Allosteric Inhibition of [^{125}I] ET-1 Binding to ET_A Receptors by Aldoxime and Hydroxamic Acid Derivatives

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Abstract: Endothelin-1 (ET-1), a potent vasoconstrictor peptide, exerts its physiological effects by binding and activating specific G protein-coupled receptors, named ET_A and ET_B. An unique property of ET-1 is its ability to bind almost irreversibly to its receptors. Aspirin and salicylic acid (SA) are allosteric inhibitors of ET-1 binding to ET_A receptors. Dihalogenated derivatives of SA have been identified as more potent allosteric inhibitors than aspirin. In this study, disubstituted benzohydroxamic acid, benzaldoximes and dihalosalicylic acid dimers were synthesized and tested as inhibitors of [^{125}I]ET-1 binding to ET_A receptors in rat embryonic cardiomyocyte (H9c2 cell) membranes. Some dihalosalicylic acid dimers **2h** showed good inhibitory activity, the most active compounds are the hydroxamic acids derived from anthranilic acid. Among these compounds, the 3, 5-diiodo-2-aminobenzohydroxamic acid e compound **2a** is three-folds more potent as inhibitor of [^{125}I] ET-1 binding to ET_A receptors than the 3; 5-diiodosalicylic acid reported in literature. Most aryl aldoximes in this study were biologically inactive as inhibitors of [^{125}I] ET-1 binding to ET_A receptors.

Key Words: Allosteric, endothelin-1, hydroxamic acid, benzaldoxime, 3, 5-diiodo-2-aminobenzohydroxamic acid, 3, 5-diiodosalicylic acid, salicylic acid dimers, ETA receptors.

INTRODUCTION

Endothelins are a family of 21-amino acid peptides exist in four distinct isoforms, ET-1, ET-2, ET-3 and Endothelin β or mouse vasoactive intestinal contract (VIC). ET-1 is the most potent vasoconstrictor discovered to date being ten-fold more potent than angiotensin II, and the duration of pressor effects is extremely long [1]. ET-1 is unusual among the mammalian bioactive peptides in being released from a dual secretory pathway [2]. Autoradiographic experiments with [^{125}I] ET-1 have demonstrated a wide distribution of endothelin receptors in different tissues [3-5]. Two endothelin receptor subtypes, termed ET_A and ET_B, have been identified [6-16]. These receptors belong to the large family of G protein coupled receptors (GPCRs). Endothelin-1 is a potent vasoconstrictor peptide [17], plays an important role in several diseases thought to be associated with vasoconstrictions. These are coronary vasospasm [18], unstable angina [19], myocardial infarction [20], cardiac insufficiency [21], and cerebral vasospasm associated with subarachnoid haemorrhage [22], and many another pathological and physiological processes [23-32]. Many agonists and antagonists of endothelin receptors are described in literature and classified to many classes according to their chemical structure and their selectivity to receptors. Like peptide [1, 33-36], and non peptide antagonist, the non peptides could be classified to sulfonamides and nonsulfonamides [1, 33, 35, 37, 38]. Recently allosteric modulation inhibition of endothelin receptors ET_A has been described in literature [39,40]. The allosteric effect is an alternative approach in principle is to target the drug to a second site that is different from the Orthosteric site, but which is conformationally linked to the Orthosteric site (i.e. an allosteric site). This provides the opportunity to be able to 'tune up' or 'tune down' a receptor response, rather than 'switching' it on (or off) by the use of an Orthosteric agonist or antagonist, respectively. Furthermore, the allosteric approach can generate novel forms of selectivity that are absent in Orthosteric ligands. Some well recognized receptors which modulated allosterically when binded to their ligands are: Muscarinic receptors, Glutamate receptors, Adenosine receptors, GABA receptors Serotonin receptors, Chemokine receptors [41]. Earlier studies indicate the presence of three subtypes of endothelin receptors and possible an allosteric interaction type, suggesting the possible existence of a specific site for BQ-123 that interacts and/or interferes with the properties of endothelin-binding sites [42]. More recent studies showed that salicylates are allosteric inhibitors of ET_A receptors [39] this led to screen a number of derivatives of salicylic and benzoic acids to obtain compounds that would be more potent. This procedure led to the identification of dihalogenated derivatives of salicylic acid that are about 50 times more potent than aspirin. Results showed that the hydroxyl group of salicylate contributes little to the effect for related molecules in the benzoic acid, and salicylic acid series are equally potent [40]. Substitution of the aromatic hydrogen ring with halogens dramatically improves activity. Actions of halogens in the benzoic acid series are according to the following rank order of potency: Br > Cl > F. All dihalogenated derivatives of salicylic acid are equipotent. 3, 5-Diisopropylsalicylic acid is almost as potent as dihalogenated derivatives. These indicate that

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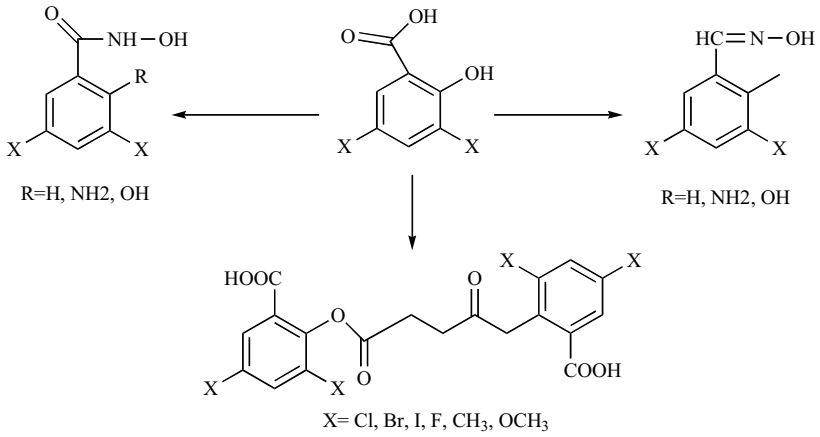


Scheme 1.

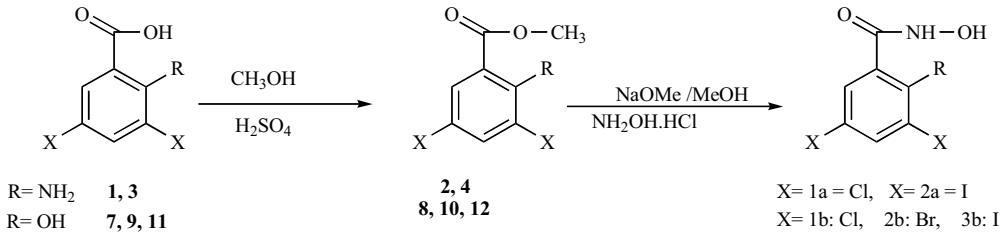
bulky groups at positions 3 and 5 of the aromatic ring of benzoic acid or of salicylic acid favour activity of these compounds [40].

