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Synthesis, antibacterial activities and molecular docking studies of peptide and Schiff bases as targeted antibiotics

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ABSTRACT

A series of peptide and Schiff bases (PSB) were synthesized by reacting salicylic acid, primary diamines with salicylaldehyde or its derivatives, and 40 of which were newly reported. The inhibitory activities against *Escherichia coli* β-ketoacyl-acyl carrier protein synthase III (ecKAS III) were investigated in vitro and molecular docking simulation also surveyed. Top 10 PSB compounds which posses both good inhibitory activity and well binding affinities were picked out, and their antibacterial activities against Gram-negative and Gram-positive bacterial strains were tested, expecting to exploit potent antibacterial agent with broad-spectrum antibiotics activity. The results demonstrate compound *N*-(3-(5-bromo-2-hydroxybenzylideneamino)propyl)-2-hydroxybenzamide (**2d**) can be as a potential antibiotics agent, displaying minimal inhibitory concentration values in the range of 0.39–3.13 μg/mL against various bacteria.

1. Introduction

During the past decades, the human population affected with life-treating infectious diseases caused by multi-drug resistant Gram-positive and Gram-negative pathogen bacteria increased an alarming level around the world[1] and represent the second leading cause of death.[2]

Type II fatty acid synthesis (FAS II) pathway has been recently reported as a attractive targeting for their efficacy against infections caused by mutiresistant Gram-positive bacteria.[3,4] Also have people not agree with this point for there are plenty of fatty acids available to the bacteria inside of the host.[5] However, FAS II it's proven to be a good target for Gram-negative.[6]

Among the related FAS II enzymes, the condensing protein, β-ketoacyl-acyl carrier protein synthase (KAS), is an essential target for novel antibacterial drug design.[7] In bacteria, there appear to be three KAS enzymes, which are denoted KAS I, KAS II, and KAS III (the *Escherichia* coli equivalents are FabB, FabF, and FabH, respectively). The initial C2–C4 step of fatty acid biosynthesis is catalyzed by KAS III; thereafter, KAS I and KAS II are involved in chain elongation.[8] Notably, KAS III, regulates the fatty acid biosynthesis rate via an initiation pathway and its substrate specificity is a key factor in membrane fatty acid composition and this protein represents a promising target for the antimicrobial drugs design.[9] Moreover, the three-dimensional structure of the protein is highly conserved in various bacteria, the active site contains a Cys-His-Asn catalytic triad,[10] and its inhibitors may thus act as potent antibiotics with broad-spectrum activity.

Recent reports demonstrated that thiolactomycin[11] and its analogues compounds[12] have low toxicity and some of which are highly active against KAS III. Acyl derivative[13] and Schiff bases were also well document as a wide variety of biological activities such as antimicrobacterial, antimicrobial, and antitumor agents, etc.[14–16] Recently, Schiff bases were reported as a potent inhibitor of *Plasmodium falciparum* KAS III[17] and ecKAS III with antimicrobial activity against various bacteria.[18] In view of this, we developed a series of new PSB compounds and suppose them possess similar or much better biological activities as salicylamide peptide or salen-type Schiff base by the fact that PSB own both peptide and Schiff base structures (Scheme 1).

In this study, our main objective is the development of potential inhibitors by newly synthesized PSB as targeted antibiotics agents, based on molecular modeling and the investigation of SAR between new inhibitors and ecKAS III. Firstly, we use structure-based design method to synthesized the PSB and test their inhibitory activity against ecKAS III. And following, top ten PSB compounds which posses good ecKAS III inhibitory activity (low IC_{50}) and well binding affinities (binding free energies, inhibition constants, hydrogen bonding) by docking into the active site of ecKAS III were picked out to test their antibacterial activities against two Gram-negative bacterial strains (*E. coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 13525) and two Gram-positive bacterial strains (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538), expecting to exploit potent antibacterial agent with broad-spectrum antibiotics activ-

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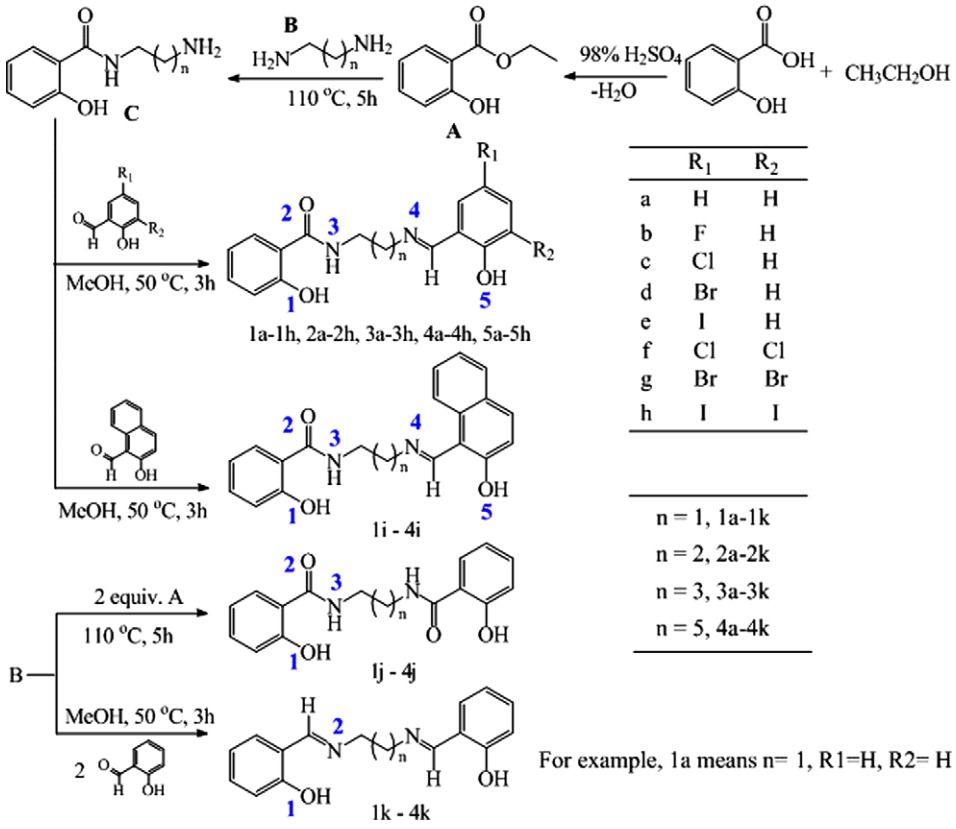

	R_1	R_2
a	H	H
b	F	H
c	Cl	H
d	Br	H
e	I	H
f	Cl	Cl
g	Br	Br
h	I	I

Scheme 1. The general procedure of the synthesized compounds. '**1**', '**2**', '**3**', '**4**', '**5**' represent the atom number bonding to the amino acid active site.

ity. As expected, the results demonstrate that the antimicrobial compound, *N*-(3-(5-bromo-2-hydroxybenzylideneamino)propyl)-2-hydroxybenzamide (**2d**), can be as an inhibitor of ecKAS III and as a potential antibiotics agent, displaying MIC values in the range 0.39–3.13 μg/mL against various bacteria. Which is much better than the best compound (YKAs3003) reported by Kim and co-workers.[18] The AutoDock study for new inhibitors docked into the active sites of ecKAS III has also been carried out.

2. Result and discussion

2.1. Chemistry

In the present study, 36 PSB were subjected by reacting salicylamide types primary amines with salicylaldehyde or its derivatives. And 8 di-peptide and di-Schiff base compounds were synthesized through primary diamines reaction with ethyl salicylate or salicylaldehyde, respectively. The general method for preparing the compounds is outlined in Scheme 1. Salicylamide types primary amines (**C**), which were not commercially available, were synthesized using modified procedures of Kido.[19] The crude products were purified using silica gel column chromatography, preparative TLC and recrystallization. All the compounds gave satisfactory chemical analyses (±0.4%). ^{1}H NMR, ESI-MS spectra and elemental analyses were consistent with the assigned structures.

Compound **1c** was successfully crystallized and its structure was determined by single-crystal X-ray diffraction analysis. The crystal data are presented in Table 1, and Figure 1 gives a perspective view of this compound together with the atomic labeling system. In the structure of compound **1c**, there are an intramolecular O–H···O (O1–H1···O2, 2.504(3) Å, 148.6°) and O–H···N hydrogen bond (O3–H3A···N2: 2.602(3) Å, 147.3°) and an intermolecular N–H···O hydrogen bond (N1–H1A···O1^i: 2.974(3) Å, 137.2°, symmetry codes: *i*, *x*, 1 + *y*, *z*) in the structure. The dihedral angle between the two benzene rings is 34.1 (0.3)°. The bond length of N1–C7 (1.330(4) Å) indicates a single bond and bond length of N2=C10 (1.266(3) Å) exhibits a double bond.

