Synthesis, