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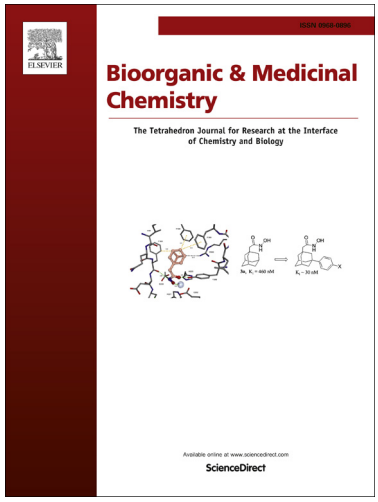

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Carbonic anhydrase inhibitors: Synthesis, molecular docking, cytotoxic and inhibition of the human carbonic anhydrase isoforms I, II, IX, XII with novel benzenesulfonamides incorporating pyrrole, pyrrolopyrimidine and fused pyrrolopyrimidine moieties.

Mostafa M. Ghorab*[a,b], Mansour S. Alsaid[a], Mariangela Ceruso [c], Yassin M. Nissan[d] and Claudiu T. Supuran*[c,e]

[a]Pharmacognosy Department, College of Pharmacy, King Saud University, P. O. Box 2457, Riyadh 11451, Saudi Arabia
[b]Drug Radiation Research Department, National Center for Radiation Research & Technology, Atomic Energy Authority, Cairo, Egypt.
[c]Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Firenze), Italy.
[d]Pharmaceutical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr Al- Aini st., P.O.Box 11562, Cairo, Egypt.
[e] Università degli Studi di Firenze, Polo Scientifico, Dipartimento Neurofaba; Sezione di Scienze Farmaceutiche e Nutraceutiche, Via Ugo Schiff 6, 50019 Sesto Fiorentino (Firenze), Italy.

*Corresponding authors: M.M. Ghorab, Professor of Applied Organic Chemistry, Pharmacognosy Department, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia. Tel.: +966 534292860; Fax: +966 01 4670560. E-mail address: mmsghorab@yahoo.com; and C.T. Supuran: Tel./Fax: +39-055-4573005;
E-mail: claudiu.supuran@unifi.it.

Abstract

A series of novel pyrroles, pyrrolopyrimidines, pyrazolopyrrolopyrimidine, triazolopyrrolopyrimidines**,** tetrazolopyrrolopyrimidine, triazinopyrrolopyrimidines and pyrrolopyrimidotriazepines bearing the biologically active benzenesulfonamide moiety were synthesized by using pyrrole-*o*-amino-carbonitrile as key intermediate. All the synthesized compounds were evaluated for their *in vitro* carbonic anhydrase (CA, EC 4.2.1.1) inhibitory effects against the human (h) isoforms hCA I, II, IX and XII. Among the tested derivatives, compounds **16**, **18** and **20-24** showed potent activity as inhibitors for the tumor associated transmembrane isoforms (hCA IX and XII) in the nanomlar and subnanomolar range, with high selectivity. All compounds underwent cytotoxic activity assays on human breast cancer cell line (MCF-7) showing effective activity, comparable to that of the clinically used drug Doxorubicin.

Key words: Pyrrolopyrimidines, sulfonamide, cytotoxic activity, carbonic anhydrase inhibitors, molecular docking.

1. Introduction

Several pyrrole and fused pyrrole derivatives have been reported as biologically active compounds with variant activities[1-6] especially anticancer activity.[7-10] In addition, sulfonamides constitute an important class of drugs with several types of pharmacological activities, including antibacterial[11,12], antiviral [13], anti-inflammatory [14] and anticancer activity.[15,16] On the other hand, pyrimidines and its fused derivatives play an essential role in several biological processes. [17-23] Also, the potential use of aromatic/ heterocyclic sulfonamides as carbonic anlydrase (CA, EC 4.2.1.1) inhibitors [24-29] has been only recently explored to date for the treatment/imaging of hypoxic tumors, considered the second cause of death for the time being.[30,31]

Fifteen isoforms of human carbonic anhydrase have been discovered, among them the transmembrane tumor associated isoforms (hCA IX and hCA XII) are over expressed in several types of tumors. Many sulfonamide derivatives have been investigated for their CA inhibition activity seeking for selective hCA IX and hCA XII inhibitors since the lack of selectivity is the major challenge for the wide use of chemotherapeutic agents in cancer therapy.[24-29]Additionally, 5,7-diphenyl-pyrrolo[2,3-d]pyrimidines were described as potent cytotoxic agents.[32-34]

In the lights of the previous facts and based on our previous work in the field of design and synthesis of sulfonamide based molecules as cytotoxic agents [35-38], we herein report the results achieved from the design and synthesis of novel pyrrole, pyrrolo[2,3-*d*]pyrimidine and fused pyrrolopyrimidine derivatives carrying a biologically active benzenesulfonamide moiety. All the newly synthesized compounds were subjected to cytotoxic screening against the breast cancer cell line MCF-7. To explore these derivatives prospective mechanism of action, assessment of their *in vitro* enzyme-inhibitory capacity against hCA I, II, IX and XII was performed and compared with the well known CAI acetazolamide (AZA).[39] Finally, molecular docking of all compounds in the active site of hCA XII was carried out in an attempt to speculate the possible binding mode of these molecules in the active site of their target enzyme.

2. Results and Discussion

2.1. Chemistry

The aim of this work was to design, synthesis some novel pyrrole, pyrrolopyrimidine and fused pyrrolopyrimidine derivatives with the biologically active benzenesulfonamide moiety

and evaluate their carbonic anhydrase inhibition as well as their cytotoxic activity. Thus, interaction of sulfanilamide **1** with 2-bromo-1-(4-bromophenyl) ethanone **2** furnished the corresponding 4-(2-oxo-2-(bromophenyl) ethylamino) sulfonamide **3**. The reactivity of compound **3** towards the active methylene compound, namely, malononitrile, was investigated. When compound **3** was refluxed with malononitrile in ethanol containing sodium ethoxide, the pyrrole derivative **5** was produced (**Scheme 1**). The formation of compound **5** was assumed to proceed via condensation of **3** with malononitrile to give the intermediate **4** followed by intramolecular cyclization to give pyrrole derivative **5**. Refluxing of compound **5** with triethylorthoformate neat or in presence of acetic anhydride gave compound **6**. When compound **6** was refluxed with hydrazine hydrate in absolute ethanol the corresponding *N*-amino-imino derivative **8** was obtained through the formation of intermediate **7**. Interaction of compound **8** with malononitrile in ethanol in presence of sodium ethoxide afforded the pyrazolo derivative **9** in good yield. On the other hand, 1, 2, 4-triazole derivatives **10**-**12** were obtained directly by heating under reflux of **8** with diethyl oxalate or diethylmalonate and/or ethylacetoacetate. Interaction of compound **8** with an equimolar ratio of ethyl chloroformate gave the corresponding 1,2,4-triazole derivative **14** through the formation of intermediate **13** followed by loss 1 mole of ethanol. Also, when compound **8** was subjected to the diazotization reaction condition, the tetrazolo derivative **15** was formed. Reaction of compound **8** with ethyl chloroacetate in refluxing sodium ethoxide yielded the triazino derivative **16** rather than its isomeric structure **17** (**Scheme 2**). Structure **16** was suggested rather than structure **17**, based on the assumption that the reaction basic condition allowed proceeding through formation of sodium salt on the less basic NH and elimination of sodium chloride followed by cyclization. In addition the IR spectrum of the isolated product showed band at 1654 cm^{-1} which was at less frequency than that expected for structure **17**. Further evidence was the ^{1}H-NMR spectrum which showed a singlet at 4.7 ppm for the methylene protons.

Treatment of compound **8** with oxalyl chloride in refluxing dry benzene afforded the corresponding dioxotriazine derivative **18**. In contrast, in our hands when the *N*-amino-imino **8** was allowed to react with acetylacetone the corresponding triazepino derivative **19** was obtained. On the other hand, the reaction of **8** with ethoxymethylenemalononitrile or ethyl ethoxymethylenecyanoacetate yielded the corresponding triazepine aminocarbonitrile **20** and aminoester **21**, respectively.

Conversely, condensation of the key compound **8** with isatin in refluxing ethanol gave the corresponding cyclocondensation product indeno-1,2,4-triazine derivative **22** (**Scheme 3**). Also, it was condensed with *N*-acetyl isatin to give the condensation product **23** in good yield. It is noteworthy that when the reaction was carried out with **8** and benzoylacetone, the product was identified as the azomethine compound **24** which could be cyclized to the corresponding triazepine **25** by heating in refluxing phosphoryl chloride (**Scheme 3**).

2.2. Carbonic anhydrase inhibition

The CA inhibitory ability of all the synthesized compounds was measured against the cytosolic isoforms hCA I and II as well as the membrane associated isoforms hCA IX and XII using stopped flow assay method and the results are displayed in **Table 1.** The well known carbonic anhydrase inhibitor acetazolamide (AZA) was used as the reference drug for this assay.[39] Data obtained from the CA inhibition assay led to the following inferences.

(i) The cytosolic isoform hCA I was moderately inhibited by the sulfonamide derivatives with k_i values in the range of 21.6- 498 nM. The most active compounds against hCA I were **8**, **14**, **23**, **19**, **11**, **25** and **15** arranged in a descending order from the most active to the least with k_i values less than 30nM. However, all the synthesized compounds were more active than AZA except compound **22** with k_i value 498 nM.

(ii) With regards to the profiling assay against hCA II, all compounds were active with k_i values in the range of 3.2-123.7 nM. Compounds **6**, **8**, **24**, **14, 5**, **10**, **9**, **16**, **20**, **15**, **23**, **25**, **21** and **22** were the most active compounds arranged in a deseeding order from the most active to the least with k_i values less than 9 nM being more active than AZA while compounds **11**, **12**, **18** and **19** were less active than AZA.