The aim of this research is the development of new compounds with potential effect at ET_A receptors, mainly derivatives of dihalosalicylic, dihaloanthranilic and dihalobenzoic acids which are designed as analogues of previously studied salicylic acid derivatives as allosteric inhibitors of ET_A receptors, therefore, the 3, 5 dihalo-substituted compounds of type a, b, c, d, e, f and dihalosailicylic acid dimers type h as described in Scheme **1** were synthesized. In compounds type a, b, c, d, e, f the carboxylic function of salicylic acid derivatives is replaced by relative groups possessing different acid properties such as hydroxamic acid moiety (compounds a, b and c) or an oxime moiety (compounds d, e, and f). Among each type the hydroxyl group (compounds b, d) replaced by isosteric amino group (compounds a, e) or hydrogen (compounds c, f) .Some salicylic acid dimer were reported in literature [43], small number of recent researches are concerned with the synthesis of bivalent ligands, we are interested to prepare such dimers of type **h**, a series of bis (3, 5-dihalosalicyl) succinate **1-3h**.

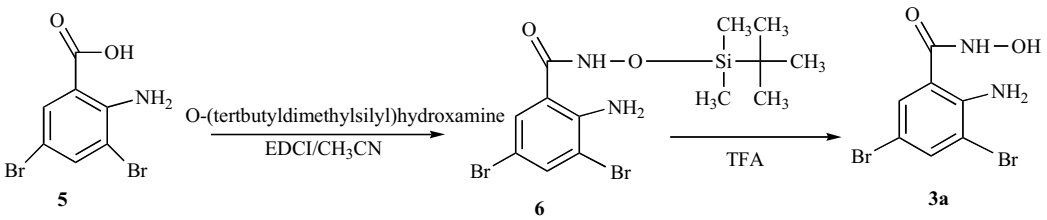
CHEMISTRY

The 3, 5- dihalogenated anthranilhydroxamic acid derivatives of type **a**, compounds; **1a**, **2a**, and 3, 5-dihalosalicyl hydroxamic acid, compounds type **b; 1b**, **2b**, and **3b** were synthesized by the reaction of corresponding 3, 5-dihaloanthranilic acid **1** and **3** or dihalo-salicylic acid **7**, **9**, **11**, with absolute methanol under reflux in presence of catalytic amount of H_2SO_4 to afford methyl anthranilate **2** and **4** or methyl salicylate esters **8**, **10**, **and 12**. These esters have been treated with $NH_2OH{\cdot}HCl$ and NaOMe [44] in methanol to obtain hydroxamic acid derivatives **1a**, **2a**, **1b**, **2b**, and **3b** Scheme **2**. Esterification of 3,5-dibromonthranilic acid **5** needs drastic condition and give very poor yield, therefore compound **3a**, have been prepared by more selective method by treating **5** with O- (tertbutyldimethylsilyl) hydroxylamine in CH_3CN in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride(EDCI) to give anthranilamide **6** which was hydrolysed in triflouroacetic acid to obtain the desired hydroxamic acid **3a**, Scheme **3**.

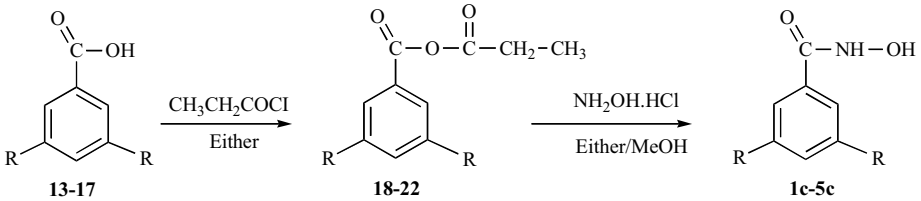
The dihalobenzohydroxamic acid derivatives type **c** gave poor yield with using previous procedure moreover due to



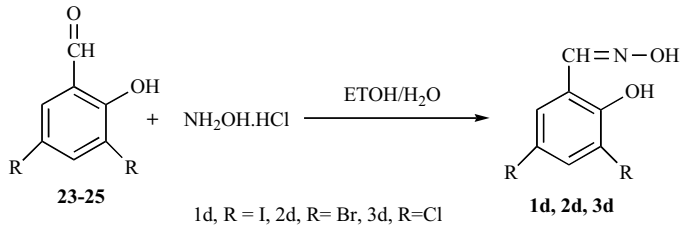
Scheme 2.



Scheme 3.



Scheme 4.



Scheme 5.

the difficulties to remove the traces of acetic acid led to use of one step synthetic procedure by treatment of 3, 5-disubstituted benzoic acids **13-17** with ethylchloroformate to obtain the oxalyl derivatives **18-22**. The reaction of intermediates (**18-22**) with hydroxylamine hydrochloride gives desired benzohydroxamic acid derivatives **1c-5c** [45] as shown in Scheme **4**.

The appearance of hydroxamic acids **1a-5c**, has been demonstrated chemically by it is reaction with $FeCl_3$ and confirmed by ^{1}H-NMR in DMSO-d_6, spectroscopy where results contain diagnostic peaks of nitrogen and hydroxyl protons of hydroxamate.

3, 5-Dihalosubstituted salicyldoximes of type **d** has been synthesized as reported in Scheme **5**. Compounds **1d**, **2d** and **3d** were obtained by treatment of disubstituted salicyldehyde **23-25** with hydroxylamine [46] in ethanol/water mixture.

3, 5-Disubstituted-2-aminobenzaldoximes of type **e** were prepared as shown in Scheme **6**. The corresponding alcohols **29-31** were obtained by reduction of 3, 5-disubstituted-2-aminobenzoic acids **26-28**, the obtained alcohols **29-31** were oxidized [47] to give 3, 5-disubstituted-2-amino-benzaldehydes **32-34**. Subsequent treatment of compounds **32-34** with hydroxylamine hydrochloride afford the desired compounds **1e-3e**, dibromo-aldehyde analogue **35** is available commercially which treated directly with hydroxylamine hydrochloride to give **4e**.

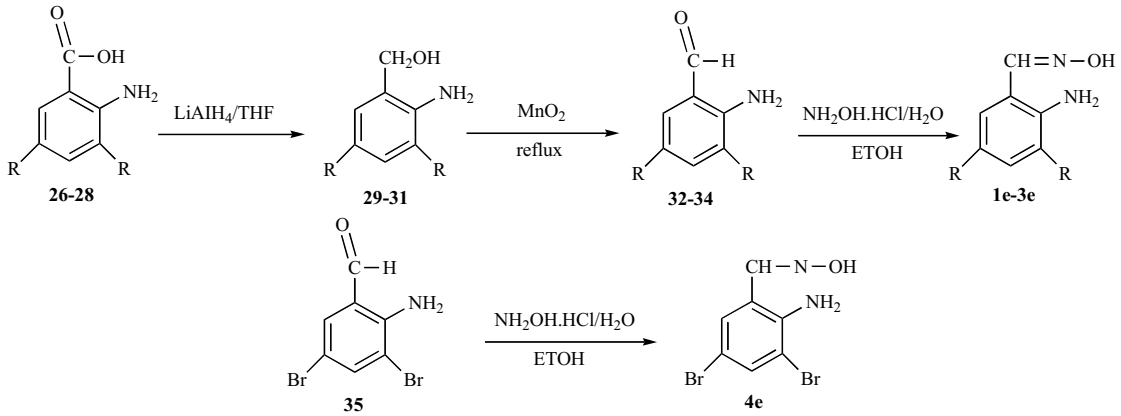
3,5-Disubstituted benzaldoximes compounds type **f**; 3,5-dimethoxylbenzaldoximes **1f**, and 3,5-dichlorobenzaldoximes **2f** have been synthesized according to the method described in Scheme **7**, starting with corresponding aldehyde **36** and **37** which treated with $NH_2OH{\cdot}HCl$ in ethanol in presence of NaOH.

