Identification of Benzothiazole–Rhodanine Derivatives as α -Amylase and α -Glucosidase Inhibitors: Design, Synthesis, In-silico and In-Vitro Analysis

Mahendra Gowdru Srinivasa

Nitte Gulabi Shetty Memorial Institute of Pharmaceutical Sciences

Natasha Naval Aggarwal

Nitte Gulabi Shetty Memorial Institute of Pharmaceutical Sciences

Banylla Felicity Dkhar Gatphoh

Nitte Gulabi Shetty Memorial Institute of Pharmaceutical Sciences

Madan Kumar S

IOE Press: UCL Institute of Education Press

Kannika B.R.

IOE: Institute of Evolution

Pujar G.V.

JSS College of Pharmacy Mysore

Sheshagiri Dixit

JSS College of Pharmacy Mysore

Subhankar P Mandal

JSS College of Pharmacy Mysore

Prashantha Kumar B.R.

JSS College of Pharmacy Mysore

Pukar Khanal

KLE University's College of Pharmacy: KLE Society's College of Pharmacy

Revanasiddappa B C (✉ revan@nitte.edu.in)

Nitte Gulabi Shetty Memorial Institute of Pharmaceutical Sciences

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Abstract

In search for possible antidiabetic agents, a new series of Benzothiazole-Rhodanine derivatives (**A1-10**) have been synthesized and characterized by spectral data (IR, $^1\text{H-NMR}$, $\text{C}^{13}\text{-NMR}$, and HR-MS). All the designed compounds were subjected to *In-silico* studies using Schrodinger software and evaluated for *In-vitro* antidiabetic activity by α -amylase and α -glucosidase inhibitory assays. Among the tested compounds **A5**, **A6** and **A9** showed good activity when compared to the standard Acarbose. Also, Molecular dynamic (MD) simulations were conducted to evaluate the stability of the ligand-protein complex by the calculation of the root mean of square deviation (RMSD), root mean square fluctuation (RMSF), and solvent accessible surface area (SASA).

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease that causes a significant challenge for the health-care system across the globe. Such a high prevalence of diabetes has boosted the search for novel alternatives. Owing to high blood glucose in diabetes, a series of complications such as blindness, high blood pressure, kidney and stroke disorders may result [1]. For controlling glycemia, there has been a development of few synthetic drugs such as Acarbose, Metformin, and Sitagliptin. However, many side effects have been resulted due to the consumption of these drugs, such as flatulence, diarrhea, and hypoglycemia. It is well known that postprandial hyperglycemia is an important contributing factor to the progression of type 2 diabetes (T2DM), therefore postprandial management of hyperglycemia is one of the therapeutic approaches to combating T2DM diabetes. α -amylase and α -glucosidase are the two main carbohydrate digestive enzymes associated with postprandial hyperglycemia in T2DM patients. The α -1, 4-glycosidic bond cleavage is catalyzed by α -amylase, in which polysaccharides are converted into smaller fragments of oligosaccharides, which are further degraded by α -glucosidase. The hydrolysis of terminal non-reducing 1,4-linked α -glucose residues is caused by α -glucosidase and monosaccharides are released into the bloodstream in an absorbable form [2]. Therefore, inhibition of these two carbohydrate digestive enzymes is essential for alleviating plasma glucose levels [3]. Rhodanines are the bioisosteres of the Thiazolidine-2,4-dione (TZD) group of molecules recorded to normalize plasma glucose levels [4]. Besides, owing to their contribution in regulating various physiological processes, TZD has been the subject of extensive research [5]. These compounds are reported to possess therapeutic actions like antimicrobial [6], anti-inflammatory [7], anticancer [8], antitubercular [9], antimalarial [10], antihyperglycemic [11]. A few well-known TZD moiety-containing drugs like pioglitazone and troglitazone are employed for treating T2DM [12]. According to the troglitazone and pioglitazone structure, rhodanines are bioisosteres of thiazolidinedione we have designed our title compounds in the present study (Figure-1).

Benzothiazoles are bicyclic compounds consist of thiazole and a benzene ring. These compounds are found to be an important active pharmacophore, exhibiting so many biological activities. This moiety has attracted considerable attention because of its wide range of pharmacological activities [13]. The designed Benzothiazole rhodanine derivatives have been active in the inhibitory potency of hydrogen bonding, necessary for the favorable orientation of the molecule towards the enzyme binding site [14]. Also, the inhibitory potential against α -amylase and α -glucosidase of benzothiazole-rhodanine derivatives has not been investigated yet. So the present investigation was designed to assess the inhibitory ability against these two enzymes. Based on the above findings, it was anticipated to synthesize a novel series of Benzothiazole-rhodanine derivatives, linking of these two active pharmacophores. The newly designed compounds were preliminarily screened for *In-Vitro* activity, followed by *In-silico* molecular docking studies and molecular dynamics simulations also have been carried out.

Results And Discussion

Chemistry

The synthesis of the titled compounds (A1-10) is represented in Scheme-01. All the compounds and intermediates were purified by successive recrystallization from ethanol. The purity of the final compounds was ensured by the TLC. The melting points and yields of the compounds were recorded and showed in the Table-1. The structures of all the compounds were established based on spectral data. (FT-IR, ^1H NMR, ^{13}C NMR, HR-MS). The title compounds benzothiazole-rhodanine derivatives (A1-10), were prepared by *Knoevenagel condensation* where N-substituted rhodanines react with substituted aromatic aldehydes in toluene medium in presence of Piperidine and glacial acetic acid as catalysts. The key intermediate N-substituted rhodanines was obtained by the procedure reported previously [15], where a freshly prepared sodium chloroacetate solution was slowly added to the dithiocarbamate solution with stirring at 0-5°C, followed by successive cyclization with Con. HCl.

In FT-IR frequencies, the compound (A1) exhibited the absorption bands at 1531 (C=C) cm^{-1} due to C=C stretching, C=N stretching at 1611 cm^{-1} and 1700 cm^{-1} due to (C=O) stretching, confirming the structures of the compound. The aromatic protons and =CH were observed as multiplets at δ 6.90-8.58 ppm. The ^{13}C -NMR spectrum of (A1) signals at δ 111.20-157.78 justified the total number of carbon atoms in the structure. The peaks at δ 176.12 and δ 202.25, were observed for the presence of (C=O) and C=S respectively. The mass spectrum of compound (A1) depicted a [M+]⁺ peak at m/z 398.98 which is under the theoretical molecular weight 398.98 ($\text{C}_{17}\text{H}_9\text{N}_3\text{O}_3\text{S}_3$). The various physicochemical characters of the synthesized compounds are summarized in Table-1.