Table 1
Crystal and experimental data for complexes **1c**

Compound	**1c**
Empirical formula	$C_{16}H_{15}ClN_2O_3$
Mr	318.75
T (K)	298(2)
Radiation (Mo Kα), λ (Å)	0.71073
Crystal shape/color	block/yellow
Crystal size (mm^3)	0.32 × 0.20 × 0.17
Crystal system	monoclinic
Space group	$P2_1/c$
a (Å)	16.527(2)
b (Å)	6.401(1)
c (Å)	14.117(1)
α (°)	90.00
β (°)	90.49(5)
γ (°)	90.00
V (Å^3)	1493.4(2)
Z	4
D_{calcd} (g/cm^{-3})	1.418
μ (mm^{-1})	0.270
F(0 0 0)	664
Goodness of fit on F^2	1.003
R_1, *wR* [$I \geqslant 2\sigma(I)$][a]	0.0487, 0.1306
R_1, *wR* (all data)[a]	0.0942, 0.1687

[a] $R_1 = \sum||F_o| - |F_c||/\sum|F_o|$, $wR_2 = [\sum w(F_o^2 - F_c^2)^2/\sum w(F_o^2)^2]^{1/2}$, $w_1 = [\sigma^2(F_o)^2 + (0.1009(F_o^2 + 2F_c^2)/3)^2]^{-1}$, $w_2 = [\sigma^2(F_o)^2 + (0.0962(F_o^2 + 2F_c^2)/3)^2]^{-1}$.

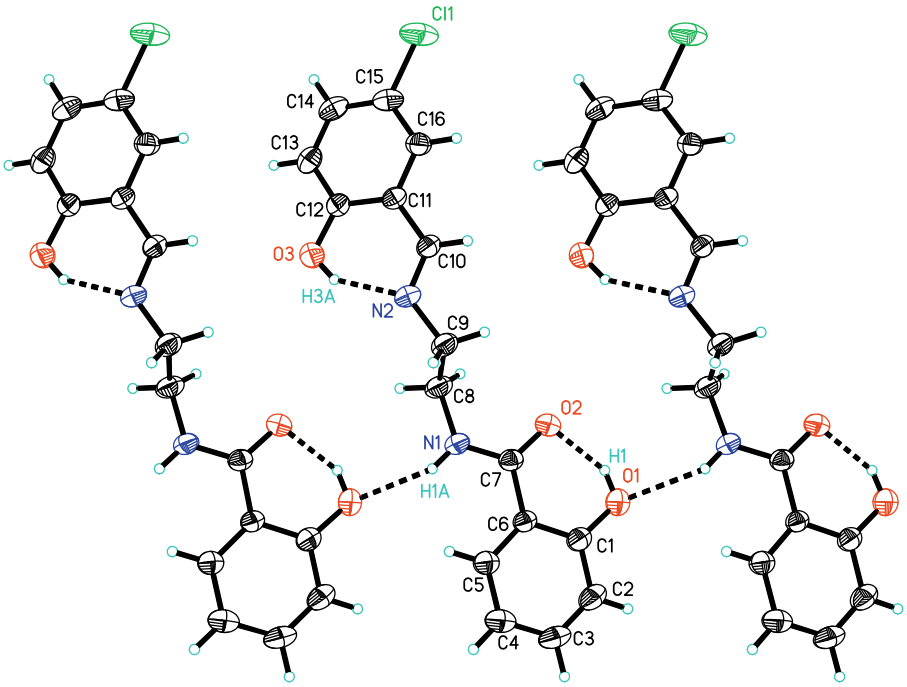

Figure 1. Molecular structure of compound **1c** with atoms and hydrogen bonds labeling.

2.2. Inhibitory activities of PSB against ecKAS III

All the synthesized compounds (**1a–1k, 2a–2k, 3a–3k** and **4a–4k**) were tested for inhibitory activity against ecKAS III. The inhibition constant (IC_{50}) of the compounds were presented in Table 2 and Figure 2 (IC_{50} more than 50 μg/mL not demonstrate in Fig. 2). It was observed that many compounds have been found to show fairly good inhibitory activity displaying IC_{50} values between 0.33 and 40.0 μg/mL.

Inspection of the chemical structure of the compounds (Scheme 1) suggested that it could be divided into two subunits: the length of aliphatic chain-*n* and the substitution on aromatic ring-R_1 and R_2. Initial SAR studies were performed by modification of the parent compound to determine how the substituents of the subunits affected the inhibitory activities. Replacement of H atom (**1a**) at R_1-position by one halogen atom or R_1 and R_2-position by two halogen atoms resulted in the improving of their inhibitory activity (**1c–1e** and **1f–1g**). Increment of the aliphatic chain from 1 (**1b** IC_{50} 10.35 μg/mL) to 2 (**2b** IC_{50} 1.76 μg/mL) also led to a similar increasing of inhibitory activity, while n increased from 2 (**2b**) to 5 (**4b** IC_{50} 40.00 μg/mL), the inhibitory activity was decrease (Fig. 2). For more extensive evidences, the substitution of H atom at R_1 (**2b–2e**, **3b–3e**, **4c–4d**), R_1 and R_2 (**2f–2g**, **3f–3h**, **4g–4h**) by halogen atoms, also produced some increment compared to the corresponding precursor **2a**, **3a** and **4a**, respectively. This suggested that compounds with electron-withdrawing groups on R_1 and R_2 and appropriate length of aliphatic chain ($n = 2, 3$) showed better inhibitory activities, excepting **1b**, **2h**, **4b**, **4e** and **4f**. The introduction naphthyl (**1i**, **2i**, **3i** and **4i**) substitute of phenyl group, led to inhibitory activity increase in **2i** (IC_{50} 3.00 μg/mL), and **3i** (IC_{50} 3.10 μg/mL). Compared to PSB, the di-peptide and di-schiff base compounds exhibit weak inhibitory activities.

2.3. Molecular docking study

Here, we investigated the AutoDock binding affinities of the synthesized PSB into ecKAS III. We defined the active site of ecKAS III, based on the center and radius of the binding substrate in an X-ray structure of ecKAS III complexed with CoA or inhibitor.[20] Towards optimization of the aforementioned compounds of the promising antimicrobial activities, the docking program AutoDock 4.0[21] was used to evaluate the binding free energies as potential inhibitors into the target ecKAS III macromolecule.

2.3.1. Validation of the accuracy and performance of AutoDock

According to the method of validation cited in literature,[22] where if the RMSD (root mean square deviation) of the best docked conformation is ⩽2.0 Å from the experimental one, the used scoring function is successful. The obtained success rates of AutoDock (Morris et al.)[21] was highly excellent as illustrated in Table 2 for the native ligand, which was docked into its KAS III (PDB code: 1HNJ).

2.3.2. AutoDock binding affinities of the synthesized and designed compounds into KAS III

The binding affinity was evaluated by the binding free energies (ΔG_b, kcal/mol), inhibition constants (K_i) and hydrogen bonding. The compounds which revealed the highest binding affinities, that is, lowest binding free energies, within KAS III and the hydrogen bond interactions into the target macromolecule are represented in Table 2. These compounds include *N*-(3-(5-bromo-2-hydroxybenzylideneamino)propyl)-2-hydroxybenzamide (**2d**), (*E*)-2-hydroxy-*N*-(4-(2-hydroxy-5-iodobenzylideneamino)butyl)benzamide (**3e**), *N*-(3-(5-chloro-2-hydroxybenzylideneamino)propyl)-2-hydroxybenzamide (**2c**), 2-hydroxy-*N*-(3-(2-hydroxy-5-iodobenzylideneamino)propyl)benzamide (**2e**) and (*E*)-2-hydroxy-*N*-(2-(2-hydroxy-5-iodobenzylideneamino)ethyl)benzamide (**1e**). Many of these derivatives exhibited one or two hydrogen bonds between O1, O2, O5 or N3 of PSB and different amino acids of the target ecKAS III including Asn 274, Asn 247, Arg 36, Arg 151, Gly 209, Trp36, Gly152, Asn 210, and Arg 249 as cited in Table 2.

The molecular docking study revealed that the majority of the compounds docked into the ecKAS III (PDB code: 1HNJ) exhibited hydrogen bonds via O2 (or O5)–NH group as illustrated for compounds **2d** in Figure 3.

Table 2
Inhibition constant (IC_{50}) against ecKAS III and docking simulation parameters