Bis (3, 5-dihalosalicyl)succinate **1-3h**, was prepared according to the method described in Scheme **8**, by treatment of salicylic acids **38**, **39**, **40** with succinylchloride and N, N-dimethylaniline in toluene to give the corresponding compounds **1-3h**.

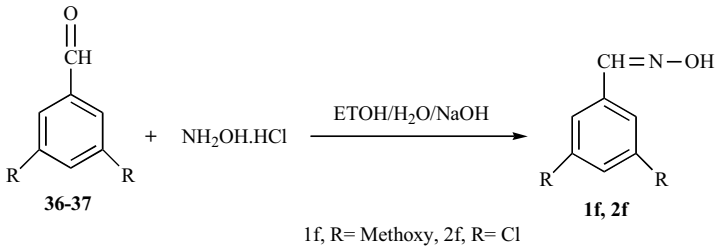
BIOLOGICAL STUDY

Materials and Methods

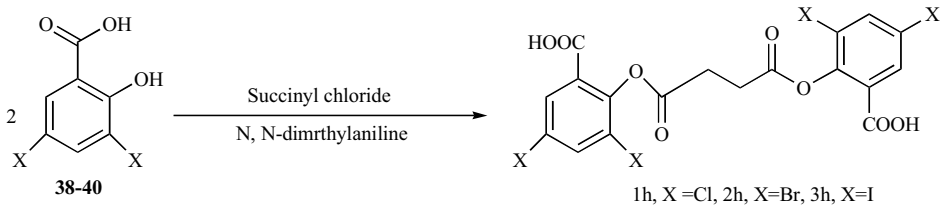
Cell culture: H9c2 rat cardioblasts were propagated in Dulbecco's modified Eagles's Medium (DMEM) supple-



Scheme 6.



Scheme 7.



Scheme 8.

mented with 10% FBS, 1 mM pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin and 0,02 mg/ml 2,4-difluoro-α,α[1]-bis(1H-1,2,4-triazol-1-ylmethyl)benzyl alcohol (fluconazole), at 37° in a humidified atmosphere containing 5% CO_2 and subcultured before confluence.

Membrane Preparation

Membrane preparation was performed as described by Ceccarelli et al. (2003) [48]. Subconfluent monolayers (passage 18-21) premium due were washed with 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 136.8 mM NaCl and 2.7 mM KCl (PBS) 3 times, harvested with a cell scraper and collected by centrifugation at 1000 g. Cells were homogenised in 10 mM Tris-HCl, pH 7.3, containing 1 mM EDTA, 160 µg/ml benzamidine, 200 µg/ml bacitracin, 0,1 mM phenylmethanesulphonyl fluoride (PMSF) and 20 µg/ml trypsin inhibitor (buffer A) using a Polytron homogeniser. The homogenate was centrifuged at 48,000 g at 4° for 30 min. The resulting pellet was resuspended in buffer A, homogenised and centrifuged as described earlier. The membrane pellet was stored in aliquots at –80° until the time of assay. Protein concentration was determined by the Coomassie Blue binding method (Bradford, 1976) [49] using bovine serum albumin (BSA) as a standard.

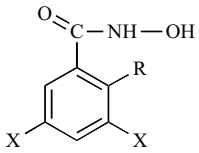
Binding Assay

[^{125}I] ET-1 binding assays were performed as described by Ceccarelli et al. [48]. Cells membrane (~ 30 µg of proteins) were incubated with [^{125}I]ET-1 (~ 20 pM) in 250 ml of 20 mM Tris-HCl buffer, pH 7.4, at 37°C, containing 2 mM EDTA, 0,1 mM bacitracin, 0,1 mM PMSF, 1 µg/ml leupeptin, 5 µg/ml aprotinin (buffer B) and 0,08 mg/ml BSA for 2 hr at 37°C. After incubation, the reaction was stopped with 3 ml of ice cold 50 mM Tris-HCl, pH 7, 3 at 4°C, containing 0, 1 mM bacitracin (buffer C). Membrane bound radioactivity was separated from the free ligand by filtration through Whatman GF/C filters that had been pre-soaked in buffer C containing 2 mg/ml BSA. The filters were washed three times with 3 ml of buffer C. Non-specific binding was defined as the binding that occurred in the presence of an excess of ET-1 (100 nM). At 20 pM [^{125}I] ET-1, specific binding was about 80% of total binding. Tested compounds were dissolved in buffer B without protease inhibitors and diluted to the desired concentration to have 10x stock solutions for the assay.

Results and Discussion

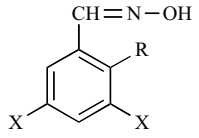
All compounds **1a-3a**,**1b-3b**, **1c-5c**, **1d-3d**, **1e-4e**,**1f-2f** and **1h-3h** were tested in a preliminary screening, at dose of 100 µM, to evaluate their ability to inhibit specific [^{125}I]ET-1 binding to H9c2 membranes, the results are showed in Tables **1**,**2**. These compounds (**1a-3a**, **1b-3b**, **1c-5c**, **1d-3d**, **1e-4e**, **1f-2f** and **1h-3h**) were designed as analogues of previously studied 3, 5-disubstitutedsalicylic acids [39, 40] with the aim to obtain potent allosteric inhibitors of ET_A receptors. As shown in Table **1**, the benzohydroxamic acid derivatives **1a-3a**, which present in their structure an amino group at position 2, are the best of the series because showed an appreciable inhibitory activity of one order of magnitude higher than that of 3, 5-disubstitutedsalicylic acids reported in the literature [40]. In fact, our compounds are active within the micromolare range of concentrations (Table **1**, **2**, **3**) while those reported in literature show activity between high micromolare and millimolar ranges [40]. Many competitive antagonists reported in literature are active in nM concentration while all allosteric inhibitors reported are active in mM concentration but even at this concentration it has great advantage that can generate new form of selectivity beside it can potentiate the competitive antagonist effects and might be of therapeutic interest to relieve tight ET-1 binding and to favor actions of receptor antagonist, this might solve the simple fact the usefulness of ET receptor antagonists against endogenous ET-1. Among the hydroxamic acid derivatives substituted with hydroxyl group at position 2 (**1b-3b**) only the bromo and iodio substituted compound shows modest inhibitory activity, compounds (**1c-5c**), which unsubstituted at position 2 only 3, 5-dibromo and 3, 5-dimethoxy derivatives showed weak inhibitory activity on ET_A receptors while other compounds 1c-3c, were completely inactive suggesting that the type of substitution in

Tables 1, 2, 3. Preliminary Screening of Compounds Synthesised (100 μM), average of three separate experiments. Values are Expressed as Percent of Inhibition of [^{125}I] ET-1 Binding to H9c2 Membranes