Table-1: Physical data of compounds (A1-10)

Comp	R-CHO	Mol. Formula	Mol. weight	Melting point (C ⁰)	Yield (%)
A1	4-NO ₂	C ₁₇ H ₉ N ₃ O ₃ S ₃	398.98	203-205	67
A2	4-Br	C ₁₇ H ₉ BrN ₂ OS ₃	433.37	245-247	72
A3	4-Cl	C ₁₇ H ₉ ClN ₂ OS ₃	388.91	273-275	69
A4	C ₆ H ₅	C ₁₇ H ₁₀ N ₂ OS ₃	354.47	259-261	65
A5	4-OH -3-OCH ₃	C ₁₈ H ₁₂ N ₂ O ₃ S ₃	400.49	214-216	67
A6	3,4 - (OCH ₃) ₂	C ₁₉ H ₁₄ N ₂ O ₃ S ₃	414.52	227-229	74
A7	4-OCH ₃	C ₁₈ H ₁₂ N ₂ O ₂ S ₃	384.50	186-188	67
A8	4-N(CH ₃) ₂	C ₁₉ H ₁₅ N ₃ OS ₃	397.04	235-237	56
A9	4-OH	C ₁₇ H ₁₀ N ₂ O ₂ S ₃	369.99	220-222	71
A10	2-OH	C ₁₇ H ₁₀ N ₂ O ₂ S ₃	369.99	192-194	52

In silico molecular docking studies

For the evaluation of the binding mode of all the synthesized compounds at the active site of target 3TOP (the crystal structures of α -amylase, α -glucosidase), molecular docking studies have been performed. Docking of the synthesized

compounds was carried out in the groove of the targetbinding site which plays an important role as a signal transducer, in diabetes and targeted to α -amylase, α -glucosidase enzymes. The binding affinity of these derivatives was expressed in terms of the G score as shown in Table-2. It is found that all derivatives had binding free energy ranging from -9.879 to -5.933. Compounds **A5** and **A9** are having the finest binding energies with a binding score of -6.676 and -6.639 respectively (Fig-2, 3). Acarbose showed the highest binding affinity towards 3TOP with binding free energies of -9.879.

Table-2: Docking results of designed molecules (A1-10)

Comp	Extra-precision docking score			
	^a Gscore (kcal/mol)	^b Gemodel (kcal/mol)	^c Gecou (kcal/mol)	^d EvdW (kcal/mol)
A1	-6.091	-56.586	-9.505	-32.99
A2	-6.069	-62.411	-5.658	-38.205
A3	-6.172	-66.669	-6.525	-41.057
A4	-5.933	-60.877	-5.678	-38.055
A5	-6.676	-59.996	-5.246	-38.558
A6	-6.604	-62.432	-10.103	-32.585
A7	-6.169	-63.518	-7.171	-39.433
A8	-5.976	-57.375	-3.922	-38.195
A9	-6.639	-60.068	-12.291	-32.386
A10	-6.049	-78.371	-8.075	-44.69
Acarbose (Std)	-9.879	-95.901	-24.29	-26.723

(Notes: ^aglide score; ^bglide model energy; ^cglide Coulomb energy; ^dglide van der Waals energy)

In compound **A5**, hydrogen bonding interactions of hydroxyl and methoxy group substitution on the phenyl ring were observed with Asp1279 and Hie1584 respectively. The pi-cation interaction is observed with Lys1460 in the benzothiazole ring (Figure-2a). The other interactions with the amino acids are Asp1157, Try1251, Ile1280, Ile1315, Trp1355, Trp1369, Trp1418, Met1421, Trp1523, Gly1525, Asp1526, Phe1550, Phe1560, and Arg1582 were observed. In compound **A9** carbonyl and hydroxyl groups have hydrogen bond interaction and are observed in Arg1510 and Asp1526 respectively (Figure -3d). The pi-cation and pi-pi bond interaction with the amino acids are Trp1369 and Lys1460 respectively. Asp1157, Pro1159, Tyr1251, Asp1279, Ile1280, Ile1351, Trp1355, Trp1418, Met1423, Arg1582 and Hie1584 also influenced for the interaction. Based on the docking scores, selected candidates are good for inhibitory activity.

Binding free energy (MM-GBSA) assay

MM-GBSA is used for estimating the relative binding affinities of the protein-ligand complex. Different drug interactions with the receptor pocket result from binding energy and these interactions include Vander walls interaction, hydrophobic interaction, polar interaction, etc. The binding free energy of compounds (A1-10) is calculated by the MM-GBSA approach, which is ranged between -71.134 to -105.14 kcal mol⁻¹ (Table 3). It is evident from results,

that van der Waals (ΔG_{vdW}) and non-polar solvation (ΔG_{Lipo}) energy terms strongly favor, while covalent energy (ΔG_{Cov}) and electrostatic solvation (ΔG_{Solv}) energy terms are not in favor of the ligand binding in the active site of 3TOP. In compounds **A5, A6, A9, and A10** coulomb energy term moderately (ΔG_{Coul} : -4.64673 to 7.015246 kcal mol⁻¹) favored the ligand binding. It is evident from the high negative values that the predominant force for the binding of compounds(A1-10)is within the catalytic pocket is ΔG_{vdW} . This can also be correlated with the docking results, where ΔG_{vdW} energy terms (-44.3913 to -56.4528 kcal mol⁻¹) also strongly favors the ligands binding. The higher values of ΔG_{vdW} and ΔG_{Lipo} are in the negative range, which shows remarkable hydrophobic interaction between target and ligands in Table-3.

Table - 3: MM-GBSA Binding free energy (kcal mol⁻¹) of compounds (A1-10)

Comp	Binding Free Energy (kcal mol ⁻¹)						
	^e ΔG_{Bind}	^f ΔG_{Coul}	^g ΔG_{Cov}	^h ΔG_{HB}	ⁱ ΔG_{Lipo}	^j ΔG_{Solv}	^k ΔG_{vW}
A1	-88.0386	7.015246	2.139889	-0.01876	-50.4151	4.507475	-50.4463
A2	-92.084	3.180458	1.717002	-0.01146	-62.2984	11.48264	-45.3457
A3	-94.8673	2.821849	0.992511	-0.01459	-62.1215	11.94102	-47.7167
A4	-71.134	1.01058	7.260379	-0.06976	-51.1803	17.31604	-44.3913
A5	-96.2461	-2.165809	0.553086	-0.12827	-56.8451	14.3328	-55.854
A6	-93.9489	-1.626818	3.761425	-0.09537	-62.4399	15.16251	-57.1502
A7	-90.0266	0.033539	-1.14307	-0.01454	-57.208	13.12495	-44.1602
A8	-105.14	5.173789	1.972509	-0.01272	-69.9424	10.42691	-52.2998
A9	-82.6684	-2.052938	3.29959	-0.03492	-56.2688	15.59313	-56.4528
A10	-91.7628	-4.64673	1.376945	-0.53416	-51.3642	16.76577	-52.7353
ABS	-73.6671	-0.95997	6.346426	-0.55324	-47.282	19.75039	-50.4028

(Notes: ^efree energy of binding; ^fCoulomb energy; ^gcovalent energy (internal energy); ^hhydrogen bonding energy; ⁱhydrophobic energy (non-polar contribution estimated by solvent accessible surface area); ^jelectrostatic solvation energy; ^kvander Waals energy.)