Compound	Inhibitory constant				Docking parameters				Total score
	IC_{50} (μg/mL)	Observed activity class	ΔG_b (kcal/mol)	K_i	ΔG and K_i score	Hydrogen bond atom of compound	Amino acids	Hydrogen bond score	
1a	7.25	++	−7.14	5.70×10^{-6}	+	O2	N–H of Asn 274	+	++++
1b	10.35	+	−7.80	1.39×10^{-6}	+	O1	N–H of Asn 247	+	+++
1c	2.77	+++	−7.5	3.18×10^{-6}	+	O5	N–H of Arg 36	+	+++++
1d	6.22	++	−6.65	1.34×10^{-5}	0	—	—	0	++
1e	1.13	++++	−9.58	9.51×10^{-8}	+++	O2	N–H of Asn 247	+	++++++++
1f	2.5	+++	−8.62	4.80×10^{-7}	++	O2	N–H of Asn 247	+	++++++
1g	0.54	+++++	−9.15	1.98×10^{-7}	+++	O2	N–H of Arg 151	+	+++++++++
1h	12.72	+	−8.85	3.27×10^{-7}	++	—	—	0	+++
1i	9.75	+	−7.79	1.96×10^{-6}	+	O5	N–H of Asn 247	+	+++
1j	16.51	0	−8.93	2.84×10^{-7}	++	O1	N–H of Asn 274	+	+++
1k	>50.00	0	−8.14	1.08×10^{-6}	++	O1	N–H of Asn 247	+	+++
2a	12.5	+	−6.94	8.22×10^{-6}	0	O2	N–H of Arg151	+	++
2b	1.76	++++	−9.35	1.40×10^{-7}	+++	O2	N–H of Asn 247	++	+++++++++
						O5	N–H of Gly 209		
2c	1.64	++++	−9.6	9.23×10^{-8}	+++	O2	N–H of Asn 247	+	++++
2d	0.33	+++++	−11.34	4.85×10^{-9}	+++	O2	N–H of Asn 247	++	++++++++++
						O5	N–H of Asn 274		
2e	1.50	++++	−9.51	1.06×10^{-7}	+++	O2	N–H of Asn 247	+	++++++++
2f	2.75	+++	−7.82	1.86×10^{-6}	+	O2	N–H of Asn 247	+	+++++
2g	6.31	++	−7.82	1.84×10^{-6}	+	O1	N–H of Gly 209	+	++++
2h	12.95	+	−6.46	1.84×10^{-8}	0	—	—	0	+
2i	3.00	+++	−8.66	4.51×10^{-7}	++	O5	N–H of Trp 36	++	+++++++
						O1	N–H of Asn 247		
2j	13.15	+	−9.71	7.63×10^{-8}	+++	O2	N-H of Asn 247	++	++++++
						O2	N–H of Asn 274		
2k	12.25	+	−9.01	2.49×10^{-7}	+++	O1	N–H of Gly 209	+	+++++
3a	13.57	+	−6.78	1.06×10^{-5}	0	O1-H	O of Gly152	+	++
3b	6.35	++	−6.19	2.91×10^{-5}	0	O2	N–H of Asn 247	+	+++
3c	2.32	+++	−9.49	1.11×10^{-7}	+++	—	—	0	++++++
3d	6.25	++	−6.32	2.33×10^{-5}	0	O5	N–H of Asn 274	+	+++
3e	0.65	+++++	−10.15	3.66×10^{-8}	+++	O5	N–H of Trp 36	++	++++++++++
						O2	N–H of Asn 247		
3f	2.95	+++	−4.67	3.74×10^{-4}	0	—	—	0	+++
3g	3.11	+++	−6.58	1.49×10^{-5}	0	O5	N–H of Asn 247	+	++++
3h	6.25	++	−9.1	2.12×10^{-7}	+++	—	—	0	+++++
3i	3.10	+++	−5.11	1.79×10^{-4}	0	O2	N–H of Asn 247	+	++++
3j	12.59	+	−7.9	1.61×10^{-6}	+	O1	N–H of Asn 210	+	+++
3k	13.04	+	−7.6	2.69×10^{-6}	+	O1	N–H of Asn 247	+	+++
4a	22.16	0	−7.27	4.72×10^{-6}	+	N3-H	O of Gly 152	+	++
4b	40.00	0	−6.06	3.60×10^{-5}	0	O2	N–H of Arg 36	+	+
4c	10.00	+	−6.16	3.06×10^{-5}	0	O2	N–H of Asn 247	+	++
4d	12.53	+	−6.76	1.11×10^{-5}	0	O2	N–H of Arg 151	+	++
4e	26.55	0	−8.79	3.63×10^{-7}	++	O5	N–H of Asn 247	+	+++
4f	>50.00	0	−6.6	1.46×10^{-5}	0	O2	N–H of Arg 249	+	+

4g	6.25	++	−7.85	1.76×10^{-6}	+	O5	N–H of Asn 247	+		++++
4h	15.28	0	−7.02	7.10×10^{-6}	+	O1	N–H of Asn 247	+		++
4i	>50.00	0	−4.77	3.17×10^{-4}	0	–	–	0		0
4j	>50.00	0	−7.75	2.07×10^{-6}	+	O2	N–H of Asn 247	+		++
4k	14.47	+	−8.04	1.28×10^{-6}	++	O1	N–H of Gly 209	+		++++

Activity level	Test		Autodocking parameters			
	MIC (μg/mL)	Symbol	ΔG (kcal/mol)	Symbol	H bond	Symbol
I	<1	+++++	<−9	+++	2 bond	++
II	1–2	++++	−9 to −8	++	1 bond	+
III	2–4	+++	−8 to −7	+	No bond	0
IV	4–8	++	>−7	0		
V	8–12	+				
VI	>12	0				

A data set, shown in Table 2 and 44 compounds were selected for future evaluation, and the inhibitory activity (IC_{50}) and Docking parameters (ΔG_b, K_i and hydrogen bond) are fifty–fifty weight. The correlation between inhibitory activities and the binding affinities predicted by AutoDock modeling was highly good for some compounds.

As IC_{50} consider, the compounds were categorized into six activity classes and based on the observed bioactivity data distribution with <1 μg/mL as very active represented by symbol +++++, 1–2 μg/mL as second active represented by symbol ++++, 2–4 μg/mL as third active represented by symbol +++, 4–8 μg/mL as fourth active represented by symbol ++, 8–12 μg/mL as fifth active represented by symbol +, and >12 μg/mL as last active represented by symbol 0. As binding free energies (ΔG_b, kcal/mol) consider, the compounds were categorized into four activity classes and based on the docking data distribution with <−9 kcal/mol as very active represented by symbol +++, −9 to −8 kcal/mol as moderately active represented by symbol ++, −8 to −7 kcal/mol as third active represented by symbol +, and >−7 kcal/mol as less active represented by symbol 0. Inhibition constants (K_i) vary tendency is according with ΔG_b, so just study one parameter is enough. Base on hydrogen bond with active sites, the compounds were categorized into three activity classes and the docking data distribution with 2 bonds as very active represented by symbol ++, 1 bond as moderately active represented by symbol +, and no bond as less active represented by symbol 0.

The global results are list in Table 2, and we selected the top 10 compounds (**2d**, **3e**, **2b**, **1g**, **2c**, **1e**, **2e**, **2i**, **3c** and **1f**) which have more '+' symbol in the 'Total Score' column to test their antibacterial activities against Gram-negative bacterial strains (*E. coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 13525) and two Gram-positive bacterial strains (*B. subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538) (Table 3), expecting to exploit potent antibacterial agent with broad-spectrum antibiotics activity. Out of the top 10 compounds, the results demonstrate that the antimicrobial compound, *N*-(3-(5-bromo-2-hydroxybenzylideneamino)propyl)-2-hydroxybenzamide (**2d**), can be as an inhibitor of ecKAS III and a potential antibiotics agent, displaying MIC of 0.39, 3.13, 1.56 and 3.13 μg/mL against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*, respectively, which was similar to the broad-spectrum antibiotic kanamycin with corresponding MIC of 3.13, 3.13, 1.56 and 1.56 μg/mL. Compounds **2b** and **1g** showed similar moderate antibacterial activity with MIC of 1.56–25.00 μg/mL against all the tested bacterial strains.

From the binding model, we can see compound **2d** (Fig. 3) (colored by element, ball and stick) is bound into ecKAS III receptor site via hydrophilic binding by hydrogen bond between O2···N–H of Asn 274 and O5···N–H of Asn 247. The ecKAS III active site generally contains a catalytic triad tunnel consisting of Cys-His-Asn,[10] effecting to these amino acid resides may greatly influence, inhibit or even stop its catalytic activity. The direct result is the fatty acid biosynthesis can't be smoothly proceeded, leading the energy supply to organism is not sufficient and could not forming the component of all cell membranes, so display its antimicrobial activity. Compound **2d** bonds two Asn active site (Asn 247 and Asn 274) of ecKAS III and fit into the mouth of the substrate tunnel (Fig. 3). This fact made **2d** had excellent inhibitory activity than other compounds and can be act as an inhibitor of KAS III and as a potential antibiotics agent in theory. The antimicrobial activity (minimum inhibitory concentrations are the average values of triplicates experiment results) against *E. coli*, *P. aeruginosa*, *B. Subtilis* and *S. aureus* proved this point in practice (Table 3). Compounds **2b** and **1g** also have moderate inhibitory activity against various bacteria.

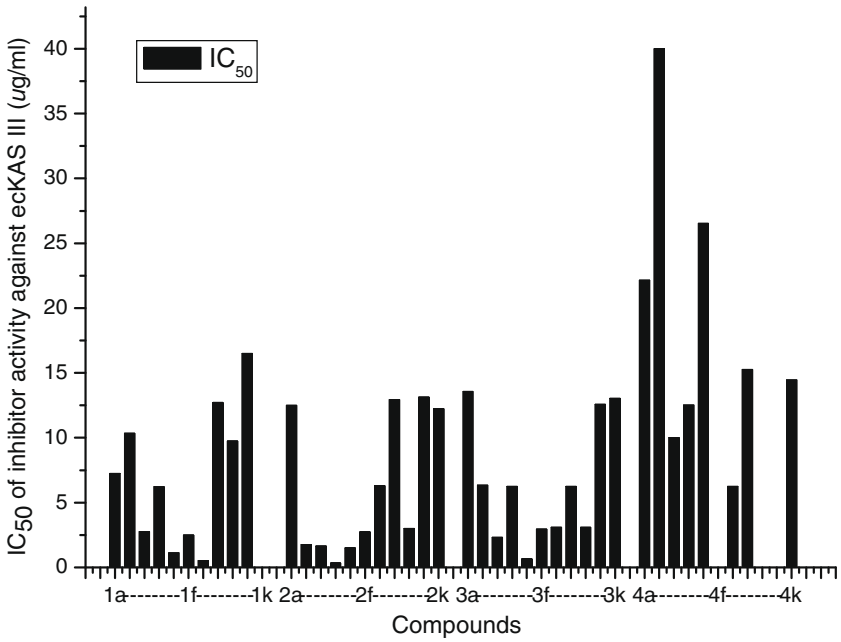

Figure 2. Inhibitory activities of the compounds. IC_{50} more than 50 μg/mL not demonstrate.