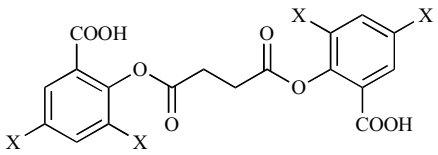
Compound	X	R	Yield%	M.P °C	M.W	M.F	% inhibition of [^{125}I]ET_A (100μM)
1a	Cl	NH_2	65	190	221	$C_7H_6Cl_2N_2O_2$	20
2a	I	NH_2	56	200-205	403.93	$C_7H_6I_2N_2O_2$	70
3a	Br	NH_2	70	196	310	$C_7H_6Br_2N_2O_2$	30
1b	Cl	OH	57	181-183	222	$C_7H_5Cl_2NO_3$	0
2b	Br	OH	60	185-187	310	$C_7H_5Br_2NO_3$	10.4
3b	I	OH	57	212-214	405	$C_7H_5I_2NO_3$	18
1c	F	H	60	151-152	173.11	$C_7H_5F_2NO_2$	0
2c	Cl	H	67	130-134	206	$C_7H_5Cl_2NO_2$,	0
3c	CH_3	H	70	142-147	165	$C_9H_{11}NO_2$	0
4c	Br	H	73	165-166	294.8	$C_7H_5Br_2NO_2$,	14
5c	OCH_3	H	80	141-143	197.18	$C_9H_{11}NO_4$	3

Table 2



Compound	X	R	Yield%	M.P °C	M.W	M.F	% inhibition of [^{125}I]ET_A (100μM)
1d	I	OH	60.0	225	389	$C_7H_5NO_2I_2$	8
2d	Br	OH	86	207-210	293	$C_7H_5NO_2Br_2$	16
3d	Cl	OH	62	195-197	205	$C_7H_5NO_2Cl_2$	10
1e	CH_3	NH_2	60	180	164	$C_9H_{16}N_2O$	0
2e	Cl	NH_2	51.54	176-177	205	$C_7H_6Cl_2N_2O$	0
3e	I	NH_2	93	210-213	387.8	$C_7H_6I_2N_2O$	0
4e	Br	NH_2	70	185	293.94	$C_7H_6Br_2N_2O$	0
1f	CH_3O	H	56	116-119	181	$C_9H_{11}NO_3$	10
2f	Cl	H	60	107-109	190	C_7H_5ClNO	8

Table 3



Compound	X	Yield%	M.P °C	M.W	M.F	% inhibition of [^{125}I]ET_A (100μM)
1h	Cl	73	175-178	496	$C_{18}H_{10}Cl_4O_8$	17
2h	Br	68	195-198	674	$C_{18}H_{10}Br_4O_8$	38.5
3h	I	69	209-211	862	$C_{18}H_{10}I_4O_8$	25.5

this position is important for their interaction with the allosteric site. As regards the halosubstituent in compounds **1a-3a** the Iodine groups (compound **2a**) were those able to confer the highest inhibitory capacity. Among compounds which present the oxime group on the aromatic moiety, those with a hydroxyl group (**1d-3d**), and those with H atom (**1f**, **2f**) in 2 positions showed some modest inhibitory activity. If the substituent in 2 positions is an amino group (**1e-4e**), independently from the halosubstituent, the compound was not able to inhibit the ET_A receptors, this may suggest the importance of carbonyl group of carboxylic and hydroxamic acid in the interaction with allosteric site. No big change in inhibitory capacity showed by dimers of salicylic acid compounds **1h-3h**, the bromo substituted compound is more active than other halogen substituted compound.

Compounds **1a**, **2a**, **3a**, **2h**, and **3h** which in the preliminary screening have shown the best inhibitory activity are retested at 10,100,500μM the obtained data are summarized in Fig. (**1**).

CONCLUSIONS

Allosteric antagonists of ET_A receptors can be effective modulators of this receptor activity and can potentiate competitive antagonist effects. Some dihalosubstituted benzohydroxamic acids, salicylaldoximes and dimers of salicylic acid synthesized in our work were more potent than salicylic acid derivatives of the literature, these compounds were designed as allosteric inhibitors of ET_A receptors. Among the benzohydroxamic acid derivatives the 2-amino-3, 5-dihalosubstituted compounds were the most potent inhibitors of ET_A receptors. Inside this series of compounds, compound **2a** (3, 5-diiodo-2-aminobenzohydroxamic acid) shows interesting activity, its three-folds more potent as inhibitor of [^{125}I] ET-1 binding to ET_A receptors than the 3; 5-dibromo and 3; 5-diiodosalicylic acid reported in literature, among salicylic acid dimers the most active compound is the 3, 5-dibromosubstituted compound **2h** which shows interesting activity in this screening. Compounds **2a**, **2h** will subject to another investigation for binding property to determine their primacy of binding to ET_A, ET_B and its effect on competitive antagonist (bosentan), dissociation, association and other allosteric inhibition properties in further extended study. We conclude also inside the series of benzohydroxamic acids and aldoximes, that the substitution at ortho position (2) and the carbonyl oxygen of the acidic moiety may play an important role in receptor binding.

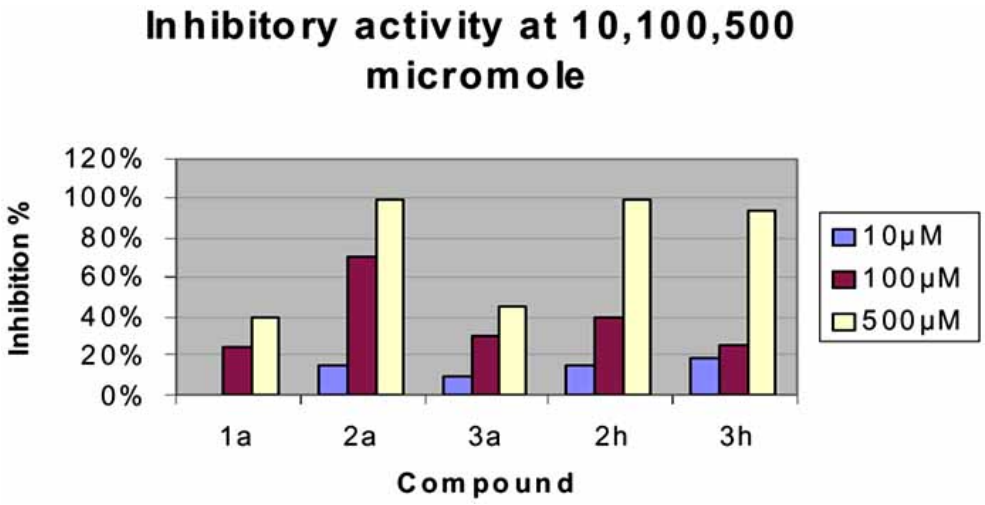


Fig. (1).

EXPERIMENTAL SECTION

General methods: Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Infrared (IR) spectra for comparison of compounds were recorded on a Mattson 1000 FTIR spectrometer. Nuclear magnetic resonance (1HNMR) spectra were recorded on a Varian Gemini 200(200MHz) in a ca.2%solution of $CDCl_3$ or DMSO-d_6 for all compounds. Peak positions are given in parts per million (ppm, δ units). The proton magnetic resonance assignments were established on the basis of the expected chemical shifts and the multiplicity of the signals. Mass spectra were recorded on a HP-5988 Spectroscopy using direct injection probe and an electron beam energy of 70eV, all isotopes were found 1:2:1 in dibromo- compounds, and 3:1 in dichloro- substituted compounds .Reactions were routinely monitored by Thin-layer chromatography(TLC) on 0.25mm silica gel plates (Merck 60F254)and Hydroxamic acids were visualized with $FeCl_3$ aqueous solution . Flash chromatography or Preparative medium pressure liquid chromatography (MPLC) were carried out through glass columns containing 40-63 μm silica gel (Machinery- Nagel Silica Gel 60). The MPLCs were performed using a chromatography apparatus consisting of a Buchi 681 pump, a Knauer differential refractometer detector and a Philips PM 8220 pen recorder. Solvents and reagents were obtained from commercial sources in the appropriate grade and were used without further purification unless otherwise indicated. Element Analysis was carried out by our analytical laboratory and was consistent with theoretical values to within ± 0.4%.