(iii) The tumor associated target isoform hCA IX was highly inhibited by sulfonamides **9**, **14**, **8**, **10**, **22**, **15**, **24**, **11**, **21**, **23**, **25**, **6** and **5** (arranged in a descending manner with respect to inhibition efficiency) showing k_i values lower than that of AZA. On the other hand, only three compounds **16**, **18** and **19** were less active than AZA.

(iv) Finally, excellent results were displayed by eight of the novel sulfonamides (**22, 5, 16**, **23**, **21**, **20**, **18** and **24** arranged in a desending order) in the profiling assay performed against the transmembrane, tumor associated isozyme hCA XII where they were potent in a subnanomolar concentration being 6 to 8.5 fold more active than AZA against the target CA isoform. Moreover, compounds **19** and **25** showed k_i values of 5.4 and 3.4 nM, respectively yet still more active than AZA. The other synthesized derivatives were less active than AZA.

Selectivity of the newly synthesized compounds towards carbonic anhydrase isoforms received great importance in order to reduce the unwanted side effects of using such candidates as anticancer agents as the cystolic isoforms hCA I and hCA II inhibition will lead to potential diueresis. Selectivity index (SI) was calculated for each compound as hCA I/ hCA XII and hCA II/ hCA XII to measure their selectivity towards the transmembranal tumor associated isoform hCA XII. Compounds **5**, **6**, **18** and **20-22** showed high selectivity for hCA XII than hCA I especially compound **22** with SI value more than 691. On the other hand, compound **18** showed high selectivity for hCA XII than hCA II with SI value more than 356.

2.3. *In vitro* cytotoxic activity

The newly synthesized compounds were evaluated for their *in vitro* cytotoxic activity against human breast cancer cell line (MCF7). Doxorubicin which is one of the most effective anticancer agents was used as the reference drug in this study. The relationship between surviving fraction and drug concentration was plotted to obtain the survival curve of breast cancer cell line (MCF7). The response parameter calculated was the IC_{50} value, which corresponds to the concentration required for 50% inhibition of cell viability. (**Table 2**) shows the *in vitro* cytotoxic activity of the synthesized compounds, where all compounds exhibited significant activity compared to doxorubicin as reference drug. All the synthesized compounds showed promising cytotoxic activity especially compounds **5-10, 14, 15, 19** and **21- 25** with better activity than Doxorubicin as positive control, while compounds **16** and **20** showed comparable activity to that of doxorubicin with IC_{50} value of 8.11 µM for each. On the other hand, compounds **11, 12** and **18** were slightly less active than doxorubicin.

The strategic starting pyrrole derivative **5** showed better activity than doxorubicin with IC_{50} value of 7.56 µM. Upon reaction of the amino group to form the imino derivative **6** the activity remained the same with no change. Cyclization of **6** to the imino-amino pyrrolopyrimidine derivative **8** had a good effect on activity as it increased to reach 7.29 µM. Adding another five-member ring to the pyrrolopyrimidine derivative **8** as in compounds **9-12**, **14** and **15** did not improve the activity with IC_{50} values of 8.94, 7.29, 7.56, 8.94, 7.56 and 7.84 µM for compounds **9-12**, **14** and **15**, respectively. The most active compound of this series was the triazolopyrrolopyrimidine derivative with an ethyl ester substituent **10** with IC_{50} value of 7.29 µM. The other cylization was performed to get an additional six member ring as in compounds **16** and **18** which did not also improve the activity with IC_{50} values of 8.11 and 8.66 µM, respectively. On our attempt to study the effect of other cyclization, seven member ring

derivatives were also synthesized and investigated for cytotoxic activity represented by compounds **19, 20, 21** and **25**. The IC_{50} values for these derivatives were 7.84, 8.11, 7.84 and 7.29 µM, respectively. The most active compound of this series was compound **25** with IC_{50} value of 7.29 µM. The final cyclization attempt was performed to get the benzopyrrolotriazine derivative **22** which showed IC_{50} value of 7.84 µM.

Moreover, two hydrazone derivatives **23** and **24** were evaluated for their cytotoxic activity with IC_{50} values of 7.56 and 7.84, respectively.

In the light of the previous biological results, we can retrieve that: the pyrrolo derivative **5** exhibited both good CA inhibitory activity and cytotoxic activity with good selectivity for inhibiting hCA XII over the other isoforms, whereas the formation of the Schiff's base derivative **6** has decreased the hCA inhibition activity. Cyclization to pyrrolopyrmidine derivative **8** also tends to decrease the activity on hCA IX and XII. However, the fused triazinopyrrolopyrimidine derivatives **16**, **18** and **24** showed potent activity towards hCA XII, in the subnanomolar range, with cytotoxic activity comparable to that of doxorubicin. Fusion of the pyrrolopyrimidine moiety with five membered rings such as pyrazole, triazole and tetrazole, in compounds **9, 10-12, 14** and **15**, respectively, tends to decrease activity towards hCA XII while still preserving good cytotoxic activity. On the other hand, fusion of pyrrolopyrimidine with triazepine moiety as in compounds **19, 20, 21** and **25** was better for hCA XII inhibitory activity, especially for **20** and **21**, which showed a subnanomolar range of activity, with cytotoxic activity comparable to doxorubicin. Fusion of the pyrrolopyrimidine scaffold with triazinoindole or substitution with an indole moiety as in compounds **23** and **22**, respectively, again was beneficial to hCA inhibitory activity with even better cytotoxic activity than doxorubicin.

2.4. Molecular Docking Results

It was of interest to perform molecular docking for the newly synthesized compounds, in order to rationalize their promising biological activity. The protein data bank file (PDB ID: 1JD0) was selected for this purpose. The file contains hCA XII co-crystallized with acetazolamide. Docking on the active site of hCA XII was performed for all synthesized sulfonamide derivatives **5, 6, 8-12, 14-16** and **18-25**. Acetazolamide interacts with the active site of hCA XII by two hydrogen bonds between Thr199 and the SO_2 group, one hydrogen bond between Thr200 and N3 of the thiadiazole ring, a water mediated hydrogen bond between Pro201 and N4 of the thiadiazole ring, and finally a metal complex between the Zn(II) ion, His94, His96

and His119 and the SO_2NH_2 group of AZA (**Figure 1**). All synthesized compounds were docked to the active site of hCA XII with good energy scores (S) supporting the observed activity of these sulfonamides as CA XII inhibitors. Energy scores (S) and amino acid interactions of the newly synthesized compounds were listed in **Table 3**.

Docking scores for the newly synthesized compounds fall in the range of (-12.7252 to -52.7571 Kcal/mol). The best docking score was assigned to compound **16** (-52.7571 Kcal/mol). On the other hand, all of the newly synthesized compounds were capable of forming a metal complex with the Zn ion through His 94, His 96 and His 119 in a similar manner to the co-crystallized ligand (AZA). It was observed from the amino acid interactions of the newly synthesized compounds that interactions with Thr 199 were achieved only by compounds **5**, **16, 18** and **20-24** in a very comparable way to that of AZA while the rest of compounds giving lower activity in the hCA XII inhibition assay failed to show interaction with this amino acid in the docking study.

In a further look to the docking results side by side to CA XII inhibition results, we can observe that the most active compounds (**5**, **16, 18** and **20-24**) have shown excellent docking scores (-41.3027, -52.7571, -51.5937, -49.5079, -46.6627, -41.5627, -40.2157 and -44.9134, respectively) in addition to their interactions with Thr 199 by at least one hydrogen bond which can give us a simulating illustration of their receptor interaction with active site of CA XII. Amino acid interactions of the most selective compounds (**16** and **18**) are illustrated in **Figures 2** and **3**, respectively.

3. Conclusions

A series of pyrroles, pyrrolopyrimidines, pyrazolopyrrolopyrimidine, triazolopyrrolopyrimidines**,** tetrazolopyrrolopyrimidine, triazinopyrrolopyrimidines and pyrrolopyrimidotriazepines bearing the biologically active benzenesulfonamide moiety were designed and synthesized as potential CAIs**.** The carbonic anhydrase inhibitory activity of these compounds was assessed against the human cytosolic isoforms hCA I and hCA II as well as the transmembrane isoforms hCA IX and hCA XII which are over expressed in various types of tumors and hence are valid targets for antitumor agents. The tested sulphonamides showed variable degrees of activities against all four evaluated hCA isoforms some being more potent than the well known CAI AZA. All of the newly synthesized compounds were more potent than AZA against the tumor associated isoform hCA IX except **18** and **19**. Most of the synthesized compounds were more potent than AZA against the tumor associated isoform hCA XII particularly, **5**, **16, 18, 20-24** exhibiting subnanomolar K_i values. These new sulfonamides

also displayed high selectivity towards the tumor associated isoform hCA XII as displayed by their hCA I/XII and hCA II/XII SI. Docking of all compounds was performed with hCA XII, and enzyme-ligand binding poses obtained displayed interactions in a fashion comparable to that of AZA especially for compounds **5**, **16, 18, 20-24**. Cytotoxic activity of the newly synthesized compounds showed better or comparable results to the reference drug Doxorubicin. These results merit further investigation of these new sulfonamides as potential antitumor agents.