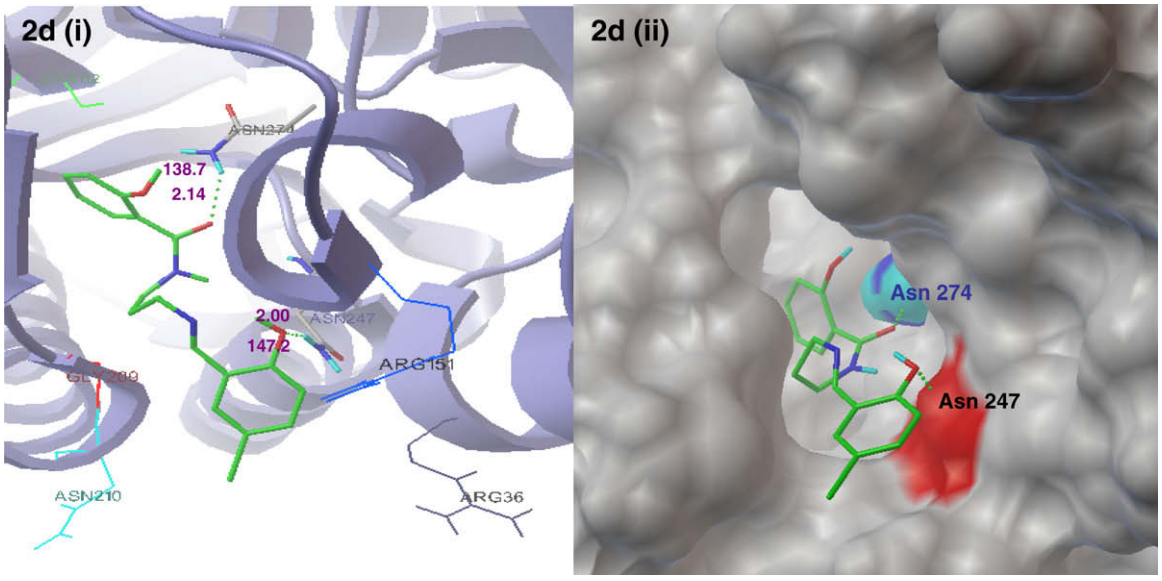

Figure 3. Compound **2d** (colored in green) is bound into ecKAS III receptor site via hydrophobic interactions and hydrophilic binding by hydrogen bond between its O2 and H–N of Asn 274 (N–H···O2: 2.14 Å, 138.7°), O5 and H–N of Asn 247 (N–H···O5: 2.00 Å, 147.2°) (2d(**i**)), and extending into the mouth of the substrate tunnel (2d(**ii**)).

Table 3
Antimicrobial activity of the top 10 compounds

Top 10 compounds	Minimum inhibitory concentrations (μg/mL)			
	Escherichia coli	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Staphylococcus aureus*
2d	0.39	3.13	1.56	3.13
3e	6.25	3.13	1.56	>50.00
2b	3.13	1.56	25.00	1.56
1g	6.25	12.50	1.56	12.50
2c	25.00	>50.00	>50.00	25.00
1e	25.00	>50.00	>50.00	>50.00
2e	3.13	6.25	25.00	>50.00
2i	>50.00	3.13	>50.00	>50.00
3c	>50.00	12.50	25.00	25.00
1f	1.56	>50.00	3.13	>50.00
Kanamycin	3.13	3.13	1.56	1.56

3. Conclusion

In summary, a series of novel PSB derivatives were prepared and tested for their inhibitory activity against ecKAS III, and the Autodock investigation also carried out by docking them into the active site of ecKAS III. Both the theory and practice demonstrate that *N*-(3-(5-bromo-2-hydroxybenzylideneamino)propyl)-2-hydroxybenzamide (**2d**), can be as a potent inhibitor of ecKAS III, displaying MIC values in the range 0.39–3.13 μg/mL against various bacteria. While a few compounds, for example **3e**, is potent inhibitors of ecKAS III but fail to inhibit *S. aureus*, suggesting that a correlation may exist. Here, the preliminary structure–activity relationships and molecular modeling study just shed a light on the further insight into interactions between the macromolecular enzyme and its inhibitor ligands.

4. Experiment

4.1. Materials

All chemicals (reagent grade) used were commercially available. All the ^{1}H NMR and spectra were recorded on a Bruker DRX 500 or DPX 300 model Spectrometer at 25 °C with TMS and solvent signals allotted as internal stands. Chemical shifts were reported in ppm (δ). ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer. Elemental analyses were performed on a CHN-O-Rapid instrument and were within ±0.4% of the theoretical values. Melting points were measured on a Boetius micro melting point apparatus.

4.2. General method of synthesis PSB

A: Concentrated sulfuric acid (1.0 mL) was added slowly with vigorous agitation to a mixture of 20.0 g (144 mmol) of salicylic acid and 63.0 mL (1.08 mol) of anhydrous ethanol. The mixture was then reflux for 15 h. Excess ethanol was removed by evaporation and the residue was washed with water. The organic layer was again treated with water and concentrated sodium bicarbonate solution to an alkaline reaction, and then again with water. The product was dried over anhydrous MgSO4, collected by filtration, and distilled (yield: 55.0%).

C: Equimolar quantities (10 mmol) of **A** and the primary diamines (1,2- diaminoethane, 1,3-diaminopropane, 1,4-diaminobuyane, 1,6-diaminohexane) were mixed and vigorously stirred at 110 °C under oil bath for 5 h to give a melicera solution. The crude product was then purified by column chromatography in 10% MeOH/CH_2Cl_2 to afford the desired compound (yield: 60.0%).

Equimolar quantities (0.5 mmol) of **C** and salicylaldehyde or its derivatives, or 2-hydroxy-1-naphthaldehyde were dissolved in methanol (10 mL) and stirred at 50 °C for 3 h to give a clear solution (**1a**–**1i**, **2a**–**2i**, **3a**–**3i** and **4a**–**4i**). The crude product was then purified by column chromatography in 25% EtOAc/hexanes to afford the desired compound. 0.5 mmol of diamines (1,2- diaminoethane, 1,3-diaminopropane, 1,4-diaminobuyane, 1,6-diaminohexane) was mixed with 2 N **A** and vigorous stirring 5 h at 110 °C under oil bath. The crude product was then purified by column chromatography in 25% EtOAc/hexanes to afford **1i**–**4j**. 0.5 mmol of **B** and 2 N salicylaldehyde were dissolved in methanol (10 mL) and stirred at 50 °C for 3 h to give a clear solution (**1k**–**4k**). The crude product was then purified by column chromatography in 25% EtOAc/hexanes to afford the desired compound.

4.2.1. General data of 1a–1k

4.2.1.1. (*E*)-2-Hydroxy-*N*-(2-(2-hydroxybenzylideneamino)ethyl) benzamide (1a). Yellow power, yield 88%, mp: 125–127 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.83 (m, 4H); 6.71 (s, 1H); 6.80 (m, 1H); 6.88 (m, 1H); 6.97 (m, 2H); 7.23 (m, 1H); 7.35 (m, 3H); 8.37 (s, 1H); 12.67 (s, 2H). ESI-MS: 285.1 ($C_{16}H_{17}N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{16}N_2O_3$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.45; H, 5.69; N, 9.79.

4.2.1.2. (*E*)-*N*-(2-(5-Fluoro-2-hydroxybenzylideneamino)ethyl)-2-hydroxybenzamide (1b). Yellow power, yield 90%, mp: 95–96 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.81 (m, 4H); 6.65 (s, 1H); 6.80 (m, 1H); 6.89 (d, J = 7.1 Hz, 1H); 6.96 (m, 1H); 7.21 (s, 1H); 7.26 (m, 1H); 7.31 (m, 1H); 7.38 (m, 1H); 8.33 (s, 1H); 12.11 (s, 1H); 13.21 (s, 1H). ESI-MS: 303.1 ($C_{16}H_{16}FN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{15}FN_2O_3$: C, 63.57; H, 5.00; N, 9.27. Found: C, 63.48; H, 4.94; N, 9.22.

4.2.1.3. (*E*)-*N*-(2-(5-Chloro-2-hydroxybenzylideneamino)ethyl)-2-hydroxybenzamide (1c). Yellow power, yield 82%, mp: 85–88 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.82 (m, 4H); 6.69 (s, 1H); 6.81 (m, 1H); 6.90 (d, J = 7.1 Hz, 1H); 6.97 (d, J = 8.4 Hz, 1H); 7.21 (s, 1H); 7.25 (m, 1H); 7.32 (m, 1H); 7.38 (m, 1H); 8.31 (s, 1H); 12.21 (s, 1H); 13.03 (s, 1H). ESI-MS: 219.1 ($C_{16}H_{16}ClN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{15}ClN_2O_3$: C, 60.29; H, 4.74; N, 8.79. Found: C, 60.35; H, 4.79; N, 8.82.

