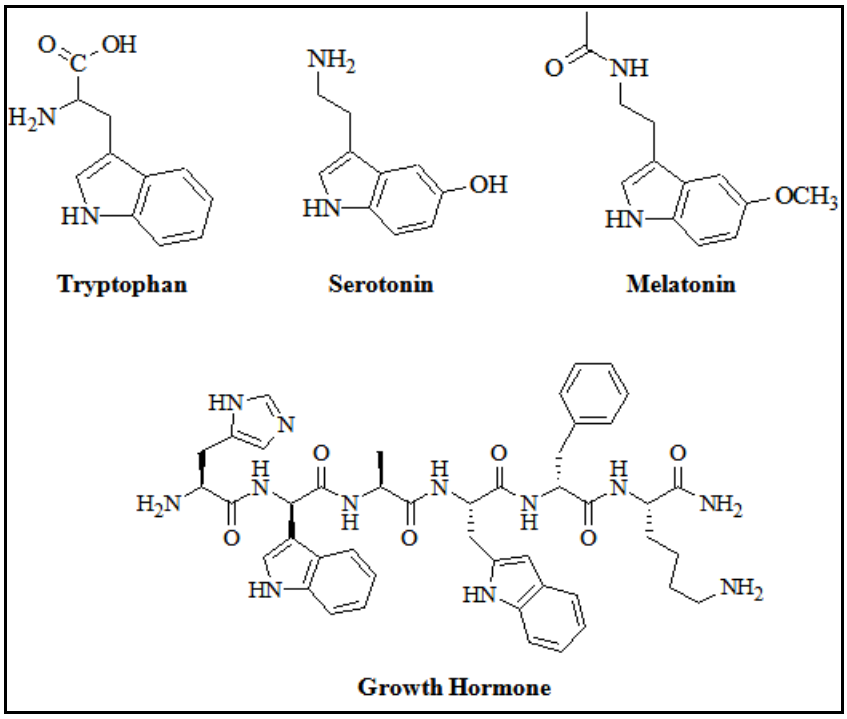


Figure 1. Naturally occurring substances having indole nucleus.

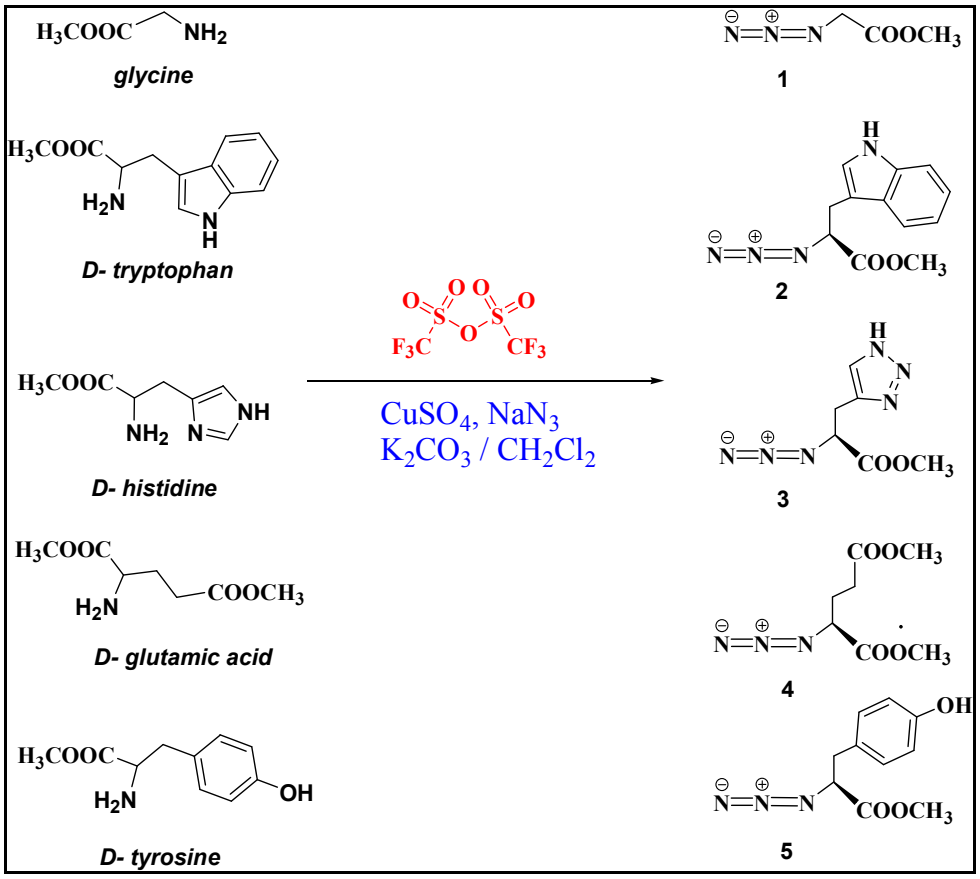
By targeting the perpetrator enzymes, a number of commercially available drugs including indomethacin, tenidap, pravadoline containing indole and targeting cyclooxygenase enzyme are well documented. Marking lipoxygenase enzyme as the potential target of anti-inflammatory drugs, MK886 and MK0591 are developed which work through inhibition of 5-LOX activating protein.[12] Montelukast and zafirlukast are effective against asthma and function by blocking leukotrienes production and acting as Leukotriene Receptor Antagonists.[13]

Principally the N1, C3 substituted indoles are well scrutinized for the purpose of developing anti-inflammatory drugs. Apart from the indoles, the use of peptides as a part of anti-inflammatory therapy has also been well studied. Peptides, being biologically acceptable, easy to administer and having minimum side effects,[14] are proved to be a better alternative to the previously used drugs against inflammation. The present set of compounds are designed to focus the inflammation causing lipoxygenase and cyclooxygenase enzymes. Indole nucleus has been appended by amino acids via triazole linker and accordingly compounds 8-17 were synthesized and checked for their activity against the 5-LOX and COX-1/2 enzymes.