Physicochemical properties

Physicochemical properties and various In-silico predictions were carried out for the evaluation of drug-likeness properties and also to know about pharmacokinetic properties of the synthesized compounds. For an orally active compound, a maximum of two violations of Lipinski's rule is acceptable in Table-4.

Table-4: Predicted Lipinski's parameter for compounds (A1-10)

Comp	Molecular weight	Donor HB	Acceptor HB	Log Po/wa ^a	#rotorb ^b	Rule of 5
Acceptable range	≤500	≤5	≤10	(−2.0 to 6.5)	≤10	≤5
A1	398.98	1.5	8	0.342	3	0
A2	433.37	0	8	-0.029	3	0
A3	388.91	0	8	0.399	3	0
A4	354.47	0	7.75	0.786	3	0
A5	400.49	0	7	1.233	2	0
A6	414.52	2	8.5	-0.068	4	0
A7	384.50	0	7	1.661	2	0
A8	397.04	0	7	1.233	2	0
A9	369.99	1	7.75	0.43	3	0
A10	369.99	1	9.75	-0.829	5	1
Acarbose (Std)	645.611	13	32.1	-7.276	22	3

^aPredicted octanol/water partition coefficient log P.

^bPredicted rotatable bonds.

Lipinski's rule of five indicates that molecular weight must be below ≤500, Log P (partition coefficient) must be ≤5 and other parameters like hydrogen bond donors as well as acceptors must be ≤5 and ≤10 respectively, distinctly however standard Acarbose won't obey the Lipinski's limit arise have additionally been anticipated in Tables 4 and 6. The absorption dynamics of the therapeutic candidates are greatly dependent upon permeability, solubility, and bioavailability. Among synthesized compounds **A5, A7, A8** has shown a considerable percentage of human oral absorption >80%. Owing to this fact, bioavailability will not be affected by the violation of Lipinski's rule of five[16]

ADME and Electronic parameters

The most intense compound **A5** demonstrated a great rate of human oral absorption (89.317%). The polar surface area (PSA) of the molecule is the ability to participate in the emergence of hydrogen bonds, which also correlates with the absorption of the drug in the human intestine. If the molecule has PSA >140 Å considered to be poor absorption of the compounds. PSA is <80 Å for 75% of the compounds, which shows that these candidates have great intestinal absorption. Some determinants like shape, flexibility, and molecular size are observed to determine the lipophilicity of the synthesized compounds. Molecular flexibility mainly depends on the total rotatable bonds present in the molecule. The recommended range of rotatable bonds is <10 but the rotatable bonds of the synthesized compounds are in the range of <5 in Table -5.

Table -5: Predicted ADME properties of compounds (A1-10)

Comp	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	Acceptable range
CNS ^a	-2	-2	-2	-1	0	-2	0	0	-2	-2	(-2 to +2)
QPPCaco (nm/sec) ^b	83.45	38.899	45.492	331.991	333.255	42.48	378.54	384.483	93.199	2.227	<25 poor >500 Great
QPlogBB ^c	-1.511	-1.878	-1.642	-0.935	-0.692	-1.837	-0.49	-0.613	-1.445	-3.404	(-3 to 1.2)
QPPMDCK ^d	63.087	27.647	68.859	280.646	695.801	30.408	1680.65	744.908	71.081	1.256	<25 poor >500 Great
QPlogKp ^e	-4.672	-5.365	-5.372	-3.547	-3.611	-5.254	-3.639	-3.463	-4.577	-7.852	(-8 to -10)
QPlogHERG ^f	-4.748	-4.811	-4.554	-4.723	-4.737	-4.545	-4.536	-4.596	-4.681	-4.773	>-5
QPlogKhsa ^g	-0.578	-0.998	-0.909	-0.926	-0.773	-0.642	-0.681	-0.786	-0.606	-0.746	(-1.5 to 1.5)
PSA ^h	128.71	147.40	147.14	110.82	102.45	145.28	102.24	102.35	124.25	208.96	≤140
% of Human Oral Absorption ⁱ	63.338	55.232	58.956	76.668	89.317	55.689	82.814	80.432	64.713	15.357	>80% High <25%Low

^aPredicted central nervous system.

^bPredicted apparent Caco-2 cell permeability in nm/s

^cPredicted brain/blood partition coefficient.

^dPredicted apparent MDCK cell permeability.

^ePredicted skin permeability.

^fPredicted IC₅₀ values to block HERG K⁺ channels

^gPrediction of binding to human serum albumin.

^hPredicted polar surface area.

ⁱPredicted percentage human absorption.

Qikprop gives the information of different molecular descriptors, for example, SASA, MW, dipole, PISA, PSA, FOSA, FISA, #rotor, volume, QPpolrz, QPlogPo/w, QPLogKhsa, QPlogBB, donorHB, acceptHB, to decide #star boundary. #star values in the range of <5, helps to conclude that the synthesized compounds have similar properties to the known drugs[17] Table-6.

Table- 6: Electronic parameters of compounds (A1-10)

Comp	#rtvFG ^a	SASA(Å ²) ^b	FOSA ^c	FISA ^d	Volume (Å ³)	QPpolrz (Å ²) ^e
A1	1	518.563	82.578	218.757	871.134	28.384
A2	1	540.22	83.042	253.713	913.332	29.941
A3	1	549.443	79.958	246.543	943.983	30.791
A4	1	536.913	175.495	155.518	914.919	30.028
A5	1	523.803	82.823	155.344	883.957	29.508
A6	1	519.061	82.644	249.68	877.882	27.721
A7	1	535.612	79.852	149.508	915.763	30.414
A8	1	515.415	79.956	148.795	879.698	29.413
A9	1	512.791	81.189	213.697	863.227	28.074
A10	1	549.443	79.958	246.543	943.983	30.791
Acarbose(Std)	3	873.172	367.771	487.408	1691.81	47.598

^aNumber of reactive functional groups

^bTotal solvent accessible surface area

^cFOSA :Hydrophobic component of the SASA (saturated carbon and attached hydrogen).