4.2.1.4. (*E*)-*N*-(2-(5-Bromo-2-hydroxybenzylideneamino)ethyl)-2-hydroxybenzamide (1d). Yellow power, yield 86%, mp: 135–137 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.62 (m, 2H); 3.80 (m, 2H); 6.86 (m, 3H); 7.36 (m, 1H); 7.46 (d, J = 8.7 Hz, 1H); 7.66 (s, 1H); 7.82 (d, J = 7.8 Hz, 1H); 8.54 (s, 1H); 8.95 (s, 1H); 12.41 (s, 1H); 13.37 (s, 1H). ESI-MS: 363.0 ($C_{16}H_{16}BrN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{15}BrN_2O_3$: C, 52.91; H, 4.16; N, 7.71. Found: C, 52.95; H, 4.09; N, 7.72.

4.2.1.5. (*E*)-2-Hydroxy-*N*-(2-(2-hydroxy-5-iodobenzylideneamino)ethyl)benzamide (1e). Yellow power, yield 92%, mp: 151–153 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.63 (m, 2H); 3.81 (m, 2H); 6.83 (m, 3H); 7.35 (m, 1H); 7.48 (d, J = 8.9 Hz, 1H); 7.64 (s, 1H); 7.81 (d, J = 8.0 Hz, 1H); 8.54 (s, 1H); 8.96 (s, 1H); 12.40 (s, 1H); 13.36 (s, 1H). ESI-MS: 410.1 ($C_{16}H_{16}IN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{15}IN_2O_3$: C, 46.85; H, 3.69; N, 6.83. Found: C, 46.93; H, 3.71; N, 6.82.

4.2.1.6. (*E*)-*N*-(2-(3,5-Dichloro-2-hydroxybenzylideneamino)ethyl)-2-hydroxybenzamide (1f). Yellow power, yield 95%, mp: 135–136 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.78 (m, 2H); 3.89 (m, 2H); 6.82 (m, 2H); 6.98 (d, J = 8.4 Hz, 1H); 7.13 (s, 1H); 7.39 (m, 3H); 8.28 (s, 1H); 12.09 (s, 1H); 14.12 (s, 1H). ESI-MS: 357.0 ($C_{16}H_{15}Cl_2N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{14}Cl_2N_2O_3$: C, 54.41; H, 4.00; N, 7.39. Found: C, 54.38; H, 3.97; N, 7.42.

4.2.1.7. (*E*)-*N*-(2-(3,5-Dibromo-2-hydroxybenzylideneamino)ethyl)-2-hydroxybenzamide (1g). Yellow power, yield 79%, mp: 145–147 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.79 (m, 2H); 3.90 (m, 2H); 6.70 (s, 1H); 6.83 (m, 1H); 6.98 (d, J = 8.2 Hz, 1H); 7.32 (m, 2H); 7.39 (m, 1H); 7.70 (s, 1H); 8.26 (s, 1H); 12.05 (s, 1H); 14.24 (s, 1H). ESI-MS: 441.9 ($C_{16}H_{15}Br_2N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{14}Br_2N_2O_3$: C, 43.47; H, 3.19; N, 6.34. Found: C, 43.48; H, 3.17; N, 6.32.

4.2.1.8. (*E*)-2-Hydroxy-*N*-(2-(2-hydroxy-3,5-diiodobenzylideneamino)ethyl)benzamide (1h). Yellow power, yield 86%, mp: 123–125 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.80 (m, 2H); 3.91 (m, 2H); 6.71 (s, 1H); 6.84 (m, 1H); 6.99 (d, J = 8.3 Hz, 1H); 7.34 (m, 2H); 7.41 (m, 1H); 7.71 (s, 1H); 8.26 (s, 1H); 12.06 (s, 1H); 14.25 (s, 1H). ESI-MS: 536.9 ($C_{16}H_{15}I_2N_2O_3^+$, $[M+H]^+$). Anal.

Calcd for $C_{16}H_{14}I_2N_2O_3$: C, 35.85; H, 2.63; N, 5.23. Found: C, 35.92; H, 3.67; N, 5.22.

4.2.1.9. (*E*)-2-Hydroxy-*N*-(2-((2-hydroxynaphthalen-1-yl)methyleneamino)ethyl)benzamide (1i). Yellow power, yield 84%, mp: 151–153 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.82 (m, 2H); 3.91 (m, 2H); 6.75 (m, 1H); 6.84 (d, J = 9.3 Hz, 1H); 6.98 (d, J = 8.4 Hz, 1H); 7.22 (m, 1H); 7.37 (m, 2H); 7.56 (m, 4H); 7.80 (d, J = 8.4 Hz, 1H); 8.80 (s, 1H); 12.17 (s, 1H); 14.44 (s, 1H). ESI-MS: 335.1 ($C_{20}H_{19}N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{18}N_2O_3$: C, 71.84; H, 5.43; N, 8.38. Found: C, 71.92; H, 5.47; N, 8.91.

4.2.1.10. *N,N′*-(Ethane-1,2-diyl)bis(2-hydroxybenzamide) (1j). White power, yield 67%, mp: 111–113 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.94 (m, 4H); 6.71 (s, 2H); 6.80 (m, 2H); 6.98 (m, 2H); 7.20 (m, 2H); 7.29 (m, 2H); 13.19 (s, 2H). ESI-MS: 301.1 ($C_{16}H_{17}N_2O_4^+$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{16}N_2O_4$: C, 63.99; H, 5.37; N, 9.33. Found: C, 64.03; H, 5.33; N, 9.91.

4.2.1.11. *N,N′*-Disalicylalethylenediamine (1k). Yellow power, yield 97%, mp: 73–75 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.91 (m, 4H); 6.86 (m, 2H); 6.95 (m, 2H); 7.21 (m, 2H); 7.31 (m, 2H); 8.36 (s, 2H); 13.21 (s, 2H). ESI-MS: 269.1 ($C_{16}H_{17}N_2O_2^+$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{16}N_2O_2$: C, 71.62; H, 6.01; N, 10.44. Found: C, 71.52; H, 5.97; N, 10.41.

4.2.2. General data of 2a–2k

4.2.2.1. 2-Hydroxy-*N*-(3-(2-hydroxybenzylideneamino)propyl)benzamide (2a). Oil, yield 83%, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 2.05 (m, 2H); 3.58 (m, 2H); 3.69 (m, 2H); 6.52 (s, 1H); 6.77 (m, 1H); 6.89 (m, 1H); 6.96 (m, 2H); 7.30 (m, 4H); 8.64 (s, 1H); 12.24 (s, 1H); 13.42 (s, 1H). ESI-MS: 299.1 ($C_{17}H_{19}N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{18}N_2O_3$: C, 68.44; H, 6.08; N, 9.39. Found: C, 68.54; H, 6.15; N, 9.37.

4.2.2.2. *N*-(3-(5-Fluoro-2-hydroxybenzylideneamino)propyl)-2-hydroxybenzamide (2b). Yellow power, yield 90%, mp: 60–63 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 2.04 (m, 2H); 3.62 (m, 2H); 3.73 (m, 2H); 6.49 (s, 1H); 6.79 (m, 1H); 6.91 (d, J = 8.53 Hz, 1H); 6.99 (d, J = 7.36 Hz, 1H); 7.25 (m, 2H); 7.42 (m, 2H); 8.35 (s, 1H); 12.28 (s, 1H); 13.28 (s, 1H). ESI-MS: 299.1 ($C_{17}H_{18}FN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{17}FN_2O_3$: C, 64.55; H, 5.42; N, 8.86. Found: C, 64.54; H, 5.36; N, 8.91.

4.2.2.3. *N*-(3-(5-Chloro-2-hydroxybenzylideneamino)propyl)-2-hydroxybenzamide (2c). Yellow power, yield 84%, mp: 98–100 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 2.06 (m, 2H); 3.59 (m, 2H); 3.71 (m, 2H); 6.44 (s, 1H); 6.79 (m, 1H); 6.88 (d, J = 8.58 Hz, 1H); 6.96 (d, J = 7.86 Hz, 1H); 7.22 (m, 2H); 7.36 (m, 2H); 8.32 (s, 1H); 12.28 (s, 1H); 13.28 (s, 1H). ESI-MS: 333.1 ($C_{17}H_{18}ClN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{17}ClN_2O_3$: C, 61.36; H, 5.15; N, 8.42; Found: C, 61.44; H, 5.15; N, 8.38.

4.2.2.4. *N*-(3-(5-Bromo-2-hydroxybenzylideneamino)propyl)-2-hydroxybenzamide (2d). Yellow power, yield 91%, mp: 74–76 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 2.06 (m, 2H); 3.60 (m, 2H); 3.71 (m, 2H); 6.43 (s, 1H); 6.81 (m, 2H); 6.97 (d, J = 8.43 Hz, 1H); 7.30 (d, J = 8.04 Hz, 1H); 7.38 (m, 3H); 8.30 (s, 1H); 12.27 (s, 1H); 13.29 (s, 1H). ESI-MS: 377.0 ($C_{17}H_{18}BrN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{17}BrN_2O_3$: C, 54.13; H, 4.54; N, 7.44. Found: C, 54.14; H, 4.63; N, 7.47.