2. Experimental

Synthesis:

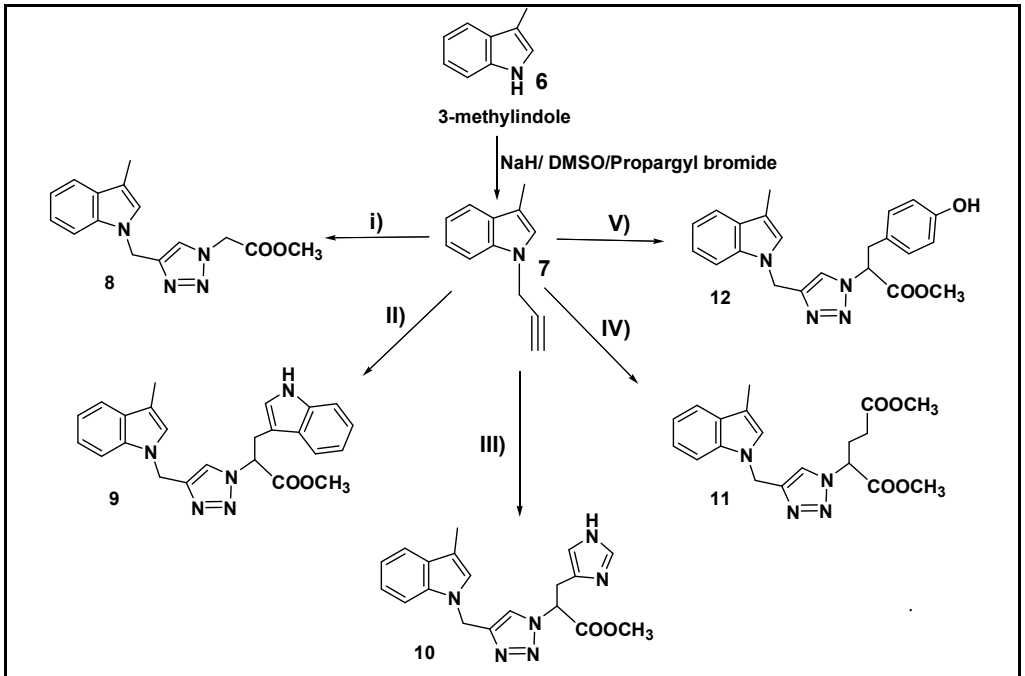
The L-amino acids (1 mmol) were dissolved in 20 ml of DCM in a round bottom flask kept on an ice bath. The mixture was stirred for a few minutes till the solution becomes clear. Activated K_2CO_3 (1.2 equivalent, activated by storing at 100 ^{0}C overnight) was then added to this solution. Afterwards trifluoromethanesulphonic anhydride (0.1 mmol) was very carefully added, dropwise to the reaction mixture. On addition, the colour of the reaction mixture immediately changes from colourless to light green. After stirring the contents of reaction mixture for 2-3 minutes $CuSO_4.5H_2O$ (2.0 mmol) was added followed by the addition of sodium azide (1.0 mmol). The progress of the reaction was monitored by thin layer chromatography. The reaction completed in about 6-8 hours. After the reaction completion, the reaction mixture was washed with 15 ml of distilled water and the product was extracted by chloroform using a separator funnel. The final product was azide adduct of the amino acid which is generally procured in a very good yield (compounds 1,2,3,4 and 5; Scheme 1).



Scheme-1

3-methylindole (6) was dissolved in 5 ml DMSO and allowed to stir for a few seconds till a clear solution is obtained. To this solution was added sodiumhydride (1.2 equivalent, washed with dry hexane to remove the paraffin coating). The reaction mixture was now stirred for 3-4 minutes till a visible change in the colour of the reaction mixture is observed. The change in colour corresponds to the generation of anion on the indole nucleus. Furthur, propargyl bromide (1.2 equivalent) was added carefully, dropwise to the reaction mixture. The completion of the reaction was monitored with the help of thin layer chromatography. Reaction completes in 25 minutes to give the product (7). The product (7) was dissolved in 9:1 solution of EtOH:H_2O to get a clear solution. To this solution was immediately added 0.02 mmol $CuSO_4.5H_2O$. After waiting for 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the azidoamino acid obtained in scheme 1 was added to the reaction mixture. The monitoring of the reaction was done by thin layer chromatography. The reaction generally completes in 10-12 hours to get the products 8-12 (scheme 2) in very good yield. The further refining of the products was done by column chromatography using ethylacetate:hexane as eluent.

The compounds 8-12 were finally hydrolysed with LiOH as base in the presence of Acetone:water (9:1) as solvent. The reaction completes within minutes to yield compounds 13-17 (scheme 3). All the reactions were performed in vaccum fume hood by using carefully cleaned oven-dried lobachemie glassware. The stirring of the reaction mixtures was done on REMI made magnetic stirrers using teflon coated magnetic beads. Drying of diethyl ether was done over activated anhydrous calcium chloride followed by passing the thin sodium wire through it. Refluxing of acetonitrile was done over anhydrous P_2O_5 followed by distillation over anhydrous K_2CO_3. The dried acetonitrile was finally stored over the activated molecular sieves of size 4 Å to prevent the infilteration of moisture. Reactions were monitored by TLC using silica gel GF254. The chromatograms thus developed were viewed in ultraviolet light and staining was done with iodine. Column chromatography was done to purify the compounds. Silica gel of 100-200 mesh size was used using hexane and ethyl acetate as preferred eluents.