^dFISA: Hydrophilic component of the SASA (SASA on N, O, and H on heteroatoms).

^ePredicted polarizability

The coefficient of blood/cerebrum (log B/B) specifies how a drug reaches the central nervous system (CNS). Synthesized compounds that are excessively polar neglect entry into the CNS. The processed CNS incentive is active at + 2 and inactive at - 2. All compounds with values below -2 are deemed to be inactive by the CNS. MadinDarby canine kidney (MDCK) is a successful adherent of the blood-brain barrier (BBB) and it anticipates BBB entrance. QPPCaCO values help to predict clear Caco-2 cell porousness in nm/sec. The gut–blood barrier model is simulated by Caco-2 cells. QikProp expectations predictions are made for transports of nonactive nature. The binding efficacy of the drug with human serum albumin is determined by LogK_{HSA}. If the imposing is less, the drug can diffuse more productively. Standard acarbose fall outside the recommended value and synthesized compounds show within the limit, indicating that compounds may strongly bind to human serum albumin. Also, Qikprop measures the number of metabolic responses, that predict if the drug can quickly enter the target site. This is meant by #metab, and the suggested range is 1–8. All subordinates fall within the limits of 2-5. The dermal penetration of the therapeutic agents is determined by LogK_p. The values observed for compounds (**A1-10**) indicate the potential dermal administration of the drug. The determined IC₅₀ of HERG K⁺ decides the poisonousness of cardiac therapeutic agents. The suggested value is >5. A value below -5 is measured to be cardio-toxic. All the compounds were found to be nontoxic, based on qualities > - 5[18].

Molecular dynamics (MD) simulation study

In an attempt to comprehend the dynamic behavior of the protein-ligand complex concerning time in a solvated system, molecular dynamic (MD) simulation studies were carried out. Hence, the most active designed ligands were used for stability mapping of complexes in simulated conditions. The simulation study analyzes the root mean square deviation (RMSD), solvent accessible surface area (SASA), gyration radius (Rg), root mean square fluctuation

(RMSF) and the number of hydrogen bonds sustained during the simulation duration and replace in the secondary structure pattern between the protein and its complexes. Six independent simulations were performed with the native protein and its ligand complex at 10ns of time length. The fundamental need for MD simulation to be conducted is to comprehend binding affinities between ligands to protein-bound complexes at time-bound stability. The RMSD plot (Figure - 4a) shows that the ligand-protein complex reached equilibrium at 2.5 to 6.5 ns time and the remaining trajectory was shown to be stable with marginal variance in the range of 0.10 to 0.15 nm RMSD, reflecting structural protein versatility reserved when complex with ligands rather than in free form. In the simulation, stable trajectories are represented by the unbound protein, while ligand-bound proteins enter equilibrium only after initial fluctuations. Considering the central rotation axis, the gyration radius (Rg) takes into account, the varied masses estimated for root mean square distances. During each time phase of the entire trajectory in the simulation, the Rg plot (Figure -4b) considers the capability, folding, and shape. Both protein entries and their respective ligand complexes show a similar Rg value pattern, deviating in the range of 0.10-0.15 nm. All the intermolecular H-bonds between ligands and proteins were considered and plotted accordingly during the study (Figure -4c). Over the total simulation time, H-bonds emerging during the molecular docking analysis were analyzed. From the plot, it is evident that the number of H-bonds remains consistent with molecular docking analysis during the simulation runs, although few bonds were broken and reconstructed at the same time. Solvent accessible surface area (SASA) measures the area between protein-ligand complexes formed around the hydrophobic center (Figure -4d). Consistent SASA values that usually fluctuate between 10-20nm² were observed. Fluctuations are due to residues evident in the RMSF plot (Figure -4e,4f) where amino acids involved in major interactions are rigidified in the complex form rather than the innate protein state.

Pharmacophore Mapping

The identified pharmacophore was represented in fig 6(a-d) with properties constitute of three hydrogen bond acceptors and two aromatic hydrophobic properties with distances ranging between 5.946 Å to 14.385 Å distances. It is interesting to observe that the features are in great accordance with the docking study (Figure - 5). The pharmacophore mapping; (a) Identified pharmacophore with all ligand structures aligned on pharmacophore, (b) superposition of most active ligand structure upon pharmacophore model (Figure -6). Distance-wise pharmacophore map; (c) distance in Å between pharmacophoric properties only representing center of the pharmacophoric properties, (d) distance of pharmacophoric properties with superposition of most active ligand.

In-vitro Antidiabetic activity

All the newly synthesized compounds were evaluated for *In-Vitro* antidiabetic potential by employing α -amylase and α -glucosidase inhibitory assay methods. Acarbose was used as standard. All the compounds were tested at a concentration range of 10-50 μ g/ml. The IC₅₀ values are summarized in Table-7.

In the α -amylase assay, compounds **A5** (IC₅₀=24.22 $\pm$ 1.65), **A6** (IC₅₀=24.64 $\pm$ 2.10), **A9** (IC₅₀=24.64 $\pm$ 1.88) showed very good inhibitory efficacy in comparison to the standard. The results are concordant with the assigned docking score values. The antidiabetic activity can be attributed to the presence of electron-donating groups which are present at the 3rd and 4th positions of the phenyl ring.

Table-7: Antidiabetic activity of compounds (A1-10)

Comp	IC ₅₀ Values	
	α -amylase (μ M)	α -glucosidase(μ M)
A1	27.59 $\pm$ 1.18	45.94 $\pm$ 2.11
A2	38.59 $\pm$ 0.85	32.40 $\pm$ 1.44
A3	36.32 $\pm$ 1.44	34.91 $\pm$ 1.16
A4	36.63 $\pm$ 1.35	31.89 $\pm$ 1.38
A5	24.22 $\pm$ 1.65	28.4 $\pm$ 0.57
A6	27.82 $\pm$ 2.10	26.37 $\pm$ 1.76
A7	28.13 $\pm$ 1.88	24.74 $\pm$ 1.63
A8	37.73 $\pm$ 1.05	29.22 $\pm$ 1.04
A9	24.64 $\pm$ 2.10	24.61 $\pm$ 1.76
A10	36.86 $\pm$ 1.42	26.22 $\pm$ 0.91
Std. (Acarbose)	24.35 $\pm$ 1.44	23.73 $\pm$ 1.22

In the α -glucosidase assay, compound **A9** (IC₅₀=24.61 $\pm$ 1.76), showed very potent activity, followed by compounds **A6** (IC₅₀=26.37 $\pm$ 1.76), **A7** (IC₅₀=24.74 $\pm$ 1.63) and compound **A10** (IC₅₀=26.22 $\pm$ 0.91), also displayed very good activity, when compared to the standard. The compounds with the electron-donating groups exhibited good activity. The compounds with electron-withdrawing groups showed weak activity in both the models.