4.2.2.5. 2-Hydroxy-*N*-(3-(2-hydroxy-5-iodobenzylideneamino)propyl)benzamide (2e). Yellow power, yield 79%, mp: 90–92 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 2.05 (m, 2H); 3.56 (m, 2H); 3.70 (m, 2H); 6.44 (s, 1H); 6.85 (m, 2H); 6.96 (d, J = 7.86 Hz, 1H); 7.28 (d, J = 8.45 Hz, 1H); 7.37 (m, 3H); 8.31 (s, 1H); 12.28 (s, 1H); 13.31 (s, 1H). ESI-MS: 425.0 ($C_{17}H_{18}IN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{17}IN_2O_3$: C, 48.13; H, 4.04; N, 6.60. Found: C, 48.14; H, 4.08; N, 6.63.

4.2.2.6. *N*-(3-(3,5-Dichloro-2-hydroxybenzylideneamino)propyl)-2-hydroxybenzamide (2f). Yellow power, yield 89%, mp: 55–57 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 2.07 (m, 2H); 3.59 (m, 2H); 3.74 (m, 2H); 6.48 (s, 1H); 6.80 (m, 1H); 6.95 (d, J = 8.22 Hz, 1H); 7.11 (s, 1H); 7.35 (m, 3H); 8.28 (s, 1H); 12.21 (s, 1H); 14.45 (s, 1H). ESI-MS: 425.0 ($C_{17}H_{17}Cl_2N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{16}Cl_2N_2O_3$: C, 55.60; H, 4.39; N, 7.63. Found: C, 55.62; H, 4.38; N, 7.65.

4.2.2.7. *N*-(3-(3,5-Dibromo-2-hydroxybenzylideneamino)propyl)-2-hydroxybenzamide (2g). Yellow power, yield 88%, mp: 163–165 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 2.08 (m, 2H); 3.60 (m, 2H); 3.74 (m, 2H); 6.44 (s, 1H); 6.81 (m, 1H); 6.95 (d, J = 8.25 Hz, 1H); 7.33 (m, 3H); 7.69 (s, 1H); 8.24 (s, 1H); 12.20 (s, 1H); 14.61 (s, 1H). ESI-MS: 456.9 ($C_{17}H_{17}Br_2N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{16}Br_2N_2O_3$: C, 44.76; H, 3.54; N, 6.14. Found: C, 44.67; H, 3.58; N, 6.15.

4.2.2.8. 2-Hydroxy-*N*-(3-(2-hydroxy-3,5-diiodobenzylideneamino)propyl)benzamide (2h). Yellow power, yield 86%, mp: 154–155 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 2.08 (m, 2H); 3.61 (m, 2H); 3.75 (m, 2H); 6.44 (s, 1H); 6.80 (m, 1H); 6.95 (d, J = 8.40 Hz, 1H); 7.35 (m, 3H); 7.67 (s, 1H); 8.24 (s, 1H); 12.20 (s, 1H); 14.60 (s, 1H). ESI-MS: 550.9 ($C_{17}H_{17}I_2N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{16}I_2N_2O_3$: C, 37.12; H, 2.93; N, 5.09. Found: C, 37.22; H, 2.95; N, 5.11.

4.2.2.9. 2-Hydroxy-*N*-(3-((2-hydroxynaphthalen-1-yl)methyleneamino)propyl)benzamide (2i). Yellow power, yield 77%, mp: 166–167 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.45 (m, 3H); 3.40 (m, 1H); 3.96 (m, 2H); 6.71 (m, 1H); 6.82 (d, J = 9.33 Hz, 1H); 6.96 (d, J = 8.40 Hz, 1H); 7.22 (m, 1H); 7.40 (m, 4H); 7.55 (d, J = 9.12 Hz, 2H); 7.78 (d, J = 8.4 Hz, 1H); 12.24 (s, 1H); 14.61 (s, 1H). ESI-MS: 349.1 ($C_{21}H_{21}N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{20}N_2O_3$: C, 72.40; H, 5.79; N, 8.04. Found: C, 72.42; H, 5.75; N, 8.06.

4.2.2.10. *N,N′*-(Propane-1,3-diyl)bis(2-hydroxybenzamide) (2j). White power, yield 93%, mp: 181–182 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.87 (m, 2H); 3.59 (m, 4H); 6.88 (m, 2H); 6.99 (d, J = 8.43 Hz, 2H); 7.27 (m, 2H); 7.41 (m, 2H); 8.49 (d, J = 8.07 Hz, 2H); 12.32 (s, 2H). ESI-MS: 316.1 ($C_{17}H_{19}N_2O_4^+$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{18}N_2O_4$: C, 64.96; H, 5.77; N, 8.91. Found: C, 65.00; H, 5.79; N, 8.88.

4.2.2.11. *N,N′*-Disalicylidenetrimethylenediamine (2k). Yellow power, yield 70%, mp: 76–77 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 2.09 (m, 2H); 3.68 (m, 4H); 6.87 (m, 2H); 6.96 (d, J = 8.3 Hz, 2H); 7.23 (d, J = 6.0 Hz, 2H); 7.29 (m, 2H); 8.35 (s, 2H); 13.44 (s, 2H). ESI-MS: 283.1 ($C_{17}H_{19}N_2O_2^+$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{18}N_2O_2$: C, 72.32; H, 6.43; N, 9.92. Found: C, 72.40; H, 6.45; N, 9.96.

4.2.3. General data of 3a–3k

4.2.3.1. (*E*)-2-Hydroxy-*N*-(4-(2-hydroxybenzylideneamino)butyl)benzamide (3a). Yellow oil, yield 82%, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.78 (m, 4H); 3.48 (m, 2H); 3.62 (m, 2H); 6.50 (s, 1H); 6.84 (m, 2H); 6.96 (m, 2H); 7.21 (m, 1H); 7.37 (m, 3H); 8.34 (s, 1H); 13.23 (s, 2H). ESI-MS: 313.1 ($C_{18}H_{21}N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{20}N_2O_3$: C, 69.21; H, 6.45; N, 8.97. Found: C, 69.32; H, 6.41; N, 8.96.

4.2.3.2. (*E*)-*N*-(4-(5-Fluoro-2-hydroxybenzylideneamino)butyl)-2-hydroxybenzamide (3b). Yellow power, yield 89%, mp: 72–74 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.78 (m, 4H); 3.51 (m, 2H); 3.64 (m, 2H); 6.41 (s, 1H); 6.80 (m, 1H); 6.91 (d, J = 8.6 Hz, 1H); 6.99 (m, 1H); 7.23 (m, 2H); 7.36 (m, 2H); 8.28 (s, 1H); 12.34 (s, 1H); 13.46 (s, 1H). ESI-MS: 331.1 ($C_{18}H_{20}FN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{19}FN_2O_3$: C, 65.44; H, 5.80; N, 8.48; Found: C, 65.42; H, 5.75; N, 8.46.

4.2.3.3. (*E*)-*N*-(4-(5-Chloro-2-hydroxybenzylideneamino)butyl)-2-hydroxybenzamide (3c). Yellow power, yield 92%, mp: 72–74 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.77 (m, 4H); 3.50 (m, 2H); 3.66 (m, 2H); 6.41 (s, 1H); 6.78 (m, 1H); 6.90 (d, J = 8.6 Hz, 1H); 6.96 (m, 1H); 7.21 (m, 2H); 7.35 (m, 2H); 8.28 (s, 1H); 12.33 (s, 1H); 13.46 (s, 1H). ESI-MS: 347.1 ($C_{18}H_{20}ClN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{19}ClN_2O_3$: C, 62.34; H, 5.52; N, 8.08. Found: C, 62.32; H, 5.55; N, 8.03.

4.2.3.4. (*E*)-*N*-(4-(5-Bromo-2-hydroxybenzylideneamino)butyl)-2-hydroxybenzamide (3d). Yellow power, yield 89%, mp: 75–77 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.77 (m, 4H); 3.49 (m, 2H); 3.65 (m, 2H); 6.40 (s, 1H); 6.83 (m, 2H); 6.97 (d, J = 8.2 Hz, 1H); 7.38 (m, 4H); 8.28 (s, 1H); 12.32 (s, 1H); 13.50 (s, 1H). ESI-MS: 391.1 ($C_{18}H_{20}BrN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{19}BrN_2O_3$: C, 55.26; H, 4.89; N, 7.16. Found: C, 55.31; H, 4.87; N, 7.11

4.2.3.5. (*E*)-2-Hydroxy-*N*-(4-(2-hydroxy-5-iodobenzylideneamino)butyl)benzamide (3e). Yellow power, yield 84%, mp: 79–81 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.78 (m, 4H); 3.48 (m, 2H); 3.66 (m, 2H); 6.40 (s, 1H); 6.85 (m, 2H); 6.96 (d, J = 8.3 Hz, 1H); 7.35 (m, 4H); 8.28 (s, 1H); 12.32 (s, 1H); 13.51 (s, 1H). ESI-MS: 339.0 ($C_{18}H_{20}IN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{19}IN_2O_3$: C, 49.33; H, 4.37; N, 6.39. Found: C, 49.31; H, 4.33; N, 6.42.