I) Azido-acetic acid (1) /(EtOH:H2O = 9:1, CuSO4.5H2O = 0.02 mmol, Sodium ascorbate = 0.05 mmol)

II) 2-Azido-3-(1H-indol-3-yl)-propionic acid (2) /(EtOH:H2O = 9:1, CuSO4.5H2O = 0.02 mmol, Sodium ascorbate = 0.05 mmol)

III) 2-Azido-3-(1H-imidazol-4-yl)-propionic acid (3) /(EtOH:H2O = 9:1, CuSO4.5H2O = 0.02 mmol, Sodium ascorbate = 0.05 mmol)

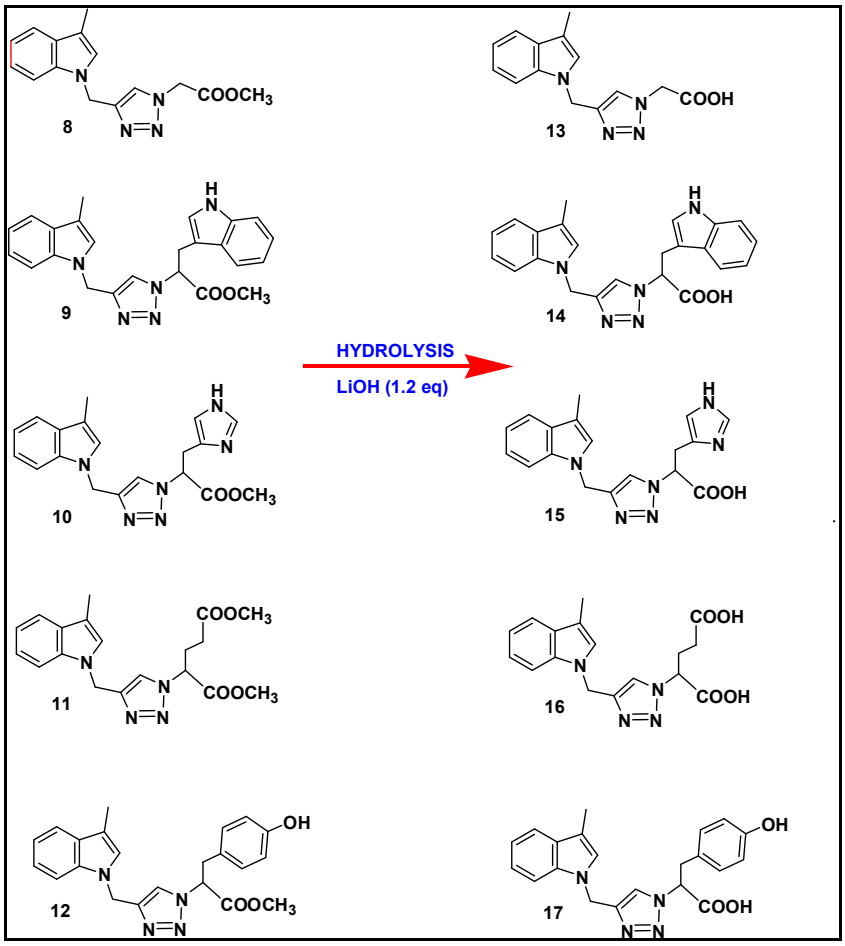
IV) 2-Azido-4-carboxy-heptanedioic acid (4) /(EtOH:H2O = 9:1, CuSO4.5H2O = 0.02 mmol, Sodium ascorbate = 0.05 mmol)

V) 2-Azido-4-(4-hydroxy-benzyl)-pentanedioic acid (5) /(EtOH:H2O = 9:1, CuSO4.5H2O = 0.02 mmol, Sodium ascorbate = 0.05 mmol)

Scheme-2

^{1}H and ^{13}C NMR spectra and DEPT-135

The spectra were recorded at a frequency 500 MHz and 125 MHz for ^{1}H and ^{13}C spectra respectively. $CDCl_3$ and DMSO were used as preferred solvents with TMS as an internal standard. The ^{1}H NMR spectral data were reported in form of chemical shifts (δ ppm), multiplicity (s = singlet, br s = broad singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet) and the coupling constant (expressed as J in Hertz). The ^{13}C and DEPT-135 NMR spectral data were represented in terms of chemical shift with +ve signals corresponding to CH_3 and CH carbons and the -ve signals corresponding to CH_2 carbons while an absent signal points towards a quaternary carbon. SP 300 PYE UNI CAM Infrared Spectrophotometer was used to record the Infrared spectra with anhydrous KBr pellets.

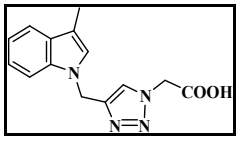


Scheme 3

Mass spectra, LC-MS procedure

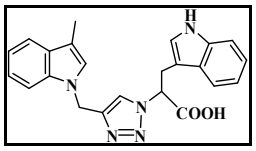
Bruker MicroTOF QII mass spectrometer (Bruker Daltonik, Bremen, Germany) machine calibrated with sodium formate (internal calibrant) was used to record the mass spectra. KdScientific automated pump with flow rate 180 µL/h was used to inject the sample to electrospray ionization source at a concentration 50 µM in acetonitrile-water-formic acid (7:2.9:0.1). Dry N_2 gas heated at 180 ^oC was used for the desolvation of the injected solution. Optimisation of the various parameters of the mass spectrometer was done for obtaining a maximum ion abundance. Specifically, the capillary voltage was adjusted at 4500 V and the vacuum was sustained at 3-4x10^{-7} mbar. For LC-MS, Dionex Ultimate 3000 system was coupled to the mass spectrometer. Chirobiotic® T 10 µm chiral HPLC column (25 cm x 4.6 mm) was used for the analysis. A solution of Acetonitrile in water (1:1) was used as eluent. 2 µL of sample (injection volume) was loaded to the column. The flow rate was maintained 0.2 ml and the absorbance was set at 200, 220 and 254 nanometers. Sodium formate was used as internal calibrant.