Methyl -3, **5-dihaloanthranilate esters**, **2**, **4;** were prepared by reaction of 3, 5-dihaloanthranilic acid (**1**, **3**; 2.42mmol) with Methanol (30 ml) in presence of catalystic amount of concentrated sulphuric acid (1 ml). Reaction has been kept under reflux, methanol was evaporated under vacuum and the residue extracted with ether (Et_2O), washed three times with a solution of 10% $NaHCO_3$ (20 ml), dried and evaporated to obtain a crude residue which was purified by column chromatography (Ethyl acetate/Hexane 30:70) to give pure products **2**,**4**. Table **4**.

Methyl -3, **5-dihalosalicylate esters**; **8**, **10**, **12;** were prepared by the reaction of (2.145 mmol) of 3, 5-dihalosalicylic acid (**7**, **9**, **11**) dissolved in 20 ml CH_3OH in presence of catalystic amount of concentrated H_2SO_4. Reaction has been left under reflux. The mixture dried under vacuum, diluted with EtoAc and washed with water (3x20 ml), then with saturated solution of $NaHCO_3$, and another once time with water. Organic phase was dried with Na_2SO_4 and evaporated to give pure esters **8**, **10**, **12**, Table **4**.

3, **5-Dichloro-2-aminobenzohydroxamic acid**, **1a;** To stirred solution of sodium metal (539.442mg, 23.454mmol) dissolved in methanol (11.7ml), a calculated amount of hydroxylamine hydrochloride (1068.6mg, 15.6 mmol) dissolved in methanol (11ml) was added. Reaction mixture was stirred for 15 minutes and the precipitate filtered off, filtrate was added freshly to solution of 3, 5-dichloro-methylanthranilate (**2**, 1720mg, 7.818mmol) in methanol (20 ml) while stirring. The reaction mixture set aside at r. t. for 5 days. Then methanol was evaporated under reduced pressure. The solid was dissolved in a minimum amount of water, filtered, and acidified cautiously with acetic acid to give a solid residue which was recrystallised from ethanol (EtOH) to afford pure **1a**.^{1}HNMR: (CD_3OD): δ, 7.76(d, J=2.6 Hz, 1H, aromatic), 7.41(d, J=2.6 Hz, 1H, aromatic). MS,m/e: 220(M^+, 12%),222(M^{+2}),224(M^{+4}),205(base peak, 100%),188,161, 124,90. (C, H, N), calculated (38.0, 2.71, 12.66), analytical (39.1, 2.65, 12.3).

2-Amino-3, **5-diiodobenzohydroxamic acid**, **2a:** by using same procedure described in **1a** (276.345 mg 12.015

Table 4.

Compound	R	X	Yield	Time	Melting Point	^{1}HNMR
2	NH2	Cl	68%	4hr	63°C	($CDCl_3$),δ,3.88(s,CH_3,ester),7.40(d, J=2.4Hz,1H,aromatic),7.79(d, J=2.4Hz, 1H, aromatic), Δv=78.2Hz.
4	NH2	I	60%	24hr	110°C	($CDCl_3$), δ, 3.89(s, CH3 ester), 8.03(d, J=2Hz, 1H aromatic), 8.16(d, J=2Hz, 1H aromatic), Δv=25.6Hz.
8	OH	Cl	65%	5 days	230-233°C	^{1}H-NMR (DMSO-d_6): δ 3.92(s, 3H, OCH_3), 7.74 (d, 1H, J=2.5 Hz, Ar), 7.91 (d, 1H, J=2.5 Hz, Ar), 10.9 (s, 1H, OH).
10	OH	Br	85%	4-days,	230-333°C	^{1}H-NMR (DMSO-d_6): δ 3.9 (s, 3H, OCH_3), 7.9 (d, 1H, J=1.4 Hz, Ar), 8.11 (d, 1H, J=1.6 Hz, Ar), 11.09 (s, 1H, OH).
12	OH	I	55%	3 days	121-124°C	^{1}HNMR (CD_3COCD_3): δ 3.99 (s, 3H, OCH_3) 8.14 (d, 1H, J=2.1 Hz, Ar), 8.21 (d, 1H, J=2.0 Hz, Ar), 11.6 (s, 1H) 3.

mmol) of sodium metal in methanol (10 ml),hydroxylamine (556.614mg, 8.01 mmol) in methanol (5.594 ml), 3,5-diiodomethylanthranilate (**4**,1614mg, 4.005mmol) in methanol (20 ml), give pure **2a**.^{1}HNMR: ($CDCl_3$), δ 8.04 (d, J=2 Hz, 1H, aromatic), 7.99 (d, J=2 Hz, 1H, aromatic), 6.80 (brs, 2H, NH_2). MS,m/e: 403(M^{-1},65%),(404, M^+, 10%), 389 (base peak,100%),374,357,344,277. (C, H, N) calculated (20.84, 1.84, 6.9), analytical (21, 1.81, 6.75).

O-tert-butyldimethylsilyl-2-amino-3,5-dibromobenzamide, **6**, 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (787 mg,4.10 mmol) (EDCI) was added portion wise to a stirred and cooled (0°C) solution of 3,5-dibromoanthranilic acid (**5**,897 mg, 3.041 mmol) and O-(tert-butyldimethylsilyl) hydroxylamine (447.95 mg,3.041 mmol) in CH_3CN (30 ml); reaction mixture was stirred at room temperature for 2 days. CH_3CN was evaporated and the resulting solid residue dissolved in ethyl acetate, washed many times with water to remove EDCI, dried and evaporated. The crude residue was purified by flash chromatography (Hexane/Ethyl acetate 70:30) to yield pure **6**. Yield; 98%, ^{1}HNMR; (DMSO) δ 8.29 (s, 1H, NH), 7.76 (d, 1H, J= 2Hz, aromatic), 7.50 (d, 1H, J = 2 Hz, aromatic), 6.1 (brs, 2H, NH_2), 0.91 (s, 9H, tertbutyl), 0.18 (s, 6H, 2 CH_3).

3, **5-Dibromo-2-aminobenzohydroxamic acid**, **3a;** A solution of O-(tertbutyldimethylsilyl) dibromoanthranilamide (**6**, 1.373 g, 3.23 mmol) in anhydrous dichloromethane (2ml) was stirred in triflouroacetic acid (18.354 g, 161.82 mmol) over the night. After this time the triflouroacetic acid was evaporated and the solid residue recrystallised ($CHCl_3$) to obtain pure **3a**. ^{1}HNMR (DMSO): δ 11.23 (s, 1H, OH), 9.15 (s, 1H, NH), 7.73 (d, 1H, J= 2Hz, aromatic), 7.52 (d, 1H, J= 2Hz, aromatic), 6.42 (brs, 2H, NH_2), MS: m/z= 308(M^+),310 (M^{+2}), 312(M^{+4}), 293, 278, 250, 79 (base peak). (C, H, N), calculated (27.27, 1.95, 9.09), analytical (27.28, 1.96, 9.1).