4.2.3.6. (*E*)-*N*-(4-(3,5-Dichloro-2-hydroxybenzylideneamino)butyl)-2-hydroxybenzamide (3f). Yellow power, yield 85%, mp 118–120 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.77 (m, 4H); 3.51 (m, 2H); 3.69 (m, 2H); 6.51 (s, 1H); 6.83 (m, 1H); 6.98 (d, J = 8.0 Hz, 1H); 7.14 (s, 1H); 7.41 (m, 3H); 8.25 (s, 1H); 12.31 (s, 1H); 14.65 (s, 1H). ESI-MS: 381.1 ($C_{18}H_{19}Cl_2N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{18}Cl_2N_2O_3$: C, 56.71; H, 4.76; N, 7.35. Found: C, 56.67; H, 4.73; N, 7.41.

4.2.3.7. (*E*)-*N*-(4-(3,5-Dibromo-2-hydroxybenzylideneamino)butyl)-2-hydroxybenzamide (3g). Yellow power, yield 91%, mp: 110–111 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.76 (m, 4H); 3.49 (m, 2H); 3.69 (m, 2H); 6.48 (s, 1H); 6.84 (m, 1H); 6.98 (d, J = 8.2 Hz, 1H); 7.57 (m, 3H); 7.69 (s, 1H); 8.21 (s, 1H); 12.30 (s, 1H); 14.79 (s, 1H). ESI-MS: 470.9 ($C_{18}H_{19}Br_2N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{18}Br_2N_2O_3$: C, 45.98; H, 3.86; N, 5.96. Found: C, 46.02; H, 3.71; N, 5.91.

4.2.3.8. (*E*)-2-Hydroxy-*N*-(4-(2-hydroxy-3,5-diiodobenzylideneamino)butyl)benzamide (3h). Yellow power, yield 90%, mp: 130–133 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.77 (m, 4H); 3.50 (m, 2H); 3.69 (m, 2H); 6.48 (s, 1H); 6.85 (m, 1H); 6.97 (d, J = 8.1 Hz, 1H); 7.55 (m, 3H); 7.70 (s, 1H); 8.21 (s, 1H); 12.31 (s, 1H); 14.80 (s, 1H). ESI-MS: 564.9 ($C_{18}H_{19}I_2N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{18}I_2N_2O_3$: C, 38.32; H, 3.22; N, 4.97. Found: C, 38.23; H, 3.19; N, 4.96.

4.2.3.9. (*E*)-2-Hydroxy-*N*-(4-((2-hydroxynaphthalen-1-yl)methyleneamino)butyl)benzamide (3i). Yellow power, yield 95%, mp: 136–137 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.80 (m, 4H); 3.52 (m, 2H); 3.68 (m, 2H); 6.74 (s, 1H); 6.81 (m, 1H); 6.92 (d, J = 9.3 Hz, 1H); 6.97 (d, J = 7.68 Hz, 1H); 7.24 (m, 1H); 7.39 (m, 3H); 7.61 (d, J = 7.1 Hz, 1H); 7.69 (d, J = 9.3 Hz, 1H); 7.85 (d, J = 8.2 Hz, 1H); 8.74 (s, 1H); 12.35 (s, 1H); 14.55 (s, 1H). ESI-MS: 363.1 ($C_{22}H_{23}N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{22}H_{22}N_2O_3$: C, 72.91; H, 6.12; N, 7.73. Found: C, 72.88; H, 6.17; N, 7.69.

4.2.3.10. *N,N′*-(Butane-1,4-diyl)bis(2-hydroxybenzamide) (3j). White power, yield 90%, mp: 165–167 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.75 (m, 4H); 3.54 (m, 4H); 6.67 (s, 2H); 6.85 (m, 2H); 6.99 (d, J = 8.3 Hz, 2H); 7.39 (m, 4H); 12.28 (s, 2H). ESI-MS: 329.1 ($C_{18}H_{21}N_2O_4^+$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{21}N_2O_4$: C, 65.84; H, 6.14; N, 8.53. Found: C, 68.73; H, 6.16; N, 8.56.

4.2.3.11. *N,N′*-Disalicylidenetetramethylenediamine (3k). Yellow power, yield 78%, mp: 131–134 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.77 (m, 4H); 3.53 (m, 4H); 6.87 (m, 2H); 6.97 (d, J = 7.8 Hz, 2H); 7.41 (m, 4H); 8.25 (s, 2H); 12.31 (s, 2H). ESI-MS: 297.1 ($C_{18}H_{21}N_2O_2^+$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{20}N_2O_2$: C, 72.95; H, 6.80; N, 9.45. Found: C, 72.89; H, 6.77; N, 9.47.

4.2.4. General data of 4a–4k

4.2.4.1. (*E*)-2-Hydroxy-*N*-(6-(2-hydroxybenzylideneamino)hexyl)-benzamide (4a). Yellow oil, yield 82%, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.45 (m, 4H); 1.69 (m, 4H); 3.46 (m, 2H); 3.59 (m, 2H); 6.43 (s, 1H); 6.84 (m, 2H); 6.96 (m, 2H); 7.24 (m, 1H); 7.33 (m, 1H); 7.36 (m, 2H); 8.33 (s, 1H); 12.70 (s, 2H). ESI-MS: 341.1 ($C_{20}H_{25}N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{24}N_2O_3$: C, 70.56; H, 7.11; N, 8.23. Found: C, 70.62; H, 7.07; N, 8.17.

4.2.4.2. (*E*)-*N*-(6-(5-Fluoro-2-hydroxybenzylideneamino)hexyl)-2-hydroxybenzamide (4b). Yellow power, yield 88%, mp: 86–89 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.42 (m, 4H); 1.69 (m, 4H); 3.45 (m, 2H); 3.59 (m, 2H); 6.41 (s, 1H); 6.84 (m, 1H); 6.88 (d, J = 8.7 Hz, 1H); 6.97 (d, J = 8.4 Hz, 1H); 7.21 (m, 2H); 7.38 (m, 2H); 8.26 (s, 1H); 12.37 (s, 1H); 13.62 (s, 1H). ESI-MS: 359.1 ($C_{20}H_{25}N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{24}N_2O_3$: C, 67.02; H, 6.47; N, 7.82. Found: C, 67.01; H, 6.43; N, 7.81.

4.2.4.3. (*E*)-*N*-(6-(5-Chloro-2-hydroxybenzylideneamino)hexyl)-2-hydroxybenzamide (4c). Yellow power, yield 87%, mp: 90–101 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.43 (m, 4H); 1.68 (m, 4H); 3.46 (m, 2H); 3.60 (m, 2H); 6.41 (s, 1H); 6.84 (m, 1H); 6.89 (d, J = 8.8 Hz, 1H); 6.98 (d, J = 8.0 Hz, 1H); 7.20 (m, 2H); 7.38 (m, 2H); 8.25 (s, 1H); 12.36 (s, 1H); 13.61 (s, 1H). ESI-MS: 375.1 ($C_{20}H_{24}ClN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{23}ClN_2O_3$: C, 64.08; H, 6.18; N, 7.47. Found: C, 64.01; H, 6.16; N, 6.21.

4.2.4.4. (*E*)-*N*-(6-(5-Bromo-2-hydroxybenzylideneamino)hexyl)-2-hydroxybenzamide (4d). Yellow power, yield 92%, mp: 90–101 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.44 (m, 4H); 1.69 (m, 4H); 3.45 (m, 2H); 3.60 (m, 2H); 6.37 (s, 1H); 6.83 (m, 2H); 6.98 (d, J = 8.2 Hz, 1H); 7.38 (m, 4H); 8.25 (s, 1H); 12.38 (s, 1H); 13.67 (s, 1H). ESI-MS: 419.1 ($C_{20}H_{24}BrN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{23}BrN_2O_3$: C, 57.29; H, 5.53; N, 6.68. Found: C, 57.31; H, 5.56; N, 6.73.

4.2.4.5. (*E*)-2-Hydroxy-*N*-(6-(2-hydroxy-5-iodobenzylideneamino)hexyl)benzamide (4e). Yellow power, yield 83%, mp: 112–114 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.43 (m, 4H); 1.67 (m, 4H); 3.45 (m, 2H); 3.61 (m, 2H); 6.37 (s, 1H); 6.84 (m, 2H); 6.97 (d, J = 8.0 Hz, 1H); 7.40 (m, 4H); 8.26 (s, 1H); 12.38 (s, 1H); 13.66 (s, 1H). ESI-MS: 467.1 ($C_{20}H_{24}IN_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{23}IN_2O_3$: C, 51.51; H, 4.97; N, 6.01. Found: C, 51.47; H, 4.51; N, 5.96.

4.2.4.6. (*E*)-*N*-(6-(3,5-Dichloro-2-hydroxybenzylideneamino)hexyl)-2-hydroxybenzamide (4f). Yellow power, yield 91%, mp: 125–127 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.43 (m, 4H); 1.65 (m, 4H); 3.44 (m, 2H); 3.62 (m, 2H); 6.45 (s, 1H); 6.82 (m, 1H); 6.97 (d, J = 7.8 Hz, 1H); 7.11 (m, 1H); 7.37 (m, 3H); 8.19 (s, 1H); 12.33 (s, 1H); 14.76 (s, 1H). ESI-MS: 409.1 ($C_{20}H_{23}Cl_2N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{22}Cl_2N_2O_3$: C, 58.69; H, 5.42; N, 6.84. Found: C, 58.49; H, 5.41; N, 6.88.