^{1}H/ ^{13}C/ DEPT-135 NMR data of the test compounds:

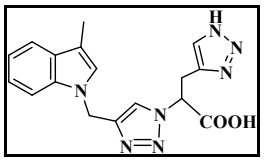


[4-(3-Methyl-indol-1-ylmethyl)-[1,2,3]triazol-1-yl]-acetic acid (8). The compound (7) was dissolved in 9:1 solution of EtOH:H_2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of $CuSO_4.5H_2O$. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the azidoacetic acid (i) synthesized in scheme 1, was added to the reaction mixture. The reaction was monitored by thin layer

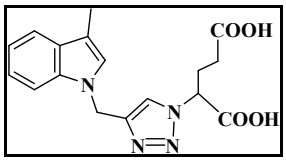
chromatography technique. The reaction generally completes in 8 hours to give a yellow coloured powdery solid, mp 90-95 ^{o}C, 80% yield, IR (KBr) νmax 3380 (NH), 1610 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 9.42 (s, 1H, COOH) 2.30 7.83-8.401(m, 3H, ArH), 7.39-7.43 (m, 2H, ArH), 4.20 (s, 2H, CH_2), 4.01 (s, 2H, CH_2), 2.25 (s, 3H, CH_3), (^{13}C NMR normal/DEPT- 135) (125 MHz, $CDCl_3$): 181.27 (C=O), 138.66 (C), 134.24 (C), 128.11(CH), 127.93 (CH), 125.74 (C), 122.87(ArCH), 115.79 (ArCH), 113.30 (ArCH), 27.48 (-ve, CH_2), 21.98 (-ve, CH_2), 21.04 (CH_3); ESI-MS (HRMS) calculated for $C_{14}H_{14}N_2O_4$ 271.1187. Found m/z 271.1181([M+H]$^+$).



3-(1H-Indol-3-yl)-2-[4-(3-methyl-indol-1-ylmethyl)-[1,2,3]triazol-1-yl]-propionic acid (9). The compound (7) was dissolved in 9:1 solution of EtOH:H_2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of $CuSO_4.5H_2O$. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Lastly, the 2-Azido-3-(1H-indol-3-yl)-propionic acid (ii) obtained in scheme 2, was added to the reaction mixture. The reaction was monitored by thin layer chromatography technique. The reaction generally completes in 8 hours to give a bright yellow coloured solid, mp 137-142 ^{0}C, 69% yield, $[\alpha]_D^{25}$ = +124 (c 0.01, MeOH), IR (KBr) νmax 3390 (NH), 3191 (NH), 1632 (C=O) cm^{-1}; ^{1}H NMR (500 MHz, DMSO d_6): δ = 8.16 (s, 1H, COOH), 6.70-7.50 (m, 9H, ArH), 6.70 (s, 1H, ArH), 4.51(t, J = 10 Hz, 1H, ArH, 1H, CH), 4.20 (s, 2H, CH_2), 3.45-3.63 (m, 2H, CH_2), 2.26 (s, 3H, CH_3) ; (^{13}C NMR normal/DEPT- 135) (125 MHz, $CDCl_3$): δ_C = 179.98 (C=O), 136.70 (C), 134.40 (C), 130.92 (ArCH), 127.47 (ArCH), 125.41 (ArCH), 123.83 (ArCH), 121.60 (ArCH), 119.44 (ArCH), 118.00 (ArCH), 114.60 (ArCH), 111.98 (ArCH), 109.85 (C), 107.07 (C), 53.15 (CH), 26.61 (CH_2), 25.45 (CH_2), 21.58 (CH_3); ESI-MS (HRMS) calculated for $C_{23}H_{21}N_5O_2$ 400.1775. Found m/z 400.1700 [M+H]$^+$.

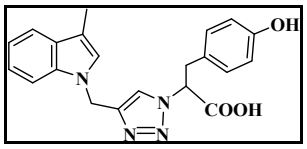


2-[4-(3-Methyl-indol-1-ylmethyl)-[1,2,3]triazol-1-yl]-3-(1H-[1,2,3]triazol-4-yl)-propionic acid (10). The compound (7) was dissolved in 9:1 solution of EtOH:H_2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of $CuSO_4.5H_2O$. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the 2-Azido-3-(1H-imidazol-4-yl)-propionic acid (iii) procured in scheme 2, was added to the reaction mixture. The reaction was monitored by thin layer chromatography technique. The reaction generally completes in 8 hours too get a grey coloured solid, mp 121-126 ^{0}C, 65% yield, $[\alpha]_D^{25}$ = +154 (c 0.01, MeOH), IR (KBr) νmax 3299 (NH), 1600 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 9.21 (s, 1H, COOH), 7.45-7.89 (m, 2H, ArH), 6.83-7.39 (m, 5H, ArH), 6.82 (s, 1H, ArH), 4.41 (t, J= 15 Hz, 1H, CH), 4.20 (s, 2H, CH_2), 3.33 (d, J= 10 Hz, 2H, CH_2), 2.27 (s, 3H, CH_3); (^{13}C NMR normal/DEPT- 135) (125 MHz, $CDCl_3$): δ_C = 171.97 (COOH), 148.95 (ArCH), 138.64 (ArCH), 136.73 (ArCH), 127.49 (C), 125.96 (C), 121.59 (ArCH), 119.05 (ArCH), 116.64 (ArCH), 111.04 (ArCH), 107.08 (C), 54.27 (CH), 28.61 (CH_2), 25.46 (CH_2), 21.24 (CH_3); ESI-MS (HRMS) calculated for $C_{17}H_{17}N_7O_2$ 352.1524 Found m/z 352.1523 [M+H]$^+$.