3, **5-Dichlorosalicylhydroxamic acid**, **1b;** To stirred solution of sodium metal 0.062 g (2.72 mmol) dissolved in methanol (2ml), a calculated amount of hydroxylamine hydrochloride 0,124 (1.809 mmol) dissolved in methanol(2ml) was added. Reaction mixture was stirred for 15 minutes and the precipitate filtered off, filtrate was added freshly to solution of 3, 5-dichloromethylsalicylate 0.200 g (0.904 mmol), **(8)** in methanol (20 ml) while stirring. The reaction mixture set aside at room temperature for 24hrs. Then methanol was evaporated under reduced pressure. The solid was dissolved in a minimum amount of water, acidified by HCl, extracted with AcOEt. The organic phase dried and evaporated, the solid residue recrystallised to give pure **1b**. m.p: 181-183°C, IR: 1651, 4 cm^{-1} (C=O). ^{1}H-NMR (DMSO-d_6): δ 7.74(d, 1H, J=2.2Hz, Ar), 7.76(d, 1H, J=2.2 Hz, Ar), 11.9(br s, 1H, OH), 9.6 (br s, 1H, NH) MS: m/e: 221(M^+), 223(M^{+2}), 225 (M^{+2}, (Base peak: 189). (C, H, N) calculated (37.8, 2.25, 6.3), analytical (37.5, 2.27, 6.5).

3, **5-Dibromosalicylhydroxamic acid**, **2b;** by using same procedure described in compound **1b**, 0.066 g (2.88 mmol) of sodium metal, methanol (2ml),hydroxylamine hydrochloride 0.132 g (1.92 mmol) in methanol (2ml),3,5-dibromomethylsalicylate A 0.300 g (0.96 mmol), **(10)** in 6 ml methanol (20ml),give pure **2b**. ^{1}H-NMR (DMSO-d_6): δ 7.79(d, 1H, J=2Hz, Ar), 8.0(d, 1H, J=2 Hz, Ar), 10.3 (br s, 1H, NH), 12.1(br s, 1H, OH). MS :m/e :308 M^+,310 (M^{+2}), 312,(M^{+4}) (Base peak: 296,100%),279(-NH-OH), (C, H, N) calculated (27, 1.6, 4.5) analytical (27.3,1.6,4.6) .

3, **5-Diiodosalicylhydroxamic acid**, **3b;** by using same procedure described in compound **1b**, 0.010 g (0.460 mmol) of sodium metal, methanol (0.2 ml), hydroxylamine hydrochloride 0.021 g (0.306 mmol) in methanol (2ml),3, 5-diiodomethylsalicylate A 0.062 g (0.153 mmol), **(12)** dissolved in 1ml methanol. give pure **3b**. IR: 1666, 3 cm^{-1} (C=O), ^{1}HNMR (CD_3COCD_3): δ 8.2 (d, 1H, J=1.6 Hz, Ar), 8.3 (d, 1H, J=2 Hz, Ar)11.7 (s, 1H, NH).MS :m/e : 404(M^+,Base peak),278,138. (C, H, N) calculated (20.74,1.23, 3.45), analytical (21, 1.25,3.6).

3,5-Diflourobenzohydroxamic acid,**1c;** Solution A: ethylchloroformate (412.4mg, 3.8 mmol) and N-methylmorpholine (415.8 mg, 4.12 mmol) were added subsequently at 0°C to a solution of 3,5-diflourobenzoic acid (**13**, 500mg, 3.162mmol) in Et_2O (20ml). The reaction mixture was stirred for 30 minutes at r. t. and filtered. Solution B: A solution of hydroxylamine hydrochloride (329.6 mg, 4.743mmol) in methanol (10 ml) was added to a solution of KOH (266 mg, 4.743mmol) in methanol (4 ml); the resulting solution was stirred at room temperature for 15 mints and filtered. The filtrate was immediately added to filtrate of solution A. The reaction mixture was stirred at room temperature for one day, evaporated and the crude solid residue, purified by flash chromatography (Hexane/Ethyl acetate 50:50), gave the pure **1c**. ^{1}HNMR: (DMSO): δ, 11.4(s, 1H, OH), 9.31(s, 1H, NH) 7.51-7.42 (m, 3H, aromatic).MS: m/z= 173(M^+), 174(M^{+1}), 158, 141(base peak), 113. (C, H, N) calculated (48.52, 2.89, 8.09), analytical (48.7, 2.77, 7.9).

3, **5-Dichlorobenzohydroxamic acid**, **2c;** by using same procedure described in compound **1c**, ethylchloroformate (1.022 g, 9.42 mmol) and N-methylmorpholine (1.032 g, 10.205mmol),3, 5-dichlorobenzoic acid (**14**, 1.5 g, 7.85 mmol) in Et_2O (20ml). hydroxylamine hydrochloride (818.24 mg, 11, 78 mmol) in methanol (10 ml), KOH (660.7 mg, 11.78 mmol) in methanol (4 ml); the reaction mixture was stirred at r. t. for two days, give the pure **2c**. ^{1}HNMR: (DMSO) δ 11.51 (s, 1 H, OH), 9.33 (s, 1 H, NH), 7.85-7.65 (m, 3H, aromatic). MS: m/z= 205(M^+), 207(M^{+2})209(M^{+4}), 188(-O), (173 base peak,100%), 145. (C, H, N) calculated (40.7, 2.42, 6.7), analytical (40.4, 2.45, 6.8).

3, **5-Dimethylbenzohydroxamic acid**, **3c;** by using same procedure described in compound **1c**, ethylchloroformate (1.301 g, 11.99 mmol) and N-methylmorpholine (1.311 g, 12.96 mmol),3,5-dimethylbenzoic acid (**15**, 1.5 g, 9.98 mmol) in Et_2O (20ml). hydroxylamine hydrochloride (1.04 g, 14.97 mmol) in methanol (10 ml), KOH (840 mg, 14.97 mmol) in methanol (4 ml); give the pure **3c**. ^{1}HNMR: (DMSO) δ 11.10 (s, 1H, OH), 8.98 (s, 1H, NH), 7.355 (d, 1H, J=0.4Hz, aromatic), 7.350 (d, 1H, J=0.4 Hz aromatic), 7.13(s, 1H, aromatic), 2.29 (s, 6H, 2CH_3). MS: m/z= 165(M^+), 150, 149, 133(base peak), 118, 105, 77. (C, H, N) calculated (65.3, 6.66, 8.4), analytical (65.9, 6.85, 8.2).

3, **5-Dibromobenzohydroxamic acid**, **4c;** by using same procedure described in compound **1c**, ethylchloroformate (698 mg, 6.43 mmol) and N-methylmorpholine (704.8 mg, 6.968 mmol), 3,5-dibromobenzoic acid (**16**, 1,5g, 5.36mmol)

in Et_2O (20ml). hydroxylamine hydrochloride (558.69 mg, 8.04mmol) in methanol (10 ml), KOH (266 mg, 4.743mmol) in Methanol (4 ml); purified by flash chromatography (Chloroform/Ethyl acetate 50:50) to give the pure **4c**.^{1}HNMR: (DMSO), δ 11.42 (s, 1H, OH), 9.33 (s, 1H, NH), 8.02 (s, 1H, aromatic), 7.91 (s, 2H, aromatic). MS: m/z= 293(M^+), 295 (M^{+2}), 197(M^{+4}), 278, 263(base peak, 100%), 156. (C, H, N) calculated (28.49, 1.69, 4.69), analytical (28.31, 1.7, 4.69).