4. Experimental

4.1. Chemistry

Melting points (^{o}C, uncorrected) were determined in open capillaries on a Gallenkemp melting point apparatus (Sanyo Gallenkemp, Southborough, UK) and were uncorrected. Pre coated silica gel plates (silica gel 0.25 mm, 60 G F254; Merck, Germany) were used for thin layer chromatography, dichloromethane/methanol (9.5:0.5) mixture was used as a developing solvent system and the spots were visualized by ultraviolet light and/or iodine. Infra-red spectra were recorded in KBr discs using IR-470 Shimadzu spectrometer (Shimadzu, Tokyo, Japan). NMR spectra (in DMSO-d_6) were recorded on Bruker AC-500 Ultra Shield NMR spectrometer (Bruker, Flawil, Switzerland, δ ppm) at 500 MHz using TMS as internal Standard and peak multiplicities are designed as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Elemental analyses were performed on Carlo Erba 1108 Elemental Analyzer (Heraeus, Hanau, Germany).

4.1.1. 4-(2-(4-Bromophenyl)-2-oxoethylamino)benzenesulfonamide 3

A mixture of sulfanilamide **1** (1.72 g, 0.01 mol.) and 2-bromo-1-(4-bromophenyl)ethanone **2** (2.77 g, 0.01 mol.) was refluxed in *N,N'*-dimethylformamide (20 mL) in the presence of catalytic amount of triethylamine for 6 h. The solid obtained was filtered off and recrystallized from ethanol to give **3**. Yield 89%, m.p. 232.6 ^{o}C, IR:υ_{max}./cm^{-1} 3358, 3255 (NH, NH_2), 3100 (CH arom.), 2970, 2863 (CH aliph.), 1685 (C=O), 1381, 1157 (SO_2).^{1}H-NMR (DMSO-d_6, D_2O) δ: 4.7 (s, 2H, CH_2), 6.6 (s, 1H, NH, D_2O exchangeable), 6.7, 7.5 (2d, 4H, Ar-H, AB system, *J*=7.1 Hz), 7.8, 8.0 (2d, 4H, Ar-H, AB system, J=6.9 Hz).^{13}C-NMR (DMSO-d_6) δ : 49.4, 111.4 (2), 127.1, 127.7, 129.9 (2), 130.7 (2), 131.8 (2), 133.9, 150.8, 195.3.Anal. Calcd. for $C_{14}H_{13}BrN_2O_3S$ (369.23): C, 45.54; H, 3.55; N, 7.59. Found: C, 45.54; H, 3.31; N, 7.24.

4.1.2. 4-(2-Amino-4-(4-bromophenyl)-3-cyano-1*H*-pyrrol-1- yl)benzenesulfonamide 5

A mixture of compound **3** (3.69 g, 0.01 mol.) and malononitrile (0.66 g, 0.01 mol.) in ethanol (20 mL) containing sodium ethoxide (0.5 g) was refluxed for 8 h. The reaction mixture was cooled and acidified with dil. HCl. The solid obtained was filtered off and recrystallized from dioxane to give **5**. Yield 78%, m.p. 221.2 ^{o}C, IR:υ_{max}./cm^{-1} 3419, 3344, 3238 (NH_2), 3095 (CH arom.), 2187 (C≡N), 1342, 1176 (SO_2).^{1}H-NMR (DMSO-d_6, D_2O) δ: 6.1 (s, 2H, NH_2, D_2O exchangeable), 7.0 (s, 1H, CH pyrrole), 7.5- 7.9 (m, 10H, Ar-H+SO_2NH_2).^{13}C-NMR (DMSO-d_6) δ : 70.5, 113.5, 117.5, 119.5, 121.3 (2), 125.2, 127.3, 131.2 (2), 131.6 (2), 132.3 (2), 139.5, 142.9, 148.5. Anal. Calcd. for $C_{17}H_{13}BrN_4O_2S$ (417.28): C, 48.93; H, 3.14; N, 13.43. Found: C, 48.71; H, 3.50; N, 13.16.

4.1.3. Ethyl *N*-4- (4-bromophenyl)-3-cyano-1-(4-sulfamoylphenyl)-1*H*-pyrrol-2-yl)-form-imidate (6)

A mixture of compound **5** (4.17 g, 0.01 mol.) and triethylorthoformate (20 mL) was refluxed for 6 h. The reaction mixture was cooled and then poured onto ice/water. The formed residue was recrystallized from ethanol to give **6**. Yield 78%, m.p. 120.2 ^{o}C, IR:υ_{max}./cm^{-1} 3151, 3136 (NH_2), 2987, 2865 (CH aliph.), 2206 (C≡N), 1629 (C=N), 1354, 1155 (SO_2). ^{1}H-NMR (DMSO-d_6, D_2O) δ: 1.0 (t, 3H, CH_3), 4.3 (q, 2H, CH_2), 7.6- 7.9 (m, 8H, Ar-H), 8.1 (s, 1H, CH pyrrole), 8.5 (s, 2H, SO_2NH_2, D_2O exchangeable), 8.7 (s, 1H, N=CH). ^{13}C-NMR (DMSO-d_6) δ: 13.6, 63.6, 79.1, 116.8, 117.4, 120.3, 122.8 (2), 125.4, 127.7, 127.8 (2), 131.5 (2), 131.8 (2), 138.5, 140.6, 145.3, 161.9. Anal. Calcd. for $C_{20}H_{17}BrN_4O_3S$ (473.34): C, 50.75; H, 3.62; N, 11.84. Found: C, 50.48; H, 3.91; N, 11.54.

4.1.4. 4-(3-Amino-5-(4-bromophenyl)-4-imino-3,4-dihydropyrrolo[2,3-d]pyrimidin-7-yl)b-enzenesulfonamide (8)

A mixture compound **6** (4.73 g, 0.01 mol) and hydrazine hydrate (1 g, 0.02 mol) was stirred in ethanol at room temperature for 1h, the solid formed was filtered, and recrystallized from ethanol to give **8**. Yield % 88, m.p. 184.5^{o}C; IR (KBr, cm^{-1}): 3331, 3296, 3230, 3132 (NH, NH_2), 1637 (C=N), 1328, 1165 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 5.6 [s, 2H, NH_2, D_2O-exchangeable], 7.3- 7.8 [m, 9H, Ar-H + NH], 7.9 [s, 1H, CH pyrimidine], 8.0 [s, 2H, SO_2NH_2, D_2O-exchangeable], 8.1 [s, 1H, CH pyrrole]. ^{13}C-NMR (DMSO-d_6) δ: 103.8, 119.8, 120.1, 121.1 (2), 124.0, 126.7, 130.8 (2), 131.1 (2), 132.9 (2), 139.6, 141.9, 142.7, 147.6, 153.0. Anal. Calcd for $C_{18}H_{15}BrN_6O_2S$ (459.32): C, 47.07; H, 3.29; N, 18.30. Found: C, 47.29; H, 3.56; N, 18.66.

4.1.5. 4-(8-Amino-9-cyano-1-(4-bromophenyl)-3*H*-pyrazolo[1,5-c]-pyrrolo[3,2-e]pyrimidin-3-yl)benzenesulfonamide (9)

A mixture of **8** (0.459 g, 0.01 mol) and malononitrile (0.079 g, 0.0012 mol) in ethanol (20 mL) containing sodium ethoxids (0.023 g, 0.01 mole) was refluxed for 6h. The reaction mixture was cooled and acidified with diluted HCl. The obtained residue was filtered and recrystallized from dioxane to give **9**. Yield % 73, m.p. 237.8 ^{o}C; IR (KBr, cm^{-1}): 3386, 3350, 3210 (NH_2), 2198 (C≡N), 1591 (C=N), 1325, 1161 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 4.2 [s, 2H, NH_2, D_2O-exchangeable], 7.2- 8.1 [m, 10H, Ar-H + SO_2NH_2], 8.1 [s, 1H, CH pyrrole], 8.6 [s, 1H, CH pyrimidine]. ^{13}C-NMR (DMSO-d_6) δ: 101.4, 109.6, 116.5, 120.5, 125.4 (2), 126.8, 128.9 (2), 129.3, 130.7 (2), 131.5, 131.9 (2), 132.2, 139.1, 140.6, 142.2, 150.0, 154.4. Anal. Calcd. for $C_{21}H_{14}BrN_7O_2S$ (508.35): C, 49.62; H, 2.78; N, 19.29. Found: C, 49.31; H, 2.48; N, 19.55.

4.1.6. Ethyl 9-(4-bromophenyl)-7-(4-sulfamoylphenyl)-7*H*-pyrrolo[3,2-e][1,2,4]triazolo-[1,5-c]pyrimidine-2-carboxylate (10)

A mixture of **8** (4.59 g, 0.01 mol) and diethyloxalate (5 mL) was refluxed for 20h. The reaction mixture was then concentrated at reduced presure and left to cool. The solid product formed was filtered off and recrystallized from ethanol to give **10**. Yield % 82, m.p. 309.2 ^{o}C; IR (KBr, cm^{-1}): 3340, 3255 (NH_2), 3076 (CH arom.), 2926, 2861 (CH aliph.), 1728 (C=O), 1622 (C=N), 1336, 1163 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 1.3 [t, 3H, CH_3], 4.3 [q, 2H, CH_2], 7.4 [s, 1H, CH pyrrole], 7.5- 8.1 [m, 10H, Ar-H + SO_2NH_2], 9.8 [s, 1H, CH pyrimidine]. ^{13}C-NMR (DMSO-d_6) δ: 14.0, 62.6, 112.7, 119.6, 124.5 (2), 124.8, 126.9 (2), 129.4, 130.6 (2), 133.6 (2), 137.3, 138.1, 142.8, 143.2, 153.7, 155.2, 157.3, 161.3. Anal. Calcd for $C_{22}H_{17}BrN_6O_4S$ (541.38): C, 48.81; H, 3.17; N, 15.52. Found: C, 48.58; H, 3.51; N, 15.19.