2-[4-(3-Methyl-indol-1-ylmethyl)-[1,2,3]triazol-1-yl]-pentanedioic acid (11). The compound (7) was dissolved in 9:1 solution of EtOH:H_2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of $CuSO_4.5H_2O$. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the 2-

Azido-4-carboxy-heptanedioic acid (iv) obtained in scheme 2, was added to the reaction mixture. The reaction was monitored by thin layer chromatography technique. The reaction generally completes in 8 hours to give a dark brown solid, mp 111-116 ^{0}C, 85% yield, $[\alpha]_D^{25}$ = +23 (c 0.01, MeOH); IR (KBr) νmax 3412 (NH), 1574 (C=O) cm^{-1}; ^{1}H NMR (DMSO d_6, 500 MHz): δ = 10.41 (s, 1H, COOH), 9.81 (s, 1H, COOH), 7.91 (s, 1H, ArH), 7.53-7.82 (m, 2H, ArH), 7.22-7.39 (m, 3H, ArH), 4.39 (t, J = 5 Hz, 1H, CH), 4.20 (s, 2H, CH_2), 2.36-2.72 (m, 2H x CH_2), 2.27 (s, 3H, CH_3); (^{13}C NMR normal/DEPT- 135) (125 MHz, DMSO d_6): δ_C = 180.03 (COOH), 179.55 (COOH), 145.55 (ArCH), 134.66 (ArCH), 130.93 (C), 127.14 (C), 123.86 (C), 122.03 (ArCH), 116.85 (C), 114.55 (ArCH), 113.53 (ArCH), 109.82 (C), 53.78 (CH), 29.57 (CH_2, -ve), 25.78 (CH_2, -ve), 25.06 (CH_2, -ve), 21.53 (CH_3); ESI-MS (HRMS) calculated for $C_{17}H_{18}N_4O_4$ 342.1408 Found m/z 352.1410 $[M+H]^+$.



3-(4-Hydroxy-phenyl)-2-[4-(3-methyl-indol-1-ylmethyl)-[1,2,3]triazol-1-yl]-propionic acid (12). The compound (7) was dissolved in 9:1 solution of EtOH:H_2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of $CuSO_4.5H_2O$. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the 2-Azido-4-(4-hydroxy-benzyl)-pentanedioic acid (v) was added to the reaction mixture. The reaction was monitored by thin layer chromatography technique. The reaction generally completes in 8 hours to get a brown solid, mp 145-153 ^{0}C, 78% yield, $[\alpha]_D^{25}$ = +35 (c 0.01, MeOH); IR (KBr) νmax 3315 (NH), 1701 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 9.15 (s, 1H, COOH), 7.73-7.94 (m, 2H, ArH), 7.53-7.63 (m, 1H, ArH), 6.87-7.52 (m, 3H, ArH), 4.42 (t, J = 15 Hz, 1H, CH), 4.35 (s, 2H, CH_2), 3.33 (d, J = 3 Hz, 2H, CH_2), 2.20 (s, 3H, CH_3); (^{13}C NMR normal/DEPT- 135) (125 MHz, $CDCl_3$): δ_C = 173.53 (COOH), 145.30 (C), 144.82 (C), 135.27 (ArCH), 134.94 (ArCH), 130.75 (C), 129.87 (ArCH), 126.81 (ArCH), 126.33 (ArCH), 124.55 (ArCH), 123.30 (ArCH), 121.36 (ArCH), 113.30 (ArCH), 109.04 (C), 107.24 (C), 58.75 (CH), 29.29 (CH_2, -ve), 25.19 (CH_2, -ve), 21.53 (CH_3); ESI-MS (HRMS) calculated for $C_{21}H_{20}N_4O_3$ 377.1615 Found m/z 377.1615 $[M+H]^+$.

The characterization of the compounds 13, 14, 15, 16 and 17 was done by mass spectroscopy which indicated the formation of these compounds in more than 95% yield through a very clean hydrolysis reaction with no formation of side products at all. The hydrolysed products were first washed with water and extracted by using ethyl acetate solvent using a separatory funnel.

3. Results and discussions.

3.1 5-lipoxygenase enzyme immunoassay:

For 5-LOX enzyme inhibition studies, the test compounds at five different concentrations of 0.01, 0.1, 1, 10 and 100 μM were prepared in DMSO. 10 μL volume of each compound from the above concentrations was added to 90 μL solution (in assay buffer) of 5-LOX enzyme taken in a 96-well plate. Each compound was tested in duplicate and the average of two values with deviation <5% was taken for calculation. Two wells were taken as blanks (assay buffer + AA) and four wells were taken as positive controls (enzyme in assay buffer + AA). The initiation of reaction was done by the adding 10 μL volume of the substrate (AA). After shaking the 96 well plate on a shaker for 5 min, addition of a 100 μL solution of the chromogen (developing reagent) was done in all the wells. Shaking of the plate was performed for 5 minutes and reading for absorbance was taken at 490 nm on a microplate scanning spectrophotometer. Percent 5-LOX inhibitory activity was determined using the mean of the two values for each sample using the formula:

[A500/min/9.47 mM $^{-1}$] x [0.21 ml/0.09 ml] x sample dilution
Where :
A500/min = A500 (sample)/min - A500 (blank)/5 min,
9.47 mM $^{-1}$ = -Extinction coefficient of chromogen,
0.21 ml = Total volume of the solution in each well and
0.09 ml = Volume of the enzyme solution used.

Percent 5-LOX inhibition = [{L.A. (P.C.) - L.A. (I.)}/L.A.(P.C.)] x 100

L.A. (P.C.) = Lipoxygenase Activity of positive control, L.A. (I.) = Lipoxygenase Activity of inhibitor. IC_{50} were determined from the graph between Percent Inhibition versus concentration of inhibitor using KaleidaGraph 3.5 software.

Compound 10 with histidine amino acid was found to be most effective 5-LOX inhibitor with IC50 = 0.43 μM. Compound 13 with glycine amino acid and a free carboxyl group was another appreciable 5-LOX inhibitor with IC50 = 0.93 μM. The other compound with tryptophan, glutamic acid and tyrosine amino acids were also having satisfactory results.