Structure Activity Relationships (SAR)

α -Amylase Inhibitory activity:

The variation in the inhibitory potential of aminobenzothiazole incorporated rhodanines was attributed due to the various groups attached to the phenyl group at position-4. The compound **A9** (24.64 $\pm$ 2.10 μ M) with the electron donating capacity (OH), compound **A7** (24.74 $\pm$ 1.63 μ M) due to the presence of methoxy group at position 4 showed very good inhibition when compared to the standard Acarbose. Similarly the presence of hydroxyl group at position-2 (ortho), compound **A10** (36.86 $\pm$ 1.49) s showed the reduction in the activity when compared to compound **A9**. The high inhibition potential also observed with the compound **A6** (26.37 $\pm$ 1.76 μ M) due to presence of two electron donating group at positions 3,4 respectively, however the incorporation of an hydroxyl group at position -3 has showed an slightly increase in the inhibition when compared to the compound **A6**. However derivatives containing Cl, Br, NO₂ groups like compounds **A1**, **A2**, **A3** (electron withdrawing groups) displayed weak to moderate activity when compared to the standard. Replacement of methyl group at position-4 of aryl part also resulted in decreased activity by the compound **A8** (29.22 $\pm$ 1.04 μ M). The standard SAR suggested that all the structural features such as aminobenzothiazole group, rodhanine group and aryl part are actively participating in the activity and the variation in the activity is mainly due to the derivative substitution group (R) on the aryl ring.

α -Glucosidase Inhibitory activity:

All the newly synthesized compounds were evaluated for their In-vitro α -Glucosidase Inhibitory activity. The structure - activity relationship of these compounds has been established. The results revealed that, some of the tested

compounds exhibited inhibitory activity in the range of $IC_{50} = 45.94 \pm 2.11 \mu M$ to $24.61 \pm 1.76 \mu M$, when compared to the standard drug acarbose ($IC_{50} = 23.73 \pm 1.22 \mu M$). The results suggested that the rhodanine group attached at the 1st position of the aminobenzothiazole group and the various substituents attached at 4th position is responsible for the variations in the inhibitory activity. The highest inhibition is observed in the compound **A9** ($IC_{50} = 24.61 \pm 1.76 \mu M$), due to the presence of electron donating group (OH) at position-4. However, a slight decrease in the activity was observed with the compound **A10** ($IC_{50} = 26.22 \pm 0.91 \mu M$) which is having the hydroxyl group at position-2(ortho).

Introduction of an electron donating groups such as methoxy (3rd position) and hydroxyl (4th position) respectively results in the good inhibitory activity by compound **A5** ($IC_{50} = 28.34 \pm 0.57 \mu M$). Similarly the introduction of methoxy groups at positions 3 & 4 showed very good inhibitory activity by the compound **A6** ($IC_{50} = 26.37 \pm 1.76 \mu M$).

Similarly the introduction of methoxy group at position-4 showed a slightly increase in the inhibitory activity by the compound **A7** ($IC_{50} = 24.74 \pm 1.63 \mu M$), when compared to the other compounds like **A5**, **A6**. Further the compounds with electron withdrawing group such as NO_2 (**A1**) ($IC_{50} = 45.94 \pm 2.11 \mu M$) showed very weak inhibitory activity. Similarly the compounds with other electron withdrawing groups like **A2** (Br), ($IC_{50} = 32.40 \pm 1.44 \mu M$) **A3** (Cl) ($IC_{50} = 34.91 \pm 1.16 \mu M$) showed moderate activity, followed by the compound **A4** ($IC_{50} = 31.89 \pm 1.38 \mu M$) with the phenyl substituent. In summary based on the obtained SAR information it is found to be useful in the future structural optimization. However the binding interaction of the molecules with the active site of α -Glucosidase were confirmed their the molecular docking studies.

Conclusion

Knoevenagel Condensation was used to synthesize a novel series of Benzothiazole-Rhodanine derivatives and evaluated the new compounds for *In-vitro* antidiabetic activity. The inhibiting enzymes such as α -amylase and α -glucosidase, which are involved in starch digestion, glucose absorption, and glucose level regulation, are some of the therapeutic approaches to combat diabetes. Among the compounds evaluated, **A5**, **A6**, and **A9** showed good inhibitory activity. The present findings suggest that these compounds are found to be good candidates in the management of diabetes. A clear approach to the development of antidiabetic drugs was supported by molecular docking and simulation studies.

Experimental

The chemicals and reagents were acquired from Sigma Aldrich, Himedia, and SD Fine Chemicals, Mumbai, India. The homogeneity of the compounds was carried out with TLC pre-coated Silica GF₂₅₄ plates employing ethyl acetate and n-hexane (4:6) as a solvent system and visualization of TLC spots was carried out in the UV chamber. Melting points were carried out in Equiptronics digital melting point apparatus and are uncorrected. FT-IR spectrum (cm^{-1}) was recorded on an Alpha Bruker IR spectrometer. Proton spectra (1H NMR) and C^{13} NMR were recorded on the Agilent 400MR DD2 FT-NMR spectrophotometer operating at 400MHz. TMS is used as an internal standard (δ ppm). Waters HR-MS mass spectrometer operating at 70eV employing ESI and TOF detector, mass spectra were recorded.

Synthesis of Dithiocarbamate

Step-(1): An equimolar solution of sodium hydroxide (0.05 mol) and 2- Aminobenzothiazole (0.05 mol) was stirred together. CS_2 (8ml) was gradually introduced to the above solution, accompanied by stirring for 15-20 min, followed by additional stirring at 0-5°C for 3hr to yield the corresponding dithiocarbamate. The mixture was then stirred at room temperature, and the precipitate was filtered, washed, and dried. Recrystallization was done using ethanol.

Preparation of sodium salt of chloroacetic acid solution

Equimolar solution of Sodium hydroxide (0.05 mol) and Monochloroacetic acid (0.05 mol) was taken in a beaker, mixed, and stirred for 30 min at 0-5°C. The solution is converted basic to litmus paper by the addition of sodium carbonate.