4.1.7. Ethyl 2-(9-(4-bromophenyl)-7-(4-sulfamoylphenyl)-7*H*-pyrrolo[3,2-e][1,2,4]triazolo[1,5-c]pyrimidin-2-yl)acetate (11)

A suspension of compound **8** (4.59 g, 0.01 mol) and diethylmalonate (10 mL) was heated under refluxed for 12h. The obtained product was filtered and recrystallized from dioxane to give **11**. Yield % 59, m.p. 268.3 ^{o}C; IR (KBr, cm^{-1}): 3348, 3234 (NH_2), 3066 (CH arom.), 2987, 2876 (CH aliph.), 1716 (C=O), 1625 (C=N), 1363, 1157 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 1.2 [t, 3H, CH_3 ethyl], 4.0 [s, 2H, CH_2], 4.1 [q, 2H, CH_2 ethyl], 7.5 [s, 1H, CH pyrrole], 7.6-8.3 [m, 10H, Ar-H + SO_2NH_2], 9.2 [s, 1H, CH pyrimidine]. ^{13}C-NMR (DMSO-d_6) δ: 14.0, 38.9, 60.8, 103.6, 117.0, 124.6 (2), 124.8, 126.8 (2), 129.2, 131.3 (2), 131.7 (2), 136.2, 139.2,

141.3, 142.7, 149.0, 152.9, 160.9, 168.6. Anal. Calcd for $C_{23}H_{19}BrN_6O_4S$ (555.40): C, 49.74; H, 3.45; N, 15.13. Found: C, 49.39; H, 3.12; N, 15.48.

4.1.8. 4-(2-Acetylmethyl-9-(4-bromophenyl)-7*H*-pyrrolo[3,2-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)benzenesulfonamide (12)

A mixture of **8** (4.59 g, 0.01 mol) and ethylacetoacetate (2.60 g, 0.02 mol) was heated at 220^{o}C in an oil bath for 4h. After cooling the product was collected by filteration and recrystallized from ethanol to give **12**. Yield % 75, m.p. 320.0 ^{o}C; IR (KBr, cm^{-1}): 3390, 3282 (NH_2), 3074 (CH arom.), 2961, 2846 (CH aliph.), 1710 (C=O), 1624 (C=N), 1336, 1163 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 2.1 [s, 3H, $COCH_3$], 4.1 [s, 2H, CH_2], 7.5 [s, 1H, CH pyrrole], 7.6- 8.2 [m, 10H, Ar-H + SO_2NH_2], 9.4 [s, 1H, CH pyrimidine]. ^{13}C-NMR (DMSO-d_6) δ: 30.9, 49.1, 103.4, 117.0, 119.9 (2), 124.0, 124.4 (2), 126.7, 129.2 (2), 131.2 (2), 135.8, 139.2, 142.5, 145.6, 149.3, 152.8, 163.5, 205.3. Anal. Calcd for $C_{22}H_{17}BrN_6O_3S$ (525.38): C, 50.29; H, 3.26; N, 16.00. Found: C, 50.56; H, 3.48; N, 16.33.

4.1.9. 4-(2-Carboethoxy-9-(4-bromophenyl)-7*H*-pyrrolo[3,2-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)benzenesulfonamide (14)

A mixture of **8** (4.59 g, 0.01 mol) and ethylchloroformate (1.4 g, 0.015 mole) in benzene (20 mL) was refluxed for 8h. The reaction mixture was then filtered off and recrystallized from ethanol to give **14**.

Yield % 82, m.p. 229.2 ^{o}C; IR (KBr, cm^{-1}): 3241, 3374 (NH_2), 3100 (CH arom.), 2966, 2817 (CH aliph.), 1732, 1645 (2C=O), 1593 (C=N), 1373, 1159 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 1.1 [t, 3H, CH_3], 4.0 [q, 2H, CH_2], 7.0 [s, 1H, CH pyrrole], 7.4- 8.1 [m, 10H, Ar-H + SO_2NH_2], 8.7 [s, 1H, CH pyrimidine]. ^{13}C-NMR (DMSO-d_6) δ: 14.0, 59.7, 100.0, 118.6, 120.8, 121.3 (2), 124.7, 126.5, 130.7 (2), 130.8 (2), 132.0 (2), 138.1, 143.2, 145.1, 147.5, 152.3, 163.2, 170.3. Anal. Calcd for $C_{22}H_{17}BrN_6O_5S$ (557.38): C, 47.41; H, 3.07; N, 15.08. Found: C, 47.09; H, 3.36; N, 15.39.

4.1.10. 9-(4-Bromophenyl)-7*H*-pyrrolo[3,2-e][1,2,3,4]tetrazolo[1,5-c]pyrimidin-7-yl)benzenesulfonamide (15)

To a cold solution of **8** (4.59 g, 0.01 mol) in acetic acid (20 mL) was added an ice-cold solution of sodium nitrite (2.07 g; 0.03 mol) in H_2O (10 mL) with stirring during 5 min. Strring was then continued for 3h. The reaction mixture was poured onto ice/ water and the solid formed was filtered off and recrystallized from dioxene to give **15**. Yield % 78, m.p. 278.3

^{o}C; IR (KBr, cm^{-1}): 3325, 3265 (NH_2), 3100 (CH arom.), 1591 (C=N), 1384, 1163 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 6.8 [s, 1H, CH pyrrole], 7.4- 8.3 [m, 10H, Ar-H + SO_2NH_2], 8.6 [s, 1H, CH pyrimidine]. ^{13}C-NMR (DMSO-d_6) δ: 106.3, 119.7, 123.2 (2), 126.3, 127.1 (2), 130.3, 131.8 (2), 132.4 (2), 136.7, 139.4, 142.2, 146.5, 155.8, 158.4. Anal. Calcd for $C_{18}H_{12}BrN_7O_2S$ (470.3): C, 45.97; H, 2.57; N, 20.85. Found: C, 45.62; H, 2.80; N, 20.54.

4.1.11. 4-(2-Oxo-10-(4-bromophenyl)-3,4-dihydropyrrolo[2,3-d]pyrimido [3,4-e][1,2,4]-triazin-8-yl)benzenesulfonamide (16)

A mixture of **8** (4.59 g, 0.01 mol) and ethyl chloroacetate (1.2 g, 0.0 mole) was refluxed in ethanol (30 mL) containing sodim ethoxide (0.23 g, 0.01 mol) for 22h, the reaction mixture was then cooled and poured onto ice water, the solid separated was recrystallized from dioxane to give **16**. Yield % 75, m.p. 292.7 ^{o}C; IR (KBr, cm^{-1}): 3323, 3296, 3110 (NH, NH_2), 3100 (CH arom.), 1654 (C=O), 1593 (C=N), 1327, 1161 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 4.7 [s, 2H, CH_2], 6.9 [s, 1H, CH pyrrole], 7.4- 8.1 [m, 10H, Ar-H + SO_2NH_2], 8.7 [s, 1H, CH pyrimidine]. ^{13}C-NMR (DMSO-d_6) δ: 55.9, 100.0, 118.6, 120.9, 121.3 (2), 124.8, 126.9, 129.3 (2), 131.3 (2), 132.0 (2), 138.1, 143.2, 147.5, 152.3, 160.9, 171.0. Anal. Calcd for $C_{20}H_{15}BrN_6O_3S$ (499.34): C, 48.11; H, 3.03; N, 16.83. Found: C, 48.46; H, 3.29; N, 16.48.

4.1.12. 4-(2,3-Dioxo-10-(4-bromophenyl)-3,4-dihydropyrrolo[2,3-d]pyrimido[3,4-e][1,2,-4]triazin-8-yl)benzenesulfonamide (18)

To a solution of **8** (4.59 g, 0.01 mol) in dry benzene (20 mL), oxalyl chloride (1.26 g, 0.01 mol) was added. The reaction mixture was heated under reflux for 12h. The solid formed was collected by filteration and recrystallized from dimethylformamide-ethanol (1: 2) to give **18**. Yield % 74, m.p. > 350; IR (KBr, cm^{-1}): 3439, 3376 (NH, NH_2), 3086 (CH arom.), 1745, 1737 (2C=O), 1627 (C=N), 1371, 1172 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 7.2 [s, 1H, CH pyrrole], 7.3-8.4 [m, 10H, Ar-H + SO_2NH_2], 9.2 [s, 1H, CH pyrimidine], 9.7 [s, 1H, NH, D_2O-exchangeable]. ^{13}C-NMR (DMSO-d_6) δ: 102.5, 119.7, 123.6, 124.7 (2), 125.5, 126.5, 129.3 (2), 130.6 (2), 135.1 (2), 136.8, 141.7, 148.3, 157.3, 160.8, 168.8, 172.9. Anal. Calcd for $C_{20}H_{13}BrN_6O_4S$ (513.32): C, 46.80; H, 2.55; N, 16.37. Found: C, 46.57; H, 2.72; N, 16.06.

4.1.13. *p*-{3-(*p*-Bromophenyl)-11,13-dimethyl-5.7.9.10.14-pentazatricyclo[7.5.0.0^{2,6}]tetra-deca-1(14),2(6),3,7,10-pentaen-5-yl}benzenesulfonamide (19)

A mixture of **8** (4.59 g, 0.01 mol) and acetylacetone (1.0g, 0.01 mol) in ethanol (30 mL) was refluxed for 4h. After cooling the solid formed was collected and recrystallized from dioxane

to give **19**. Yield % 54, m.p. 293.0 ^{o}C; IR (KBr, cm^{-1}): 3392, 3284 (NH_2), 3078 (CH arom.), 2976, 2836 (CH aliph.), 1624 (C=N), 1334, 1165 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 1.0 [s, 3H, CH_3], 1.9 [s, 3H, CH_3], 7.4 [s, 1H, CH pyrrole], 7.5- 8.3 [m, 10H, Ar-H + SO_2NH_2], 8.4 [s, 1H, CH triazepine], 9.4 [s, 1H, CH pyrimidine]. ^{13}C-NMR (DMSO-d_6) δ: 19.3, 29.0, 103.5, 114.2, 117.0, 120.0, 124.0 (2), 124.5, 126.8, 129.2 (2), 131.3 (2), 131.7 (2), 135.9, 141.3, 142.5, 148.9, 154.6, 158.8, 163.6. Anal. Calcd for $C_{23}H_{19}BrN_6O_2S$ (523.41): C, 52.78; H, 3.66; N, 16.06. Found: C, 52.46; H, 3.39; N, 16.37.