3.2 Anti-cyclooxygenase activities:

For studying the COX-1/2 inhibitory activities of the test compounds, various reagents were prepared as per the protocol of the assays. The background samples were prepared for both COX-1 (ovine) and COX-2 (human recombinant) by taking 20 μL of each enzyme in separate test tubes and keeping them in boiling water for a time period of 3 minutes. Inactivated enzymes were used to generate background values. In two test tubes named background COX-1 and background COX-2, 970 μL of reaction buffer, a 10 μL solution of heme and a 10 μL volume of inactive COX-1 or COX-2 were added. 100% Initial activity tubes were prepared for both COX-1 and COX-2 by adding 950 μL solution of reaction buffer, 10 μL solution of heme and 10 μL volume of COX-1 or COX-2. Inhibitor tubes were prepared for compounds 8–17. The addition of a 950 μL reaction buffer, 10 μL of heme, 10 μL of COX-1 or COX-2 enzyme and 20 μL of the inhibitor solution in each sample tube was done. All the solutions were thereby incubated at 37 ^{0}C for a time period of 10 minutes. After incubation, a 10 μL volume of the substrate was added to all the test tubes and vortexing was performed. Incubation was done again for another 2 minutes. Afterwards, an addition of 50 μL solution of 1M Hydrochloric acid was done to each one of the test tubes in order to cease the reaction. Furthur, an addition 100 μL solution of stannous chloride solution was carried out to each one of the test tubes followed by vortexing. After another incubation for 5 minutes, the test tubes were kept at 0–4 ^{0}C. Prostaglandin screening standards were prepared as test tubes S1–S8. Addition of 800 μL of EIA buffer was done to the test tube S1 and 500 μL of the same was added to the test tubes S2–S7. Then 200 μL of bulk standard (10 ng/ml) was added to tube S1 and mixed thoroughly. The standards were diluted serially by removing 500 μL from tube S1 and placing it in tube S2 and mixed thoroughly. Same process was repeated from S2–S3, S3–S4 up to S7–S8. To make dilutions for COX reactions, two test tubes named BC1 and BC2 were prepared. To each test tube was added, 990 μL solution of EIA (enzyme immunoassay) buffer and 10 μL solution of background COX-1 or COX-2 and the contents were mixed thoroughly. COX 100% initial activity samples were prepared as three test tubes for COX-1 and COX-2 both and numbered as IA1–IA3. For each sample, aliquot 990 ll of EIA buffer to IA1, 950 ll of EIA buffer to IA2 and 500 μL of EIA buffer to IA3. A 10 μL solution of the enzymes COX-1 or COX-2 100% initial activity sample was added to IA1 and mixed thoroughly. Aliquot, 50 μL of tube IA1 and added to tube IA2 and mixed thoroughly. Again aliquot 500 μL from test tube IA2 and added to test tube IA3 and mixed well. In the same manner, COX inhibitor samples were prepared by further dilutions and named C1–C3 for each concentration. After preparing all the dilutions, theywere introduced on 96 well plate. The wells were distributed as blank-1A, NSB (nonspecific binding)-1B and Bo (maximum binding)-1C. Well 1H was named as TA (Total activity well).Wells 2A–2H were used for S1–S8 and 3A–3H were used for S1–S8 duplicate. Wells 4A and 5A were prepared as BC1 and its duplicate. Similarly, for BC2 wells 4B and 5B were prepared. Remaining wells were used for inhibitor samples for COX-1 and COX-2. The addition of the reagents on 96-well plate was performed as follows: Addition of a 100 μL solution of EIA buffer was done to the NSB well and 50 μL of solution of EIA buffer to the Bo well. 50 μL of prostaglandin screening standard was added to the respective wells S1–S8 from their respective test tubes S1–S8 and duplicated. 50 μL of BC1 and BC2 were added per well and in duplicate. 50 μL of 100% initial activity samples were added per well and only IA2 and IA3 were assayed in duplicate for both COX-1 and COX-2. 50 μL of COX inhibitor sample was added per well from their respective dilutions (only C2 and C3 were assayed). Except for the TA and Blank well, an addition of 50 μL solution of PG screening AChE tracer was done in each well. At last, except for TA, NSB and blank wells, a 50 μL solution of PG screening EIA antiserum was done in each of the wells. Eventually, the plate was covered with a cellulose film and incubated for a period of 18 hours at RT. After incubation, plate was developed by emptying the wells and rinsing the wells with wash buffer for five times. After washing the wells, a 200 μL solution of Ellman's reagent was added to each one of the wells followed by an addition of 5 μL of

the tracer to the Total activity wells. This plate was covered with a cellulose film and was kept for a period of 60–90 minutes. Before reading the plate, it was wiped from bottom to remove any fingerprints and finally read at 420 nm. Calculation of % inhibition and IC50 values: %B/Bo value for each sample was determined from the absorbance values attained after reading the 96 well plate at 420 nm according to the calculation strategy provided in the manufacturer's protocol for inhibition assay.

It was seen that the tst compounds were effective against the COX-2 enzyme were failed to effective against the COX-1 enzyme. Compound 8 with glycine substitution had Selectivity index of 748.33 for COX-2 enzyme. Compound 10 with histidine substitution was found to have a selectivity index of 1173.6 for COX-2 enzyme, highest among all the test compounds. Compound 17 with tyrosine substitution and a free carboxyl group has selectivity index 757.35.

Both the immunoassays indicate the dual effectiveness of the test compounds against the 5-LOX and COX-2 enzymes.

Table 1. 5- LOX and COX-1/2 enzyme immunoassay

Compound	**IC50 (µM)**			
	5-LOX	**COX-2**	**COX-1**	**SI***
8	1.81	0.03	22.45	748.33
9	3.61	2.12	102.21	48.21
10	0.43	0.05	58.68	1173.6
11	9.21	2.56	45.54	17.78
12	8.80	1.11	31.45	28.33
13	0.93	0.09	21.30	236.67
14	1.31	1.99	39.09	19.64
15	4.21	0.08	10.09	126.125
16	2.34	1.01	21.23	21.01
17	1.21	0.087	65.89	757.35

SI* = selectivity index = IC50 (COX-1)/ IC50 (COX-2)