Procedure for the synthesis of Benzothiazole incorporated N-substituted rhodanines

Step-(2): A solution of freshly prepared sodium chloroacetate was introduced to the solution containing dithiocarbamate (**1**) (0.01 mol) with stirring at 0-5°C. The stirring was further continued for 3 hr, followed by cyclization with concentrated HCl (65-70°C, 30 min). The formation of the product was monitored with the help of thin layer chromatography (TLC). The reaction mixture is allowed to cool at RT, followed by filtration and recrystallization using ethanol.

General procedure for the synthesis of benzothiazole substituted rhodanines by *Knoevenagel condensation* (A1-10)

N-substituted rhodanines (**2**) (0.01 mol) and aromatic aldehydes (0.01 mol) were dissolved in 15-20 ml of toluene in a 500ml round bottom flask. Piperidine and glacial acetic acid were added in catalytic amounts and the contents were refluxed for 8-10 hrs. (100-110°C). The formation of the product was monitored with the help of TLC and followed by cooling the reaction mixture. The precipitated compound is filtered, washed with water, and recrystallization was done using ethanol [15]. The physical data of compounds (**A1-10**) is given in Table-1.

(Z)-3-(benzo[d]thiazol-2-yl)-5-(4-nitrobenzylidene)-2 thioxothiazolidin-4-ones (A1): Yellow solid. Yield: 67%, melting point (MP). 233-35°C, FT-IR (cm⁻¹): 3065 (Ar,C-H), 1700 (C=O), 1611 (C=N), 1531(C=C). ¹H-NMR (400 MHz, DMSO-d₆, δ, ppm): 7.228-7.963 (m, Ar, 8H), 8.03(s, =CH, 1H). ¹³CNMR (400MHz, DMSOd₆, δ, ppm): 113.42, 115.97, 118.13, 118.23, 122.05, 125.23, 126.23, 127.34, 140.01, 143.82, 145.78, 154.01, 155.89, 157.78, 162.71, 176.10 and 202.24. HR-MS (m/z): calculated for C₁₇H₉N₃O₃S₃ is 398.98; found: 398.9896 (M+).

(Z)-3-(benzo[d]thiazol-2-yl)-5-(4-bromobenzylidene)-2-thioxothiazolidin-4-one (A2): Yellow solid. Yield 72%, MP. 245-47°C, FT-IR (cm⁻¹): 3048 (Ar, C-H), 1725 (C=O), 1655 (C=N), 1593 (C=C). ¹H-NMR (400 MHz, DMSO-d₆, δ, ppm): 6.98-7.51 (m, Ar, 8H), 7.61 (s, =CH, 1H). ¹³C-NMR (400MHz, DMSO-d₆, δ, ppm): 111.16, 115.84, 116.53, 120.50, 121.56, 121.85, 123.00, 125.39, 126.47, 129.10, 131.24, 131.60, 131.79, 148.47, 154.10, 164.00 and 192.02. HR-MS (m/z): calculated for C₁₇H₉BrN₂OS₃ is 433.91; found: 433.9170(M+).

(Z)-3-(benzo[d]thiazol-2-yl)-5-(4-chlorobenzylidene)-2-thioxothiazolidin-4-one (A3): Yellow solid. Yield 69%, MP. 273-75°C, FT-IR (cm⁻¹): 3048 (Ar, C-H), 1655 (C=O), 1593 (C=N), 1516 (C=C). ¹H-NMR (400 MHz, DMSO-d₆, δ, ppm): 6.97 - 7.62 (m, Ar, 8H), 7.6 (s, =CH, 1H). ¹³C-NMR (400MHz, DMSO-d₆, δ, ppm): 115.11, 117.68, 118.11, 119.91, 121.29, 122.38, 122.71, 123.42, 125.87, 126.05, 132.32, 132.41, 142.13, 146.47, 153.52, 179.51 and 196.23. HR-MS (m/z): calculated for C₁₇H₉ClN₂OS₃ is 354.47; found: 354.4713 (M+).

(Z)-3-(benzo[d]thiazol-2-yl)-5-benzylidene-2-thioxothiazolidin-4-one (A4): Yellow solid. Yield 65%, MP. 259-61°C; FT-IR (cm^{-1}): 3010 (Ar, C-H), 1723 (C=O), 1631 (C=N), 1531 (C=C). $^1\text{H-NMR}$ (400 MHz, DMSO-d_6 , δ , ppm): 6.97 - 7.47 (m, Ar, 8H), 7.63 (s, =CH, 1H). $^{13}\text{C-NMR}$ (400MHz, DMSO-d_6 , δ , ppm): 116.32, 117.68, 118.11, 119.91, 121.29, 122.38, 122.71, 123.42, 125.87, 126.05, 132.32, 132.41, 142.13, 146.47, 153.52, 169.43 and 196.23. HR-MS (m/z): calculated for $\text{C}_{17}\text{H}_{10}\text{N}_2\text{OS}_3$ is 354.47; found: 354.47 (M+).

(Z)-3-(benzo[d]thiazol-2-yl)-5-(4-hydroxy-3-methoxybenzylidene)-2-thioxothiazolidin-4-one (A5): Yellow solid. Yield 87%, MP. 214-16°C, FT-IR (cm^{-1}): 3176 (OH), 3064 (Ar, C-H), 1722 (C=O), 1639 (C=N), 1530 (C=C). $^1\text{H-NMR}$ (400 MHz, DMSO-d_6 , δ , ppm): 4.20 (s, OCH_3 , 3H), 6.99 - 7.53 (m, Ar-H, 7H), 7.63 (s, =CH, 1H), 9.98-9.82 (s, OH, 1H). $^{13}\text{C-NMR}$ (400MHz, DMSO_6 , δ , ppm): 40.09, 114.47, 115.84, 117.96, 121.42, 121.47, 125.98, 130.95, 143.39, 143.84, 148.10, 150.10, 150.24, 150.24, 150.30, 150.79, 151.03 168.54 and 191.54. HR-MS (m/z): calculated: for $\text{C}_{19}\text{H}_{14}\text{N}_2\text{O}_3\text{S}_3$ is 400.50; found: 400.5043 (M+).