3, **5-Dimethoxybenzohydroxamic acid**, **5c;** by using same procedure described in compound **1c**, Ethylchloroformate (357.4mg, 3.29mmol) and N-methylmorpholine (360.296 mg, 3.562mmol), 3,5-Dimethoxylbenzoic acid (**17**, 500mg, 2.74mmol) in Et_2O (20ml). Hydroxylamine Hydrochloride (286.646 mg, 4.125mmol) in Methanol (10 ml), KOH (230mg, 4.125mmol) in Methanol (4 ml); purified by Flash chromatography (Chloroform /Ethyl acetate 50:50), to give the pure **5c**. ^{1}HNMR: (DMSO), δ 11.19 (s, 1H, OH), 9.06 (s, 1H, NH), 6.90 (d, 2H, J=2.2Hz aromatic), 6.62 (s, 1H, aromatic), 3.76(s, 6H, 2OCH3). MS: m/z= 197(M^+), 182, 181, 166, 165(base peak) 150, 149, 137, 133, 77. (C, H, N) calculated (54.8, 5.54, 7.1), analytical (54.65, 5.56, 6.97).

3, **5-Diiodosalicyladoxime**, **1d;** A solution of hydroxylamine hydrochloride (150.6mg, 2.168mmol) in a little amount of water (1ml) was added to 3, 5-diiodosalicylaldehyde (**23**, 500 mg, 1.337 mmol) dissolved in ethanol (19.2ml); the reaction has been kept under reflux for 3 hours and controlled by TLC. After this time, the reaction mixture was filtered, and the crude solid residue was collected. Pure **1d** has been obtained by recrystallization ($CHCl_3$). ^{1}HNMR :(DMSO), δ 7.77(d, J= 2Hz, 1H aromatic proton), 7.99(d, J= 2Hz, 1H aromatic proton), 8.31(s, CH=N), 11.15(s, OH aromatic hydroxyl group), 11, 93(s, OH oxime hydroxyl group). MS: m/z = 389(M^+, base peak), 343,244,189, 164,127, 62. (C, H, N) calculated (21.59, 1.28, 3.68), analytical (21.57, 1.27, 3.7).

3, **5-Dibromosalicyldoxime**, **2d;** by applying procedure described in compound **1d**, A hydroxylamine hydrochloride (150.6 mg, 2.16 mmol) in water (1.9ml), 3, 5-dibromosalicylaldehyde (**24**, 500 mg, 1.78 mmol) in ethanol (19.5 ml), reaction mixture stirred at r.t. for one hour, recrystallization ($CHCl_3$) give pure **2d**. ^{1}HNMR :(CD_3OD), δ, 8.21(s, CH=N), 7.63(d, J=2.4Hz, 1H aromatic), 7.46(d, J= 2.4Hz, 1H aromatic). MS, :m/z= 293(M^+),295(M+2),297(M^{+4}),277 (base peak),249,223, 196,170,143,88,62. (C, H, N), calculated (28.6, 1.7, 4.77), analytical (28.4, 1.66, 4.71).

3, **5-Dichlorosalicyldoxime**, **3d;** by using the procedure described in compound **1d**, hydroxylamine hydrochloride (200.6 mg, 5.17 mmol) in water (1.9 ml),3, 5-dichlorosalisyldehyde (**25**, 500 mg, 3.6 mmol) in ethanol (19.2ml), give pure **3d**.^{1}HNMR: (CD_3OD), δ, 8.24(s, CH=N), 7.371(d, J= 2.4 Hz, 1 H aromatic), 7.30(d, J= 2.4Hz, 1H aromatic). MS: m/z, 205(M^+),207(M^{+2}),209(M^{+4}), 187(base peak, 100%), 159, 133, 124, 97, 88, 62. (C, H, N) calculated (40.89, 2.43, 6.81), analytical (40.6, 2.5, 6.6).

2-Amino 3, 5 dimethylbenzaldehyde, 32; $LiALH_4$ (344.73 mg, 9.09mmol) was added to a solution of 3, 5-dimethylanthranilic acid (**26**, 1.0 g, 6.06 mmol) in anhydrous THF (30 ml) stirred at 0°C degree under nitrogen atmosphere. The resulting mixture was stirred at room temperature one hour and then under reflux 30 min. After this time the reaction mixture was cooled and, a mixture of THF: Water (2:1, 5ml) was added to finish the reaction, filtered, and to the filtrate MnO_2 (670.8 4mg, 18 mmol) was added; the reaction has been left under reflux for tow days. The organic phase, dried and evaporated afforded the crude aldehyde which was purified by flash chromatography (Hexane/Ethyl Acetate 80:20) to give pure (**32**) .Yield= 80%, ^{1}HNMR (DMSO): δ 9.94 (s, 1H, CHO), 7.26 (s, 1H, aromatic), 7.18 (s, 1H, aromatic), 6.15 (brs, 2H, NH_2), 2.36 (s, 3H, CH_3), 2.24 (s, 3H, CH3).

2-Amino-3, **5-dimethylbenzaldoxime**, **1e;** A solution of hydroxylamine hydrochloride (407.21 mg, 5.86 mmol) in little amount of water (1.9 ml) was added drop wise to a solution of 2-Amino-3, 5-dimethylbenzaldehyde (**32**, 438 mg, 2.93 mmol) in ethanol (19 ml) in presence of K_2CO_3 (809.91 mg, 5.86mmol). The reaction has been left under reflux for 4 hours, filtered, extracted with Et_2O, and the organic layers dried and evaporated afforded a solid residue which was crystallized ($CHCl_3$) to give pure **1e**.^{1}HNMR (DMSO): δ 8.21 (s, 1H, CH=N), 6.91 (s, 1H, aromatic), 6.82 (s, 1H, aromatic), 2.22 (s, 3H, CH_3), 2.16 (s, 3H, CH_3). MS: m/z= 164(M^+, base peak), 165(M^{+1}), 147, 132, 120. (C, H, N) calculated (65.8, 7.3, 17), analytical (65.7, 7.25, 16.8).

2-Amino-3, **5-dichlorobenzaldehyde**, **33;** by using same procedure described in compound **32**, $LiALH_4$ (110.44mg, 2.9mmol), 3, 5-dichloroanthranilic acid (**27**, 400mg, 1.94 mmol) in anhydrous THF (30 ml), MnO_2 (400mg, 10.82 mmol), give pure (**33**). Yield= 85%, ^{1}HNMR ($CDCl_3$): δ 9.80 (s, 1H, CHO), 7.44 (d, 1H, J=2.4 Hz aromatic), 7.41 (d, 1H, J=2.4 Hz aromatic), 6.61 (br s, 2H, NH_2).

2-Amino-3, **5-dichlorobenzaldoxime**, **2e;** by using same procedure described in compound **1e**, hydroxylamine hydrochloride (136.98 mg, 2 mmol) in water (1 ml), 2-Amino-3,5-dichlorobenzaldehyde (**33**, 190mg, 1mmol) in ethanol (19 ml), K_2CO_3 (276 mg, 2mmol), give pure **2e**.^{1}HNMR ($CDCl_3$): δ 8.15 (s, 1H, CH=N), 7.27 (d, 1H, J=2.4Hz, aromatic), 7.03 (d, 1H, J=2.4, aromatic), 6.01 (brs, 2H, NH_2). MS: m/z; 205(M^+), 207(M^{+2}), 208(M^{+4}), 204(base peak), 188, 174. (C, H, N) calculated (40.9, 2.92, 13.65), analytical (40.7, 2.89, 13.5).