3.3 Isothermal titration calorimetry:

The compound solution was titrated into the sample cell (containing the enzyme) using a 250 µL rotating stirrer syringe set at 500 rpm. The reference cell contained HEPES buffer. Each experiment consisted of 19 consecutive injections of 2 µL of 10 µM of the compound to the enzyme contained in the sample cell after regular time intervals of 120 s to guarantee the equilibrium in each titration point. Control experiments were performed for comparison. The total heat Q produced or absorbed in the active cell volume V_0 determined at fractional saturation Θ after the i^{th} injection is given by equation 1

$$Q = n \Theta M_t \Delta H V_0 \quad (1)$$

Where, M_t is the total concentration of the macromolecule
n is the total number of binding sites in the macromolecule and
ΔH is the molar heat of ligand binding

The enthalpy change for the x^{th} injection ΔH(i) for an injection volume dVx is then given by the following equation 2.

$$\Delta H(x) = Q(x) + dV_i/V_0 [Q(x) – Q(x-1)/2] – Q(x-1) \quad (2)$$

The control titrations consisting in identical titrant solution with the same cell filled just with the buffer solution and also the successive buffer additions to the enzyme solution were carried out to determine the background heat which was to be subtracted from the main experiment. The origin 7.0 software provided by Microcal was used to determine the titration heat profiles for determining the binding parameters. The data fitted well to the single binding site model.

The test compounds indicated appreciable binding interactions with COX-2 enzyme. Compound8, 10 and 17 showed a higher values of gibbs free energy of association with COX-2 enzyme. The ΔH and TΔS were also most favourable for these compounds. The various thermodynamic parameters are given in table 2, figure 2.

Table 2. Isothermal calorimetric data of compounds 5 (a-e) for COX-2 enzyme.

Cmpd	N	$K_{asso.}$ (M^{-1})	ΔH (kJ mol^{-1})	TΔS (kJ mol^{-1})	ΔG (kJ mol^{-1})
8	1.11±0.25	$(8.99\pm0.81).10^5$	-31.96±0.6	4.44±0.8	-36.40±1.5
10	1.23±0.18	$(1.99\pm0.79).10^6$	-29.79±0.4	2.98±0.7	-32.77±1.1
13	1.03±0.09	$(2.99\pm0.99).10^5$	-21.71±0.7	2.92±0.65	-24.63±0.8
15	0.80±0.09	$(6.99\pm1.09).10^4$	-18.74±2.5	6.25±1.33	-24.99±2.2
17	0.88±0.11	$(0.89\pm1.09).10^5$	-21.84±2.5	6.44±1.33	-28.28±2.2

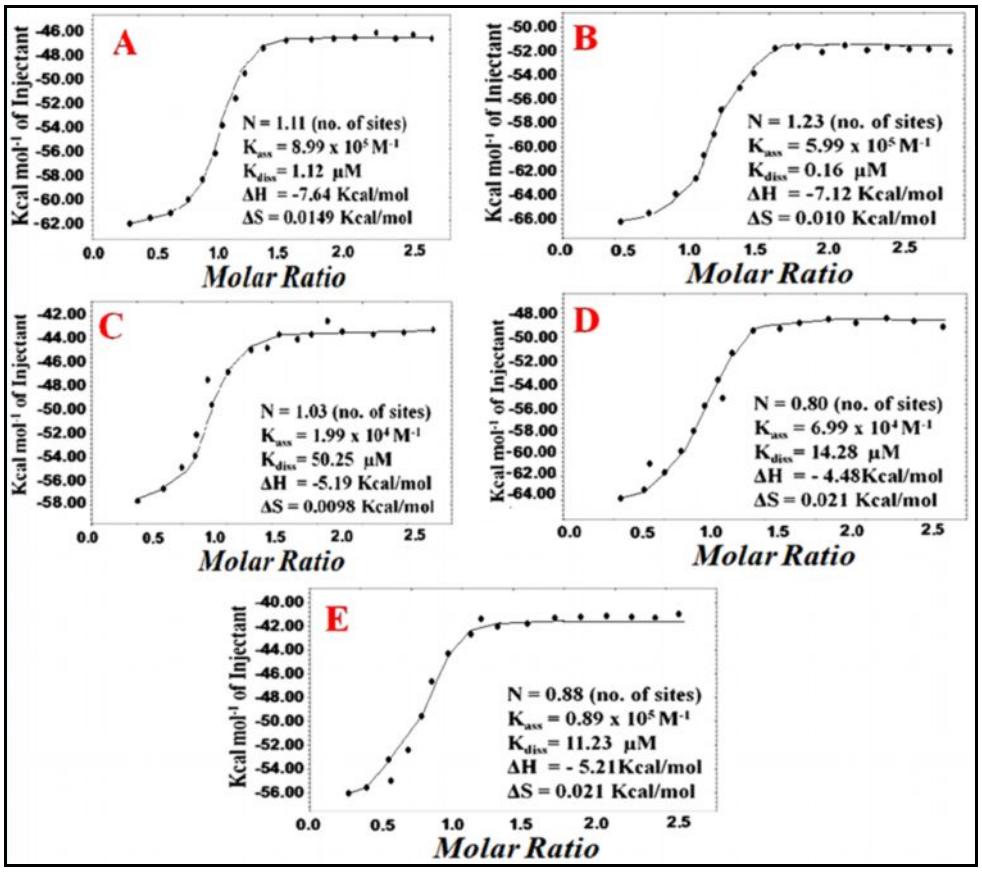


Figure 2. Isothermal titration calorimetric data for A. Compound 8, B. Compound 10, C. Compound 13, D. Compound 15, E. Compound 17

3.4. Cell viability assay

Performing the experiment in triplicate, HeLa cells were taken in a 96 well plate (4000 cells/well) and were incubated at 37 ^oC in a humidified chamber in the presence of 100 µg/ml concentration of the test compounds with final volume of 200 µL in each well. Cells with solvent only (DMSO) were taken as a control. After a certain fixed intervals of time, a 20 µL volume of MTT solution (5 mg/ml in PBS) was added to each one of the wells and eventually the incubation was done for another 4 hours. At the end of 4 hours, the supernatant was removed and the resulting crystals of formazan were dissolved in a 200 µL volume of DMSO. The absorbance (A) was measured at a wavelength of 570 nm by using a microplate reader. The percentage viability of the cells was calculated by using the following equation.