4.2.4.7. (*E*)-*N*-(6-(3,5-Dibromo-2-hydroxybenzylideneamino)hexyl)-2-hydroxybenzamide (4g). Yellow power, yield 87%, mp: 130–131 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.44 (m, 4H); 1.65 (m, 4H); 3.44 (m, 2H); 3.63 (m, 2H); 6.38 (s, 1H); 6.83 (m, 1H); 6.98 (d, J = 8.0 Hz, 1H); 7.28 (m, 1H); 7.37 (m, 3H); 8.16 (s, 1H); 12.35 (s, 1H); 14.91 (s, 1H). ESI-MS: 499.0 ($C_{20}H_{23}Br_2N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{22}Br_2N_2O_3$: C, 48.22; H, 4.45; N, 5.62. Found: C, 48.09; H, 4.41; N, 5.58.

4.2.4.8. (*E*)-2-Hydroxy-*N*-(6-(2-hydroxy-3,5-diiodobenzylideneamino)hexyl)benzamide (4h). Yellow power, yield 85%, mp: 143–145 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.43 (m, 4H); 1.66 (m, 4H); 3.44 (m, 2H); 3.65 (m, 2H); 6.39 (s, 1H); 6.85 (m, 1H); 6.97 (d, J = 7.9 Hz, 1H); 7.28 (m, 1H); 7.38 (m, 3H); 8.17 (s, 1H); 12.31 (s, 1H); 14.90 (s, 1H). ESI-MS: 592.9 ($C_{20}H_{23}I_2N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{22}I_2N_2O_3$: C, 40.56; H, 3.74; N, 4.73. Found: C, 40.59; H, 3.71; N, 4.68.

4.2.4.9. (*E*)-2-Hydroxy-*N*-(6-((2-hydroxynaphthalen-1-yl)methyleneamino)hexyl)benzamide (4i). Yellow power, yield 82%, mp: 139–141 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.49 (m, 4H); 1.77 (m, 4H); 3.47 (m, 2H); 3.62 (m, 2H); 6.79 (m, 1H); 6.89 (s, 1H); 6.95 (m, 2H); 7.23 (m, 1H); 7.42 (m, 3H); 7.61 (d, J = 7.8 Hz, 1H); 7.68 (d, J = 9.1 Hz, 1H); 7.84 (d, J = 8.4 Hz, 1H); 8.69 (s, 1H); 12.44 (s, 1H); 14.55 (s, 1H). ESI-MS: 391.2 ($C_{24}H_{27}N_2O_3^+$, $[M+H]^+$). Anal. Calcd for $C_{24}H_{26}N_2O_3$: C, 73.82; H, 6.71; N, 7.17. Found: C, 73.77; H, 6.65; N, 7.21.

4.2.4.10. *N,N′*-(Hexane-1,6-diyl)bis(2-hydroxybenzamide) (4j). White power, yield 86%, mp: 171–173 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.44 (m, 4H); 1.68 (m, 4H); 3.63 (m, 4H); 6.39 (s, 2H); 6.91 (m, 2H); 7.22 (m, 2H); 7.60 (d, J = 7.3 Hz, 2H); 7.85 (m, 2H); 12.41 (s, 2H). ESI-MS: 357.2 ($C_{20}H_{25}N_2O_4^+$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{24}N_2O_4$: C, 67.40; H, 6.79; N, 7.86. Found: C, 67.52; H, 6.81; N, 7.88.

4.2.4.11. *N,N′*-Disalicylidene-1,6-hexanediamine (4k). Yellow power, yield 81%, mp: 133–134 °C, ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.26 (m, 4H); 1.54 (m, 4H); 3.63 (m, 4H); 6.85 (m, 2H); 6.95 (m, 2H); 7.22(m, 2H); 7.85 (m, 2H); 8.72 (s, 2H); 13.61 (s, 2H). ESI-MS: 345.2 ($C_{20}H_{25}N_2O_2^+$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{24}N_2O_2$: C, 74.04; H, 7.46; N, 8.64. Found: C, 74.12; H, 7.42; N, 7.51.

4.3. *E. coli* KAS III purification and activity assay

Native *E. coli* KAS III protein was expressed in *E. coli* strain BL21(DE3) using the pET30 vector. Harvested cells containing KAS III were lysed by sonication in 20 mM Tris, pH 7.6, 5 mM imidazole, 0.5 M NaCl and centrifuged at 20,000 rpm for 30 min. The supernatant was applied to a Ni-NTA agarose column, washed, and eluted using a 5–500 mM imidazole gradient over 20 column volumes. Eluted protein was dialyzed against 20 mM Tris, pH 7.6, 1 mM DTT, and 100 mM NaCl. Purified ecKAS III were concentrated up to 2 mg/mL and stored at −80 °C in 20 mM Tris, pH 7.6, 100 mM NaCl, 1 mM DTT, and 20% glycerol for enzymatic assay.

In a final 20 μL reaction, 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.0, 0.5 mM DTT, 0.25 mM $MgCl_2$, and 2.5 μM holo-ACP were mixed with 1 nM ecKAS III, and H_2O was added to 15 μL. After 1 min incubation, a 2 μL mixture of 25 μM acetyl-CoA, 0.5 mM NADH, and 0.5 mM NADPH was added for ecKAS III reaction for 25 min. The reaction was stopped by adding 20 μL of ice-cold 50% TCA, incubating for 5 min on ice, and centrifuging to pellet the protein. The pellet was washed with 10% ice-cold TCA and resuspended with 5 μL of 0.5 M NaOH. The incorporation of the ^{3}H signal in the final product was read by liquid scintillation. When determining the inhibition constant (IC_{50}), inhibitors were added from a concentrated DMSO stock such that the final concentration of DMSO did not exceed 2%.

4.4. Antimicrobial activity

The antibacterial activity of the synthesized compounds was tested against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* using MH medium (Mueller–Hinton medium: casein hydrolysate 17.5 g, soluble starch 1.5 g, beef extract 1000 mL). The MICs of the test compounds were determined by a colorimetric method using the dye MTT.[23] A stock solution of the synthesized compound (50 μg/mL) in DMSO was prepared and graded quantities of the test compounds were incorporated in specified quantity of sterilized liquid medium (MH medium for antibacterial activity and RPMI-1640 medium for antifungal activity). A specified quantity of the medium containing the compound was poured into microtitration plates. Suspension of the microorganism was prepared to contain approximately 10^5 cfu/mL and applied to microtitration plates with serially diluted compounds in DMSO to be tested and incubated at 37 °C for 24 h for bacterial. After the MICs were visually determined on each of the microtitration plates, 50 μL of PBS (Phosphate Buffered Saline 0.01 mol/L, pH 7.4: $Na_2HPO_4{\cdot}12H_2O$ 2.9 g, KH_2PO_4 0.2 g, NaCl 8.0 g, KCl 0.2 g, distilled water 1000 mL) containing 2 mg of MTT/mL was added to each well. Incubation was continued at room temperature for 4–5 h. The content of each well was removed, and 100 μL of isopropanol containing 5% 1 mol/L HCl was added to extract the dye. After 12 h of incubation at room temperature, the optical density (OD) was measured with a microplate reader at 550 nm. The observed MICs were presented in Table 3.

4.5. Experimental protocol of docking study

The automated docking studies were carried out using AutoDock version 4.0. First, AutoGrid component of the program pre-calculates a three-dimensional grid of interaction energies based on the macromolecular target using the AMBER force field. The cubic grid box of 60 Å size (x, y, z) with a spacing of 0.375 Å and grid maps were created representing the catalytic active target site region where the native ligand was embedded. Then automated docking studies were carried out to evaluate the binding free energy of the inhibitors within the macromolecules. The GALS search algorithm (genetic algorithm with local search) was chosen to search for the best conformers. The parameters were set using the software ADT (Auto-Dock Tool Kit) on PC which is associated with Auto-Dock 4.0. For all docking parameters, default values were used with 20 independent docking runs for each docking case. The AutoDock performs the task of the docking, where the ligand moves randomly in any one of six degrees of freedom, and the energy of the new ligand 'state' is calculated. If the energy of the new state is lower than that of the old state, the new one is automatically accepted as the next step in docking.

4.5.1. Preparation of ligands and target ecKAS III

The compounds were studied for their binding activities into protein ecKAS III. The three-dimensional structures of the aforementioned compounds were constructed using Chem 3D ultra 11.0 software [Chemical Structure Drawing Standard; Cambridge Soft corporation, USA (2009)], then they were energetically minimized by using MOPAC with 100 iterations and minimum RMS gradient of 0.10. The Gasteiger–Hückel charges of ligands were assigned. The crystal structures of protein ecKAS III (PDB code: 1hnj) complex were retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). All bound waters and ligands were eliminated from the protein and the polar hydrogens and the Kollman-united charges were added to the proteins. The amino acids of the ligand-target binding site were defined using data in pdbsum (http://www.ebi.ac.uk/pdbsum/).

4.5.2. Molecular modeling and analysis of the docked results

The predicted binding free energy that includes the intermolecular energy and torsional free energy was used as the criterion for ranking. Furthermore, the intermolecular hydrogen bonds, whose effect has already been counted in the binding energy, were also investigated in order to find useful information for drug design. Cluster analysis was performed on the docked results using a root mean square (RMS) tolerance of 0.5 Å. Each of the clusters that exhibited significant negative interaction energies was examined by Accelrys, DS visualizer 2.0 [Accelrys Inc., San Diego, CA (2007)] to determine their binding orientations, molecular modeling, evaluation of the hydrogen bonds.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.10.037.

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