(Z)-3-(benzo[d]thiazol-2-yl)-5-(3,4-dimethoxybenzylidene)-2-thioxothiazolidin-4-one (A6): Yellow solid. Yield 74%, MP. 227-29°C, FT-IR (cm^{-1}): 3274 (OH), 3007 (Ar, C-H), 1726 (C=O), 1641 (C=N), 1460 (C=C). $^1\text{H-NMR}$ (400 MHz, DMSO-d_6 , δ , ppm): 3.82 (s, OCH_3 , 3H), 4.26 (s, OCH_3 , 3H), 6.97-7.49 (m, Ar-H, 7H), 7.53 (s, =CH, 1H). $^{13}\text{C-NMR}$ (400MHz, DMSO_6 , δ , ppm): 55.96, 56.32, 109.92, 111.72, 118.10, 121.30, 121.59, 125.87, 126.54, 130.09, 131.26, 144.92, 149.62, 152.98, 154.64, 166.96, 168.54, 177.92 and 191.80. HR-MS (m/z): calculated for $\text{C}_{19}\text{H}_{14}\text{N}_2\text{O}_3\text{S}_3$ is 414.52; found: 414.5277 (M+).

(Z)-3-(benzo[d]thiazol-2-yl)-5-(4-methoxybenzylidene)-2-thioxothiazolidin-4-one (A7):

Yellow solid. Yield 67%, MP. 266-68°C; FT-IR (cm^{-1}): 3211 (OH), 3014 (Ar, C-H), 1725 (C=O), 1632 (C=N), 1531 (C=C). $^1\text{H-NMR}$ (400 MHz, DMSO-d_6 , δ , ppm): 3.85 (s, OCH_3 , 3H), 6.99 - 7.21 (m, Ar-H, 8H), 7.31 (s, =CH, 1H). $^{13}\text{C-NMR}$ (400MHz, DMSO-d_6 , δ , ppm): 56.12, 113.82, 114.94, 115.02, 118.14, 121.25, 121.28, 125.86, 130.10, 131.32, 132.24, 148.21, 153.16, 164.66, 166.89, 168.55, 179.92 and 191.71. HR-MS (m/z): calculated for $\text{C}_{18}\text{H}_{12}\text{N}_2\text{O}_2\text{S}_3$ is 384.50; found: 384.5043 (M+).

(Z)-3-(benzo[d]thiazol-2-yl)-5-(4-(dimethylamino) benzylidene)-2-thioxothiazolidin-4-one (A8): Yellow solid. Yield 56%, MP. 235-37°C; FT-IR (cm^{-1}): 2912 (Ar, C-H), 1725 (C=O), 1649 (C=N), 1529 (C=C). $^1\text{H-NMR}$ (400 MHz, DMSO-d_6 , δ , ppm): 3.01 (s, 2 x CH_3 , 6H), 6.77 - 7.64 (m, Ar-H, 8H), 7.66 (s, =CH, 1H). $^{13}\text{C-NMR}$ (400MHz, DMSO-d_6 , δ , ppm): 40.33, 40.53, 111.49, 118.11, 121.30, 124.95, 125.87, 131.26, 131.98, 132.24, 139.12, 140.21, 141.12, 152.99, 154.64, 154.86, 166.95, 168.53 and 190.30. HR-MS (m/z): calculated for $\text{C}_{19}\text{H}_{15}\text{N}_2\text{OS}_3$ is 397.54; found: 397.5439 (M+).

(Z)-3-(benzo[d]thiazol-2-yl)-5-(3, 4-dichlorobenzylidene)-2-thioxothiazolidin-4-one (A9): Yellow solid. Yield 79%, MP. 217-19°C, FT-IR (cm^{-1}): 3194 (-OH), 2996 (Ar, C-H), 1725 (C=O), 1640 (C=N), 1532 (C=C). $^1\text{H-NMR}$ (400 MHz, DMSO-d_6 , δ , ppm): 6.99 - 7.64 (m, Ar-H, 8H), 7.66 (s, =CH, 1H), 9.78 (s, OH, 1H). $^{13}\text{C-NMR}$ (400MHz, DMSO-d_6 , δ , ppm): 114.52, 116.29, 117.86, 121.47, 121.53, 126.01, 130.77, 122.51, 130.77, 132.51, 150.14, 151.21, 154.38, 155.78, 156.15, 167.10, 168.52, 169.93 and 191.36. HR-MS (m/z): 369.96 (M+1 found) and calculated: 369.9819 (M+).

(Z)-3-(benzo[d]thiazol-2-yl)-5-(2-hydroxybenzylidene)-2-thioxothiazolidin-4-one (A10):

Yellow solid, Yield 52%, MP. 192-94°C; FT-IR (cm⁻¹): 3293 (OH), 3064 (Ar, C-H), 1725 (C=O), 1639 (C=N), 1529 (C=C). ¹H-NMR (400 MHz, DMSO-d₆, δ, ppm): 6.94 - 7.49 (m, Ar-H, 8H), 7.62 (s, =CH, 1H), 10.26 (s, OH, 1H). ¹³C-NMR (400MHz, DMSO-d₆, δ, ppm): 117.88, 118.11, 119.91, 121.29, 122.30, 122.71, 123.42, 125.87, 126.79, 129.70, 131.27, 136.82, 153.02, 161.17, 166.93, 168.53 and 192.31. HR-MS (m/z): 369.96 (M+1 found) and calculated: 369.9178 (M+).

***In-silico* studies:**

In-silico studies were performed by determining ADMET properties, Lipinski's rule of five, docking studies, free energy calculation, and toxicity profile, which is mainly focused to determine drug-likeness and preferred orientation of the molecule.

Drug likeness evaluation of benzothiazole–rhodanine derivatives

Determination of Lipinski's rule of five, physicochemical parameters ADMET properties and evaluation of toxicity of the Benzothiazole–Rhodanine derivatives

Lipinski's rule of five is mainly used to verify molecular properties [19] which are essential and related to PK of the drug molecule, which is carried out with Schrodinger 2018-3 suite device Maestro 11.7.012[20].

Qikprop(<https://www.schrodinger.com>) is a rapid, precise, and accessible to predict ADME properties [21]. In stages of drug development, toxicity must be done and in many cases, toxic effects are identified in the later stage of the development. So in drug development, prior precise toxicity assessments are very important.

Molecular docking studies

To rationalize the results from the *In-Vitro* antidiabetic studies, we have further carried out flexible ligand docking using the Glide module. Molecular docking was performed by utilizing the Glide module of Schrodinger 2018-3 suite device Maestro 11.7.012 [22] (<https://www.schrodinger.com>) Crystal structures with good resolution of all the target proteins were taken from RCSB PDB (<https://www.rcsb.org>)[23]

Binding free energy (MM-GBSA) calculation

Binding free energy (MM-GBSA) calculations were performed by prime-MM-GBSA Schrodinger 2018-3 suite devices Maestro 11.7.012. Scores were taken from XP docking for free energy calculation [24,25].