2-Amino-3, **5-diiodobenzaldehyde**, **34;** by using same procedure described in compound **32**, LiALH4 ((196.89mg, 5.19mmol), 3, 5-diiodoanthranilic acid (**28**, 1 g, 3.461mmol) in anhydrous THF (30 ml), MnO_2 (455mg, 12.32mmol), give pure (**34**). Yield= 90%, ^{1}HNMR ($CDCl_3$): δ 9.67 (s, 1H, CHO), 8.10 (d, 1H, J=2Hz, aromatic), 7.98 (d, 1H, J=2Hz, aromatic), 7.14 (br s, 2H, NH_2).

2-Amino-3, **5-diiodobenzaldoxime**, **3e;** by using same procedure described in compound **1e**, hydroxylamine hydrochloride (100mg, 0.7mmol) in water (1 ml),2-amino-3,5-diiodobenzaldehyde (**34**, 131mg, 0.352mmol) in ethanol (19 ml), K_2CO_3 (97.29mg, 0.704mmol), give pure **3e**.^{1}HNMR (DMSO): δ 11.37 (s, 1H, OH), 8.19 (s, 1H, CH=N), 7.83 (d, 1H, J= 2 Hz, aromatic), 7.58 (d, 1H, J= 2Hz, aromatic), 6.61 (brs, 2H, NH_2).MS: m/z; 388($M^{+)}$, 389(M^{+1}), 375(base peak,100%), 371, 357, 261, 244, 134. (C, H, N) calculated (21.6, 1.55, 7.2), analytical (21.7, 1.58, 7.2).

2-Amino-3, **5-dibromobenzaldoxime**, **4e;** by using same procedure described in compound **1e**, hydroxylamine hydrochloride (399mg, 5.7mmol) in (1 ml) 2-amino-3,5-dibromobenzaldehyde (**35**, 500mg, 1.79mmol) in ethanol (19 ml), K_2CO_3 (397.35mg, 2.87mmol), give pure **4e**. ^{1}HNMR: (DMSO): δ 11.43 (s, 1H, OH), 8.27 (s, 1H, CH=NH), 7.59 (d, 1H, J=2.2Hz, aromatic), 7.49 (d, 1H, J= 2.2Hz, aromatic), 6.68 (brs, 2H, NH_2).MS: m/z = 292(M^+),294(M^{+2}), 296 (M^{+4}), 278, 277(base peak,100%),280, 215, 198. (C, H, N) calculated (28.66, 2.04, 9.55), analytical (28.4, 1.99, 9.55).

3, **5- Dimethoxybenzaldoxime: (1f):** 0.660 g of NaOH and 0.714 g of hydroxylamine hydrochloride were dissolved in little amount of water (2 ml) added to 1.000 g (6.020 mmol) of 3, 5-dimethoxybenzaldahyde **36** dissolved in 10 ml of ethanol. The mixture had been left under magnetic agitation at room temperature for 18 hrs before it was dried under vacuum, then diluted with water and acidified to pH = 5 with HCl 1N, precipitate was filtered, collected and dried. The white solid was purified by recrystallization from EtoAc/ Hexane to give pure **1f**. m.p: 116-119°C, M.W: 181. IR: 1567 cm^{-1} (C=N).^{1}H-NMR (DMSO): δ 11.23(s,1H,OH), 8.05(s,1H,CH=N), 6.75(s,2H,Ar), 6.50(s,1H,Ar), 3.74(s,6H, 2OCH_3). M.S: m/z, 181(M^+, base peak), 182(M^{+1}), 164(-OH).Elemental analysis: (C, H, N) calculated (59.66, 6.07, 7.73), analytical (59.6, 6.04, 7.70).

3, **5- Dichlorobenzaldoxime: (2f):**By using same procedure described in compound **1f**, 0.120 g (3 mmol) of NaOH and 0.135 g of hydroxylamine hydrochloride in 0.2 ml of water, 0.200 g (1.142 mmol) of 3, 5-dichlorobenzaldahyde **37** in 6 ml of ethanol, give pure **2f**. m.p: 107-109°C, M.W: 190.IR: 1565, 45 cm^{-1} (C=N),^{1}H-NMR (DMSO): δ 11.67(s, 1H, OH), 8.15(s, 1H, CH=N), 7.62(s, 3H, Ar).M.S: m/z, 190(M^+, base peak), 192(M^{+2}), 194(M^{+4}), 154(-Cl), 146. (C, H, N) calculated (44.2, 2.3, 7.4), analytical (44.3, 2.3, 7.5).

Bis (3, **5-dichlorosalicyl) succinate (1h):**1.23 ml (9.66 mmol) of N,N-dimethylaniline and 0.26 ml (2.415 mmol) of succinylchlorid was added drop by drop to 1.00 g (4.83 mmol) of 3,5-dihclorosalicylic acid **38** in 30 ml of toluene .The solution has been left under magnetic agitation at room temperature for 4 days. Acidic water and AcOEt was added to the reaction mixture at 0°C, water layer removed and the organic layer washed with water many times then evaporated, obtained residue treated with triturate AcOEt until gave white solid which purified by recrystallization (ethanol) to give pure 1h. m.p: 175-178°C,^{1} H-NMR (DMSO-d_6): δ 3.03 (s, 4H, -CH_2-CH_2-), 7.89 (d, 2H, J= 2.6 Hz, Ar), 8.07 (d, 2H, J= 2.6 Hz, Ar).M.S: m/z, 496(M^+), 206(base peak) 160. (C, H, N) calculated (43.5, 2.01, 0), analytical (43.7, 2.03, 0).

Bis (3, **5-dibromosalicyl) succinate (2h):** By using same procedure described in compound **1h**, 0.86 ml (6.765 mmol) of N,N-dimethylaniline and 0.185 ml (1.689 mmol) of succinylchlorid,1.00 g (3.378 mmol) of 3,5-dibromosalicylic acid **39** in 30 ml of toluene, give pure **2h**. m.p: 195-198°C ^{1} H-NMR (DMSO-d_6): δ 3.02 (s, 4H, -CH_2-CH_2-), 8.04 (d, 2H, J= 2.2 Hz), 8.27 (d, 2H, J= 2.2 Hz). M.S: m/z, 674(M^+), 296 (base peak), 278($C_7H_3Br_2O_2$), 250. (C, H, N) calculated (32, 1.4, 0), analytical (32.1, 1.5, 0).

Bis (3, **5-diiodosalicyl) succinate (3h):** By using same procedure described in compound **1h**, 0.3 ml (2.564 mmol) of N,N-dimethylaniline and 0.071 ml (0.641 mmol) of succinylchlorid,0.500 g (1.282 mmol) of 3,5-diiodosalicylic acid **40** in 15 ml of toluene . Reaction mixture stirred for 1days, give pure **3h**. m.p: 209-211°C ^{1}H-NMR (DMSO-d_6): δ 3.01 (s, 4H, CH_2CH_2), 8.17 (d, 2H, J=2.0 Hz, Ar), 8.45 (d, 2H, J=2.0 Hz, Ar). M.S: m/z, 862(M^+), 815(base peak), 418($C_8H_4O_2I_2$), 390, 292. (C, H, N) calculated (25.05, 1.1, 0), analytical (25, 1.1, 0).

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