Percentage of cell viability = (absorbance of test samples/absorbance of the control sample) x100

The cell viability assay indicated that the most active compounds 10 and 17 did not affect the viability of the cells even at very high doses of 250 mg of the test compounds (figure 3). The cell viability results were better than indomethacin which was used as control.

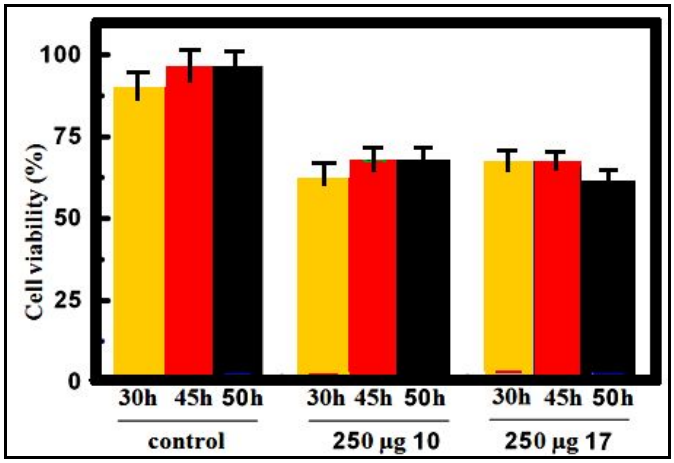


Figure 3. Cell viability assay

3.5. Docking

Docking procedure: The compounds were drawn by utilizing the molecule building toolskit of the ArgusLab 4.0.1 software. The software utilizes a semi-empirical quantum mechanical method, PM3 for performing the energy minimization on the test compounds. The crystal coordinate structure of the enzyme 15-lipoxygenase (pdb ID 3V99) was downloaded from the protein data bank in the form of a pdb file. This enzyme has a monomeric structure with arachidonic acid i.e 3-(2-oct-1-ynylphenyl)-acrylic acid as a substrate (ligand) in its binding site. This is considered as the active site of the enzyme which is marked 15 Å around the vicinity of the ligand when observed in the molecular tree view. The energy minimised test compound was passed in the work space accompanied by the crystal structure of the enzyme in whose active site the docking of this compound is to be performed. The application of a well organized grid dependent docking algorithm which estimates a comprehensive investigation in the free volume of the binding site cavity of the enzyme forms the basis of the docking protocol of the ArgusLab 4.0.1 software. The identification of the conformational space was done by the geometry optimization of the flexible ligand (rings were treated to be rigid) allied with the augmented edification of the ligand torsions. The docking thus explicitly occurs between the flexible parts of the compound with that of the enzyme. The orientation of the ligand in the enzyme premises was ascertained by a shape scoring function based on Ascore. The final positions were classified by the lowest energy of interaction values. Hydrogen bonding and hydrophobic interactions between the test compounds and the enzyme were explored by this method. The docking chatracteristics of the test compound has been indicated in Table 3 and Table 4.

Table 3. Docking of test compounds in 5LOX enzyme

Cmpd.	Docking Score (Kcal/mol)	H-bond interactions with amino acid residues			
8	-18.57	H367(2.99Å) C=O	Q557(2.71 Å) $CONH_2$	-	-
9	-17.43	Q363(2.99 Å) $CONH_2$	-	-	-
10	-18.57	F177(2.99 Å) C=O	N554(1.19 Å) C=O	-	-
11	-16.57	F177(2.82 Å) C=O	F177(2.08 Å) C=O	-	-
12	-27.79	H367(2.47 Å) COOMe	-	-	-
13	-20.0	N554 (2.59 Å) COOH	Q557(2.96 Å) $CONH_2$	N554(2.59 Å) C=O	-
14	-29.0	F177(2.47) C=O	-	-	-
15	-21.5	N554(2.97 Å) C=O	H367(2.71 Å) COOMe	-	-
16	-23.0	N554(2.28 Å) COOMe	Q557(2.60 Å) C=O	-	-
17	-22	F177(1.82 Å) C=O	Q557(1.96 Å) $CONH_2$	H367(1.01 Å) COOH	

Table 4. Docking of test compounds in COX-2 enzyme

Compd.	Docking Score (Kcal/mol)	H-bond interactions with amino acid residues			
8	-16.66	S516 (1.21 Å), C=O			-
9	-19.12	Y341 (2.12 Å), COOMe	Y371 (1.84 Å), N(indolic)	-	-
10	-18.11	S516 (1.14 Å), $CONH_2$	G512 (1.11 Å), $CONH_2$	Y371 (3.17 Å), C=O	-
11	-16.23	S516 (3.14 Å), COOMe		-	-
12	-19.99	Y341 (1.10 Å), COOMe			-
13	-22.33	R106 (2.92 Å), COOH	-	-	-
14	-21.11		-	-	-
15	-22.81	R106 (2.14 Å), COOH	Y341 (1.89 Å), C=O		-
16	-26.21	Y371 (1.11 Å), C=O	S516 (2.90 Å), C=O		-
17	-24.84	S516 (1.81 Å), COOH	G512 (1.71 Å), COOH	R106 (1.92 Å), C=O	

4. Conclusion

Indole nucleus appended with amino acids via a triazole linker showed interesting inhibitory activities against the inflammation causing enzymes. The in vitro enzyme immunoassays indicated compound 10 to be the most effective inhibitor of these enzymes. The in vitro results were well supported by kinetic analysis by isothermal titration calorimetry.

5. Acknowledgements

PP thanks the lovely faculty of physical sciences for providing the requisite resources for the synthesis of compounds. The state of the art instrumental facility at GURU NANAK DEV UNIVERSITY is highly acknowledged for characterization of the compounds. Special thanks to the faculty of biotechnology, lovely professional university for providing the instruments to carry out the invitroenzyme immunoassay studies.

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