4.1.14. *p*-{13-Amino-3-(*p*-bromophenyl)-12-cyano-5, 7, 9, 10, 14-pentazatricyclo-[7.5.-0-.0^{2,6}]-tetradeca-1(14),2(6),3,7,10,12-hexaen-5-yl}benzenesulfonamide (20)

A mixture of **8** (4.59 g, 0.01 mol) and ethoxymethylenemalononitrile (1.22 g, 0.01 mol) in absolute ethanol (20 mL) was refluxed for 8h. The precipitate formed after cooling was filtered off and recrystallized from acetic acid to give **20**. Yield % 67, m.p. 356.8 ^{o}C; IR (KBr, cm^{-1}): 3400, 3265, 3130 (NH_2), 3066 (CH arom.), 2193 (C≡N), 1618 (C=N), 1371, 1165 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 6.7 [s, 2H, NH_2, D_2O-exchangeable], 7.5 [s, 1H, CH pyrrole], 7.6- 8.4 [m, 10H, Ar-H + SO_2NH_2], 8.5 [s, 1H, CH triazepine], 9.4 [s, 1H, CH pyrimidine]. ^{13}C-NMR (DMSO-d_6) δ: 73.9, 103.9, 117.0, 119.9, 124.4, 124.7 (2), 126.7, 127.1, 129.0 (2), 130.6 (2), 131.9 (2), 136.2, 139.2, 148.2, 154.1, 166.8, 176.9, 178.3. Anal. Calcd for $C_{22}H_{15}BrN_6O_2S$ (535.38): C, 49.36; H, 2.82; N, 20.93. Found: C, 49.08; H, 2.49; N, 21.29.

4.1.15. *p*-{13-Amino-3-(*p*-bromophenyl)-12-ethoxycarbonyl- 5, 7, 9, 10, 14- pentazatricyclo [7.5.0.0^{2,6}]tetradeca-1(14),2(6),3,7,10,12-hexaen-5-yl}benzenesulfonamide (21)

A mixture of **8** (4.59 g, 0.01 mol) and ethyl ethoxymethylenecyanoacetate (1.6 g, 0.01 mol) in absolute ethanol (30 mL) was refluxed for 8h. After cooling the solid precipitate was filtered off and recrystallized from dioxane to give **21**. Yield % 90, m.p. 368.7 ^{o}C; IR (KBr, cm^{-1}): 3400, 3267, 3130 (NH_2), 3082 (CH arom.), 2976, 2863 (CH aliph.), 1733 (C=O), 1620 (C=N), 1383, 1165 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 1.0 [t, 3H, CH_3], 4.4 [q, 2H, CH_2], 7.5 [s, 1H, CH pyrrole], 7.8- 8.4 [m, 10H, Ar-H + SO_2NH_2], 8.5 [s, 1H, CH triazepine], 9.5 [s, 1H, CH pyrimidine]. ^{13}C-NMR (DMSO-d_6) δ: 18.5, 56.0, 93.0, 104.0, 117.0, 119.9, 123.8 (2), 124.4, 126.7, 129.0 (2), 130.6 (2), 131.5 (2), 136.2, 139.2, 148.2, 154.1, 161.6, 165.8, 167.2, 172.1. Anal. Calcd for $C_{24}H_{20}BrN_7O_4S$ (582.43): C, 49.49; H, 3.46; N, 16.83. Found: C, 49.12; H, 3.81; N, 16.54.

4.1.16. *p*-{5-(*p*-Bromophenyl)-2, 7, 9, 11, 12, 20- hexazapentacyclo[11.7.0.0^{3,11}.0^{4,8}.0^{14,19}]-icosa-1(20), 2, 4(8), 5, 9, 12, 14(19), 15, 17-nonaen-7-yl}benzenesulfonamide (22)

A mixture of **8** (4.59 g, 0.01 mol) and isatin (1.5 g, 0.01 mol) in absolute ethanol (20 mL) was refluxed for 12h. The product which was separated during reflux was filtered off and recrystallized from acetic acid to give **22**. Yield % 82, m.p. 342.4 ^{o}C; IR (KBr, cm^{-1}): 3354, 3290 (NH_2), 3061 (CH arom.), 1629 (C=N), 1330, 1157 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 7.3 [s, 1H, CH pyrrole], 7.4- 8.1 [m, 14H, Ar-H + SO_2NH_2], 9.4 [s, 1H, CH pyrimidine]. ^{13}C-NMR (DMSO-d_6) δ: 107.6, 119.6, 120.0, 120.4 (2), 122.1, 123.1, 124.7, 126.3, 126.9, 129.1 (2), 130.6 (2), 131.4, 131.9 (2), 132.9, 133.2, 142.7, 145.6, 148.2, 162.6, 164.2, 166.7, 170.1. Anal. Calcd for $C_{26}H_{16}BrN_7O_2S$ (570.42): C, 54.75; H, 2.83; N, 17.19. Found: C, 54.45; H, 2.43; N, 17.51.

4.1.17 4-(3-(1-Acetyl-2-oxoindolin-3-ylideneamino)-5-(4-bromophenyl)-4-imino-3,4-dihydropyrrolo[2,3-d]pyrimidin-7-yl)benzenesulfonamide (23)

A mixture of **8** (4.59 g, 0.01 mol) and *N*-acetylisatin (1.75 g, 0.01 mol) in absolute ethanol (30 mL) was refluxed for 10h. After cooling the product was filtered off and recrystallized from dioxane to give **23**.

Yield % 77, m.p. 340.3 ^{o}C; IR (KBr, cm^{-1}): 3298, 3210 (NH, NH_2), 1691, 1683 (2C=O), 1585 (C=N), 1338, 1161 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 2.1 [s, 3H, $COCH_3$], 6.6 [s, 1H, CH pyrrole], 7.2- 8.6 [m, 14H, Ar-H + SO_2NH_2], 9.4 [s, 1H, CH pyrimidine], 10.5 [s, 1H, NH, D_2O-exchangeable]. ^{13}C-NMR (DMSO-d_6) δ: 18.5, 110.1, 117.3, 118.1, 120.1, 120.8, 122.9 (2), 123.2, 124.5, 124.8, 127.0 (2), 130.1, 130.7 (2), 131.6, 131.9 (2), 132.3, 138.2, 141.5, 143.1, 144.0, 160.0, 164.3, 167.8, 173.8. Anal. Calcd for $C_{28}H_{20}BrN_7O_4S$ (630.47): C, 53.34; H, 3.20; N, 15.55. Found: C, 53.10; H, 3.57; N, 15.21.

4.1.18 4-(5-(4-Bromophenyl)-4-imino-3-(4-oxo-4-phenylbutan-2-ylidene-amino)-3,4-dihydropyrrolo[2,3-d]pyrimidin-7-yl)benzenesulfonamide (24)

A mixture of **8** (4.59 g, 0.01 mol) and benzoylacetone (1.62 g, 0.01 mol) in absolute ethanol (30 mL) was refluxed for 15h. The solvent was evaporated under reduced pressure and the solid formed was collected and recrystallized from dioxane to give **24**. Yield % 86, m.p. 249.4 ^{o}C; IR (KBr, cm^{-1}): 3327, 3286 (NH, NH_2), 3084 (CH arom.), 1653 (C=O), 1591 (C=N), 1328, 1161 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 0.9 [s, 3H, CH_3], 2.8 [s, 2H, CH_2], 6.6 [s, 1H, CH pyrrole], 7.5- 8.3 [m, 15H, Ar-H + SO_2NH_2], 9.2 [s, 1H, CH pyrimidine], 9.6 [s, 1H, NH, D_2O-exchangeable]. ^{13}C-NMR (DMSO-d_6) δ: 18.6, 102.6, 109.0, 118.9, 120.9, 121.0 (2), 124.6, 125.0, 125.3 (2), 126.8 (2), 129.3 (2), 130.7 (2), 131.4 (2), 131.7, 138.5, 142.9, 144.5, 147.5,

152.5, 164.3, 165.8, 193.2. Anal. Calcd for $C_{28}H_{23}BrN_6O_3S$ (603.49): C, 55.73; H, 3.84; N, 13.93. Found: C, 55.46; H, 3.50; N, 13.66.

4.1.19. *p*-{3-(*p*-Bromophenyl)-11-methyl-13-phenyl-5, 7, 9, 10, 14-pentazatricyclo-[7.5.0.0^{2,6}] tetradeca-1(14),2(6), 3, 7, 10,12- hexaen-5-yl}benzenesulfonamide (25)

A solution of **24** (0.603 g, 0.001 mol) in $POCl_3$ (20 mL) was heated under reflux for 6h. After cooling the reaction mixture was poured onto a mixture of ice water and neutralized with ammonium hydroxide solution. The solid formed was collected and recrystallized from ethanol to afford **25**. Yield % 69, m.p. 219.4 ^oC; IR (KBr, cm^{-1}): 3460, 3331 (NH_2), 3061 (CH arom.), 2970, 2926 (CH aliph.), 1627 (C=N), 1338, 1161 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 0.9 [s, 3H, CH_3], 5.9 [s, 1H, CH triazipene], 6.8 [s, 1H, CH pyrrole], 7.1- 8.2 [m, 15H, Ar-H + SO_2NH_2], 9.4 [s, 1H, CH pyrimidine]. ^{13}C-NMR (DMSO-d_6) δ: 19.1, 108.9, 112.7, 119.6, 121.4, 122.6 (2), 124.7, 127.0, 127.4 (2), 128.0 (2), 128.6, 128.8 (2), 131.4 (2), 132.0 (2), 132.7, 134.2, 140.1, 143.2, 152.7, 157.4, 165.6, 168.8. Anal. Calcd for $C_{28}H_{21}BrN_6O_2S$ (585.47): C, 57.44; H, 3.62; N, 14.35. Found: C, 57.70; H, 3.29; N, 14.03.