Molecular dynamics (MD) simulation study

On GROMACS 5.1.5, performed with CHARMM27 all-atom force field, MD simulation analyses were carried out on native protein structures (PDB ID: 3TOP) in innate as well as docked complex form with ligand structure. Externally, all atomic force fields were generated using the SwissParam server (<https://www.swissparam.ch/>) using CHARMM to create the topology files for the ligand. For calculating the Van der Waal interactions, a distance cut-off of 1.0 nm was maintained. Partial Mesh Ewald (PME) summation for long-range electrostatics was applied to calculate columbic interactions (1nm cut-off). To maintain the electro-neutrality of the system, counter ions were included. Using the TIP3P water model, the system was solvated and simulations were carried out in a triclinic box wherein the protein

atoms were removed from the box wall dimensions at 1.0 to 1.5 nm, keeping intact the conditions of the periodic boundary. Structure minimization was done foremost using the steepest descent algorithm and a 1000 KJ/mol/nm tolerance. Position restraints were applied on the complex to equilibrate the system. Simulations were also performed by employing canonical NVT and NPT ensembles. At a temperature of 300 K and a pressure of 1 bar, both equilibria were carried out at 200 ps each. Initial velocities were yielded following Maxwell distribution and coupling of temperature was implemented using velocity rescaling (coupling constant of 0.1 ps). Extended ensemble Parrinello–Rahman algorithm helped in temperature pressure coupling (coupling constant of 2 ps). The equilibrated system was further exposed to a production run of 10 ns and 2 fs time step integration. XMGRACE-5.1.22 and GROMACS analysis tools program were employed to analyze the saved trajectories. (<https://plasma-gate.weizmann.ac.il/Grace/>).

Pharmacophore Mapping

The pharmacophore mapping identifies the molecular properties in 2D and 3D terms. The present title compounds were initially aligned to a common scaffold and prepared a dataset. The pharmacophore could be generated from several algorithms of which common feature pharmacophore application is one such. The application identifies molecular features out of the several structural properties and the individual features were kept a maximum of 2.5 Å distance with a maximum of six features as a single pharmacophore set. The best pharmacophore thus generated was selected best on fitness.

In-vitro Antidiabetic activity

α -amylase inhibitory assay

20 μ L of α -amylase solution (0.5 mg/ml) was added to phosphate buffer (pH 6.9, 0.02 M, 200 μ L). 250 μ L of test samples (10–50 μ g/mL) was added, to all the above solutions and kept for incubation for 10 min. 1% starch solution (200 μ L) was further added and again kept for incubation for 10 min at a temperature of 25°C. Termination of the reaction was affected by the addition of 400 μ L of 3, 5-dinitrosalicylic acid (DNS) reagent. Lastly, it was incubated in water (at 70°C, 5 min). The absorbance was recorded using an ELISA microplate reader at 540 nm. Acarbose was served as the standard. All the reactions were carried out in triplicates. The reaction mixture, devoid of the test sample, served as control. The following formula was employed to calculate percentage inhibition. The IC₅₀ values are tabulated in table-7[26].

$$\% \text{ Inhibition} = [(Ac - As) / Ac] \times 100$$

Ac-Control absorbance, As-Standard absorbance

α -glucosidase inhibitory assay

10 μ L of the α -glucosidase enzyme, 20 μ L of test samples (10–50 μ g/mL) and 50 μ L of 0.1M phosphate buffer (pH=6.8), were incubated at 37°C for 15 min in a 96-well plate. 20 μ L of p-nitrophenyl–D-glucopyranoside solution was further added as a substrate and again incubated for 20 min at 37°C. Sodium carbonate (50 μ L of 0.1M) was added to terminate the reaction. P-nitrophenol was liberated, during the reaction which was measured at 405 nm using an ELISA microplate reader. Acarbose served as the standard. A control was prepared under similar conditions by omitting test samples. All the experiments were carried out in triplicates. The percentage inhibition was calculated by the formula. The IC₅₀ values are tabulated in table-7.26

$$\% \text{ Inhibition} = [(Ac - As) / Ac] \times 100$$

Ac-absorbance for control, As-absorbance for standard

Declarations

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Author contributions

GSM, NNA and BFDG performed all experiments, purified all compounds, analyzed the data and summarized the results. GSM tested all compounds for their antibacterial activity. MKS and KBR characterized all compounds for NMR and Mass. PK and helped in compiling the data of the manuscript. RBC conceived and designed this research and wrote the manuscript. All authors have contributed to the final version and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical statement

This study doesn't include any animal or human study.

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Figures

Figure 1

Strategy applied in designing the target molecules

Figure 2

Interaction of A5 with 3TOP

Figure 3

Interaction of A9 with 3TOP

Figure 4

Analysis of RMSD, Rg, hydrogen bond, SASA and RMSF of 3TOP (protein) with ligand complexes at 10000 ps. (a) Time evolution of backbone RMSD of the CLPro protein alone and with ligand complex structures. (b) Radius of gyration (Rg) of the protein backbone in its free and complex form over the entire simulation time. The ordinate is Rg(nm) and abscissa is time (ps) interval. (c) SASA is indicated, where ordinate is SASA (nm) and abscissa is time (ps). (d) Hydrogen bonds occurring over the time of simulation between protein and ligand (e and f). Residue wise average RMSF plot of protein and ligand.

Figure 5

The pharmacophore mapping; (a) Identified pharmacophore with all ligand structures aligned on pharmacophore, (b) superposition of most active ligand structure upon pharmacophore model.

Figure 6

Distance wise pharmacophore map; (c) distance in Å between pharmacophoric properties only representing center of the pharmacophoric properties, (d) distance of pharmacophoric properties with superposition of most active ligand.

Figure 7

scheme-1: Outline for the synthesis. Reagents and conditions: a) CS_2 and NaOH , ice-cold condition, $0-5^\circ\text{C}$, stir, 3hr b) Sodium chloroacetate solution, ice-cold conditions with stirring, c) $60-70^\circ\text{C}$, Conc. HCl , d) Aromatic aldehydes, glacial acetic acid, piperidine, toluene, $100-110^\circ\text{C}$, reflux, 10hr. A1: 4- NO_2 , A2: 4- Br , A3: 4- Cl , A4: C_6H_5 , A5: 3- OCH_3 -4- OH , A6: 3,4- $(\text{OCH}_3)_2$, A7: 4- OCH_3 , A8: 4- $\text{N}(\text{CH}_3)_2$, A9: 4- OH , A10: 2- OH .

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