4.2. Carbonic anhydrase inhibition assay

An Applied Photophysics stopped flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-ionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were pre-incubated together for 15 min at room temperature or 4°C prior to assay, in order to allow for the formation of the E-I complex. Data from **Table 1** were obtained after 15 minutes incubation of enzyme and inhibitor.[40] The inhibition constants were obtained by non-linear least squares methods using PRISM 3, as reported earlier [41] and represent the mean from three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier. [40, 42-47]

4.3. *In vitro* cytotoxic activity

Human tumor breast cell line (MCF-7) was used in this study. The cytotoxic activity was measured *in vitro* for the newly synthesized compounds using the Sulfo-Rhodamine-B stain (SRB) assay using the method of Skehan *et al*.[48] The *in vitro* anticancer screening was done by the pharmacology unit at the National Cancer Institute, Cairo University. Cells were plated in 96-multiwell plate (10^4 cells/ well) for 24 h before treatment with the compound(s) to allow attachment of cell to the wall of the plate. Test compounds were dissolved in dimethylsulfoxide. Different concentrations of the compound under test (10, 25, 50, and 100 µM) were added to the cell monolayer. Triplicate wells were prepared for each individual concentration. Monolayer cells were incubated with the compound(s) for 48 h at 37 ^{o}C and in atmosphere of 5% CO_2. After 48 h, cells were fixed, washed and stained for 30 min with 0.4% (wt/vol) SRB dissolved in 1% acetic acid. Excess unbound dye was removed by four washes with 1% acetic acid and attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve for breast tumor cell line after the specified time. The molar concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and compared to the reference drug Doxorubicin (CAS, 25316-40-9). The surviving fractions were expressed as means ± standard error.

4.4. Molecular Docking Study

All the molecular modeling studies were carried out on an Intel Pentium 1.6 GHz processor, 512 MB memory with Windows XP operating system using Molecular Operating Environment (MOE, 10.2008) software. All the minimizations were performed with MOE until a RMSD gradient of 0.05 $Kcalmol^{-1}Å^{-1}$ with MMFF94X force field and the partial charges were automatically calculated. The X-ray crystallographic structure of CA isozyme XII complexed with acetazolamide (PDB ID: 1JD0) was obtained from the protein data bank. The enzymes were prepared for docking studies where: (i) The co-crystallized ligand molecule (AZA) was removed from the enzyme active site. (ii) Hydrogen atoms were added to the structure with their standard geometry. (iii) MOE Alpha Site Finder was used for the active sites search in the enzyme structure and dummy atoms were created from the obtained alpha spheres. (iv) The obtained model was then used in predicting the ligand enzymes interactions at the active site. (v) Docking protocol was verified by re-docking of the co-crystallized ligand (acetazolamide) in the vicinity of the active site of the enzyme with energy score (S) = -36.3568 Kcal/ mol and root mean standard deviation (RMSD) = 1.1976.

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Table 1. hCA I, II, IX and XII inhibition data of compounds **5-25** and acetazolamide as standard.

Compound	K_i* (nM)				SI**	
	hCA I	hCA II	hCA IX	hCA XII	hCA I/XII	hCA II/XII
5	84.7	4.6	6.3	0.74	114.46	6.22
6	159.6	3.2	3.9	31.6	5.05	0.12
8	21.6	3.8	2.7	211	0.10	0.01
9	32.4	4.9	2.4	70.8	0.46	0.03
10	66	4.7	2.7	365	0.18	0.01
11	27.6	24.5	3	184	0.15	0.02
12	33.2	123.7	3.1	79.2	0.42	0.04
14	23.2	4.5	2.6	9.3	2.49	0.28
15	28.4	5.3	2.8	9.2	3.09	0.30
16	94.1	4.9	11.7	0.76	123.82	15.39
18	69.1	64.3	325	0.912	75.77	356.36
19	25.4	18.2	49.6	5.4	4.70	9.19
20	80.4	5.2	3.1	0.9	89.33	3.44
21	126	7.3	3	0.83	151.81	3.61
22	498	8.5	2.7	0.72	691.67	3.75
23	25.1	5.6	3.1	0.81	30.99	3.83
24	16.3	3.9	2.8	0.94	17.34	2.98
25	28.2	7.1	3.6	3.4	8.29	1.06
AZA	250	12.1	25.0	5.7	43.86	2.12

AZA (Acetazolamide), a well known CAI, was used as a standard for comparison.
* K_i presented is the mean from 3 different assays; errors are in the range of ± 5-10% of the reported values (data not shown).
** SI (Selectivity index) is a ratio between the K_i values observed for two hCA isoforms; low value index is indicative of weak selectivity.

Table 2

In vitro anticancer screening of the synthesized compounds against the human breast cancer cell line MCF7.

Compound	Compound concentration (µM)				IC_{50} (µM)
	10µM	25µM	50µM	100µM	
	Surviving fraction (Mean ± S.E.)*				
Doxorubicin	0.314±0.032	0.309±0.016	0.251±0.023	0.266±0.032	8.02
5	0.327±0.121	0.273±0.043	0.233±0.011	0.255±0.020	7.56
6	0.340±0.090	0.294±0.021	0.246±0.110	0.256±0.002	7.56
8	0.330±0.211	0.309±0.016	0.227±0.110	0.268±0.132	7.29
9	0.347±0.218	0.292±0.100	0.213±0.152	0.221±0.101	7.56
10	0.305±0.113	0.281±0.100	0.274±0.109	0.309±0.001	7.29
11	0.426±0.100	0.410±0.121	0.348±0.133	0.335±0.104	8.94
12	0.400±0.011	0.399±0.110	0.365±0.021	0.350±0.002	8.94
14	0.361±0.003	0.311±0.011	0.232±0.021	0.216±0.011	7.56
15	0.348±0.110	0.294±0.021	0.254±0.100	0.216±0.111	7.84
16	0.393±0.014	0.358±0.001	0.337±0.111	0.362±0.001	8.11
18	0.436±0.021	0.366±0.136	0.350±0.011	0.358±0.011	8.66
19	0.380±0.002	0.285±0.018	0.332±0.006	0.375±0.001	7.84
20	0.382±0.021	0.380±0.031	0.401±0.058	0.379±0.110	8.11
21	0.372±0.001	0.377±0.012	0.353±0.032	0.391±0.011	7.84
22	0.347±0.111	0.338±0.035	0.315±0.012	0.381±0.008	7.84
23	0.342±0.022	0.287±0.018	0.259±0.002	0.326±0.001	7.56
24	0.381±0.001	0.333±0.008	0.251±0.001	0.301±0.039	7.84
25	0.303±0.002	0.306±0.006	0.277±0.011	0.280±0.011	7.29

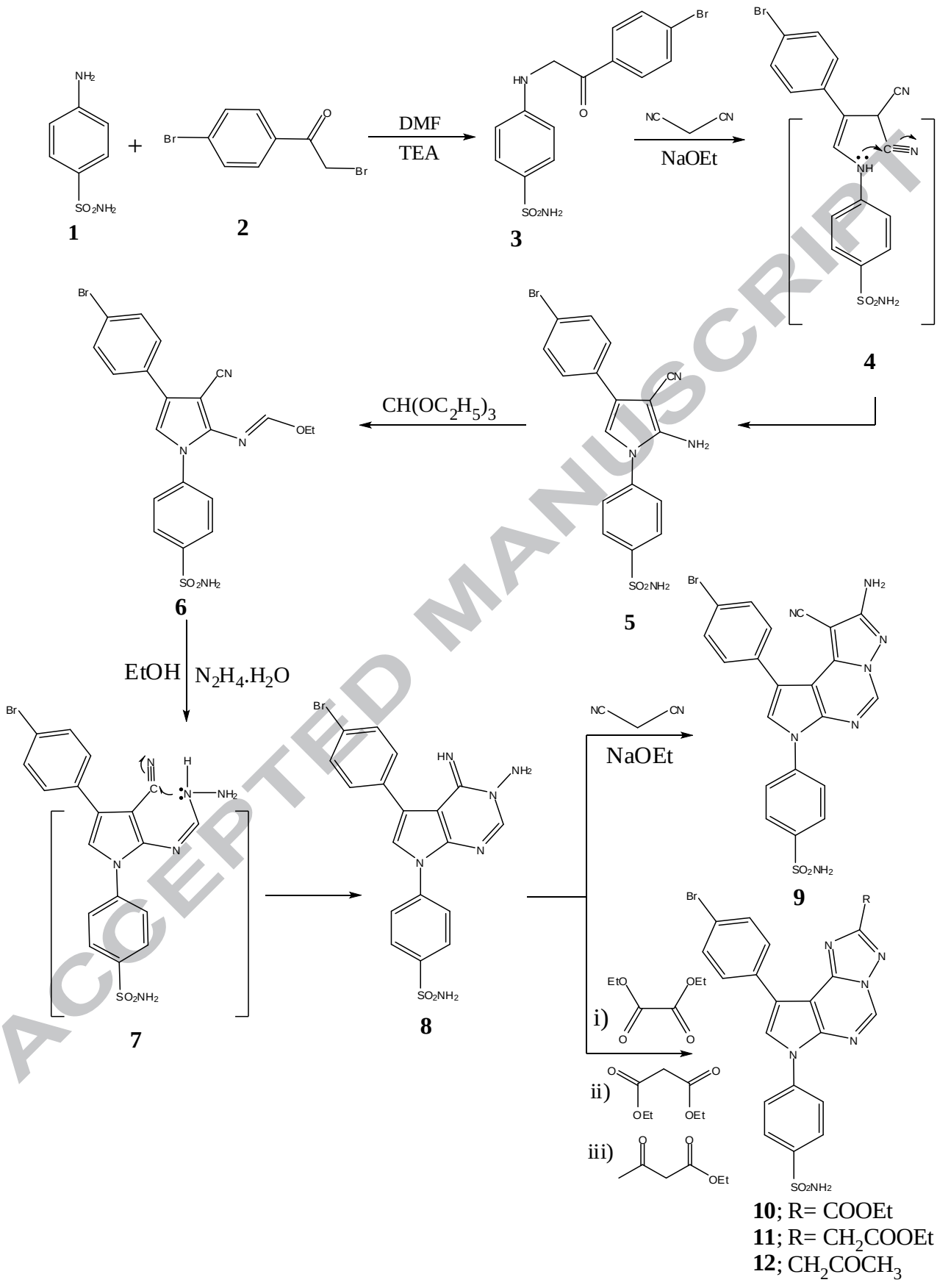
* Each value is the mean of three values ± Standard Error.

Table 3. Docking results of the synthesized sulfonamide derivatives with CA XII using MOE software version 2008.10.

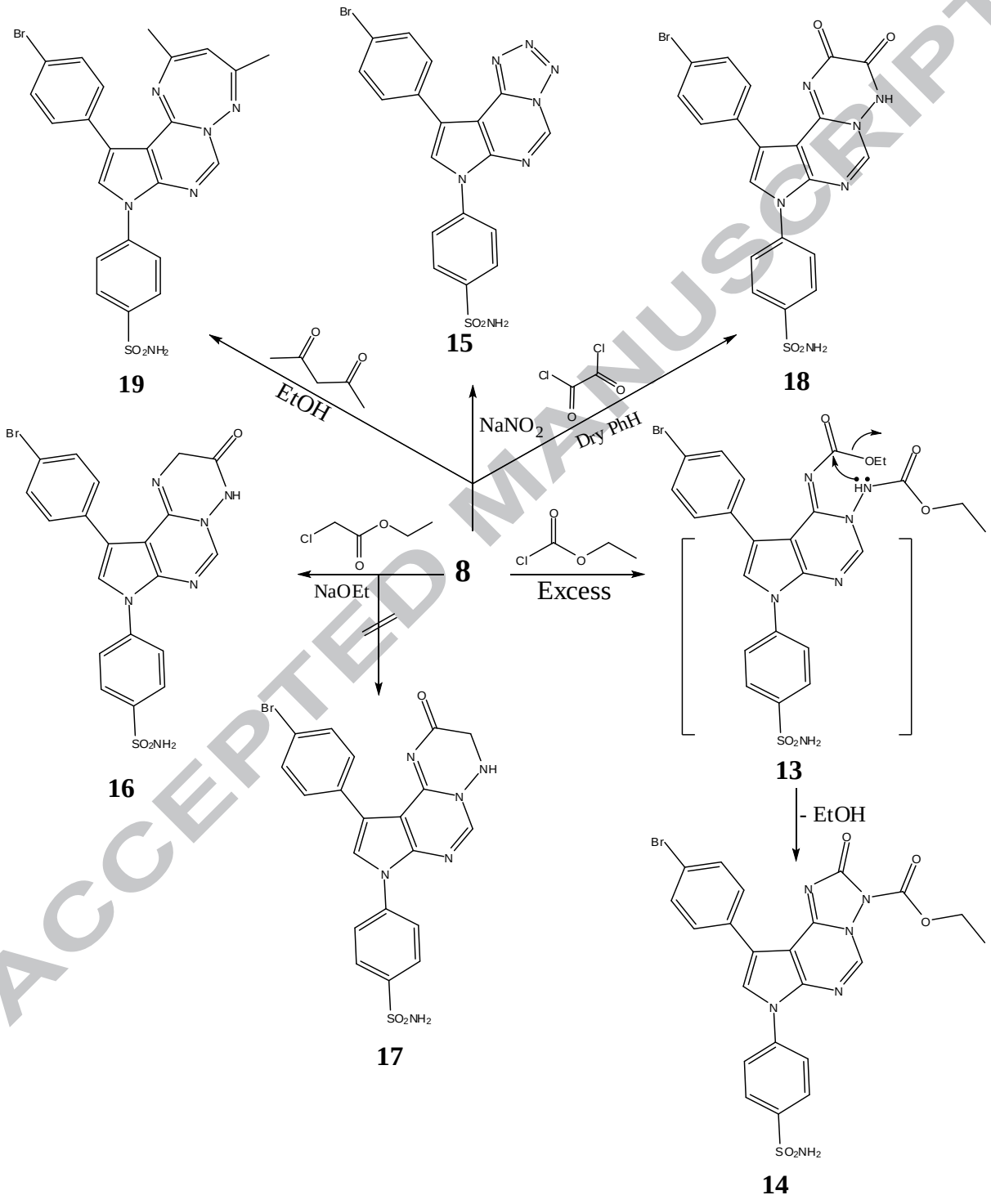
Compound	S (Kcal/mol)	Amino acids	Interacting groups	Type of interaction	Length (A^o)
5	-41.3027	Thr199	SO_2	H-bond (acceptor)	2.41
		Thr199	SO_2	H-bond (acceptor)	3.00
		Thr199	NH_2	H-bond (donor)	1.49
		His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
		His199	SO_2	H-bond (acceptor)	2.73
		Thr91	CN	Solvent (H_2O)	2.90
		Gln92	CN	Solvent (H_2O)	2.90
6	-19.9791	His94	SO_2NH_2	Metal complex (Zn)	2.06
		His 6	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
8	-13.0846	His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
9	-23.6440	His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
10	-12.7252	His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
11	-15.3873	His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
12	-13.2802	His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
14	-35.6833	His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
15	-35.7456	His94	SO_2NH_2	Metal complex (Zn)	2.06

		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
16	-52.7571	Thr199	SO_2	H-bond (acceptor)	3.57
		His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
		His119	SO_2	H-bond (acceptor)	2.93
		Thr200	NH	Solvent (H_2O)	3.44
		Asn62	C=O	Solvent (H_2O)	2.42
18	-51.5937	Thr199	SO_2	H-bond (acceptor)	2.64
		Thr199	SO_2	H-bond (acceptor)	2.93
		Thr199	NH_2	H-bond (acceptor)	1.35
		His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
		His96	NH_2	H-bond (acceptor)	2.34
		His119	SO_2	H-bond (acceptor)	3.10
		Thr91	C=O	H-bond (acceptor)	2.47
		Gln92	C=O	Solvent (H_2O)	2.58
19	-32.2959	His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
20	-49.5079	Thr199	SO_2	H-bond (acceptor)	2.87
		His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
21	-46.6627	Thr199	SO_2	H-bond (acceptor)	2.76
		Thr199	NH_2	H-bond (donor)	1.68
		His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
22	-41.5627	Thr199	SO_2	H-bond (acceptor)	3.70
		His119	SO_2	H-bond (acceptor)	3.20
		His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04

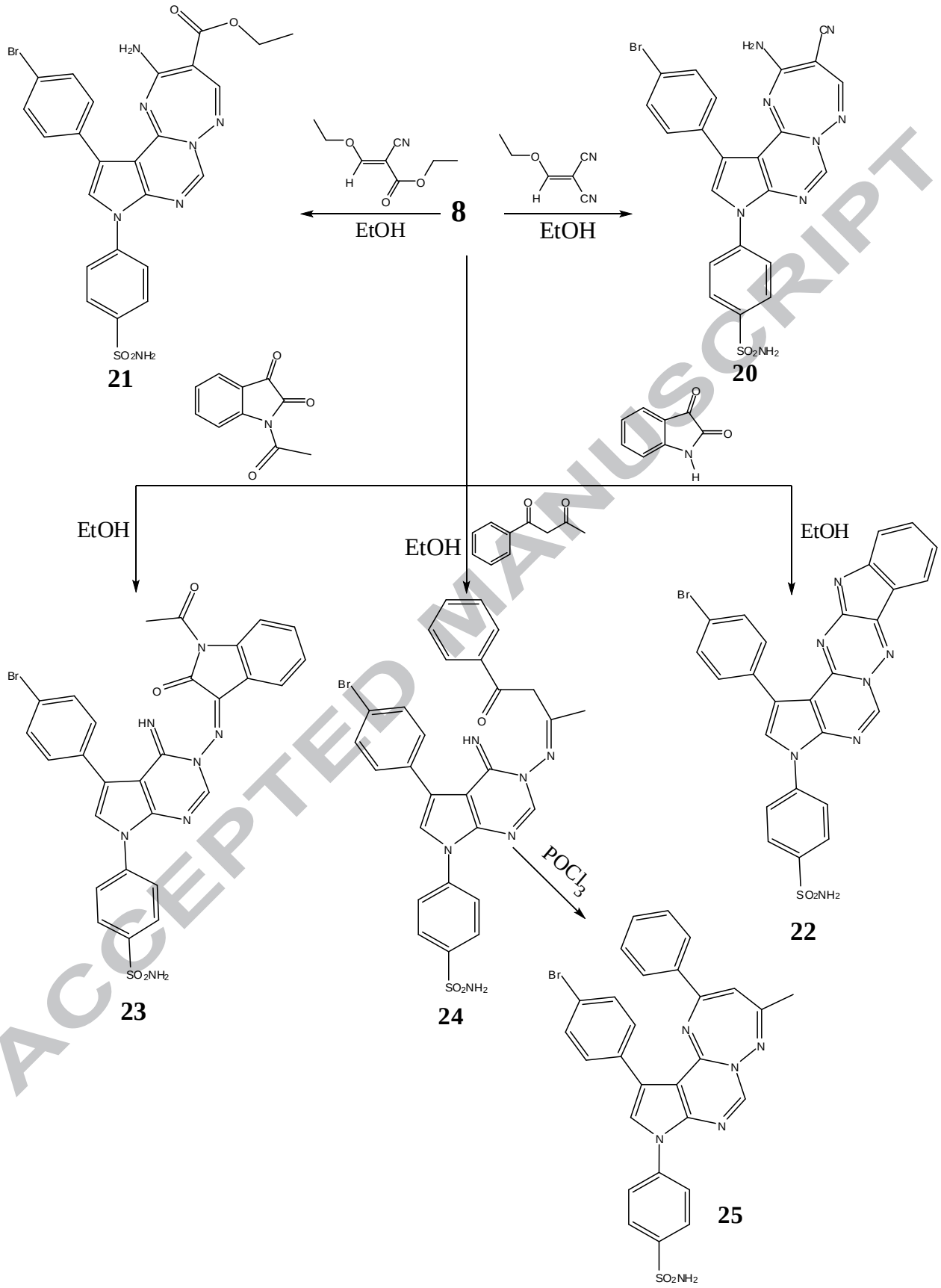
23	-40.2157	Thr199	SO_2	H-bond (acceptor)	3.70
		His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
24	-44.9134	Thr199	SO_2	H-bond (acceptor)	3.66
		His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04
25	-36.0029	His94	SO_2NH_2	Metal complex (Zn)	2.06
		His96	SO_2NH_2	Metal complex (Zn)	2.03
		His119	SO_2NH_2	Metal complex (Zn)	2.04



Scheme 1: Synthesis of pyrrole and pyrrolopyrimidine derivatives 5- 12.



Scheme 2: Synthesis of triazolo, tetrazolo, triazino, and triaziepenopyrrolopyrimidine derivatives.



Scheme 3: Synthesis of triaziepeno, triazinopyrrolopyrimidine and pyrrolopyrimidine derivatives

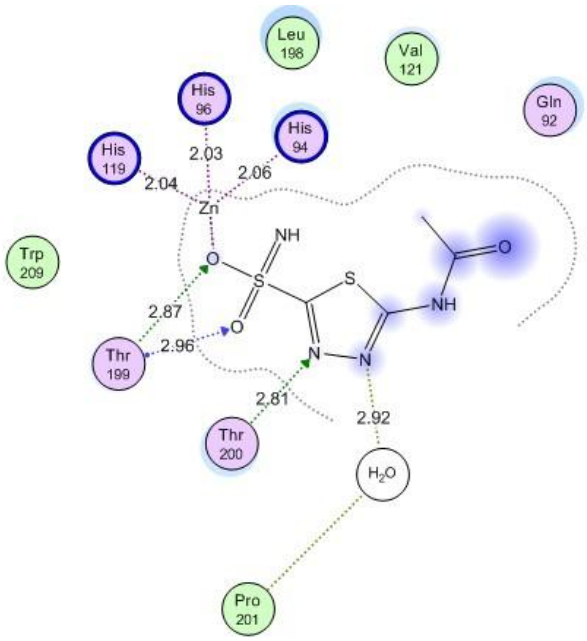


Figure 1. 2D interaction of AZA with the active site amino acids of CA XII: (S) = -36.3568 Kcal/ mol (RMSD) = 1.1976.

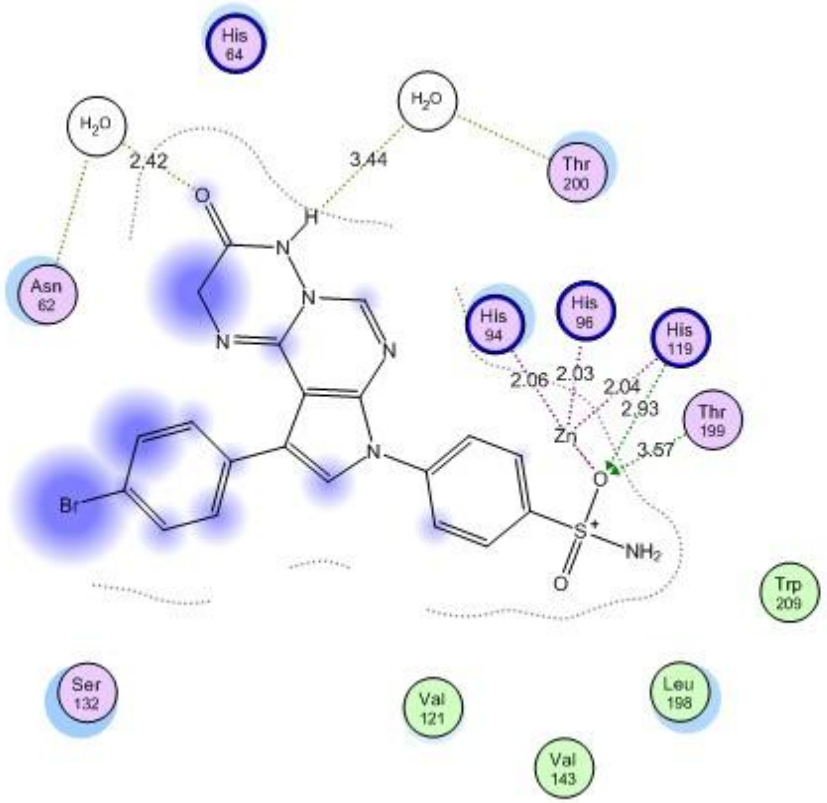


Figure 2. 2D enzyme-ligand interaction of compound **16** with CA XII: energy score (S) = -52.7571 Kcal/ mol.

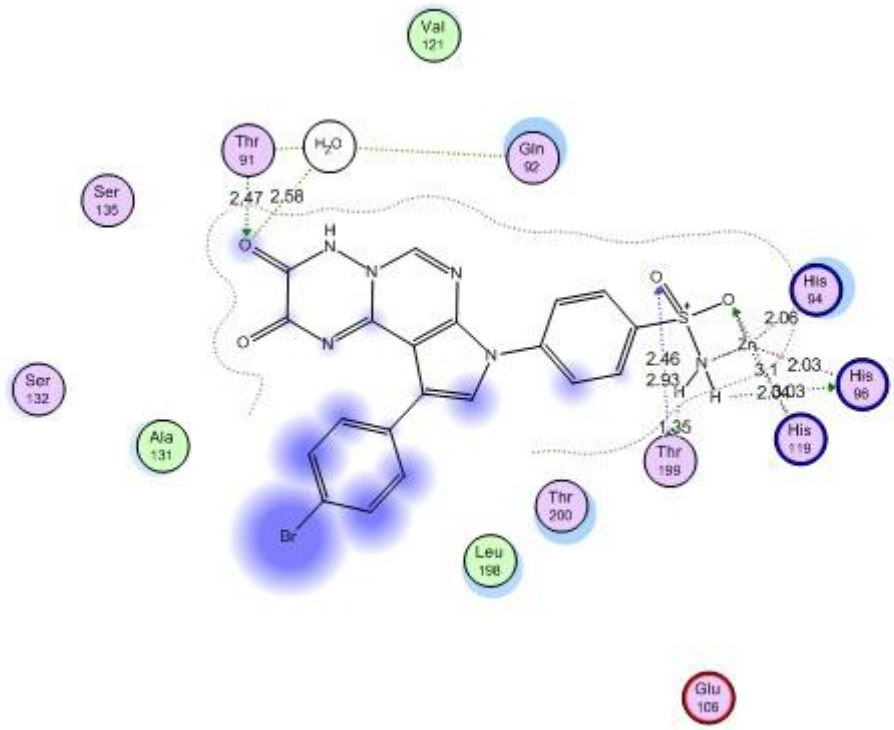
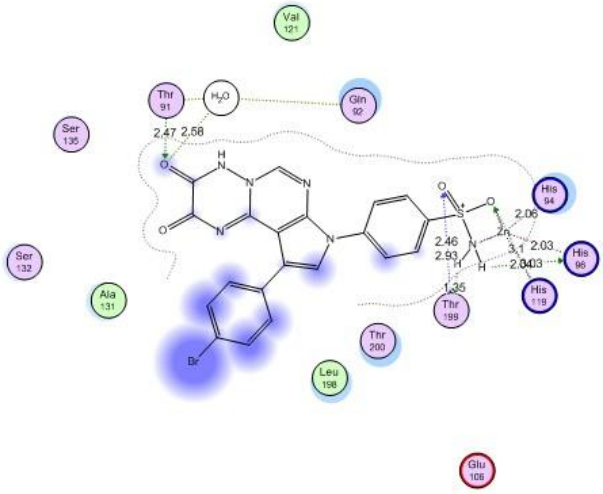
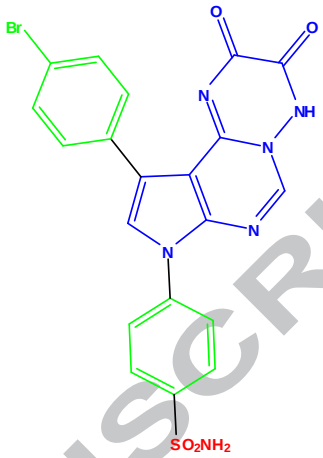


Figure 3. 2D enzyme-ligand interaction of compound **18** with CA XII: energy score (S) = -51.5937 Kcal/ mol.





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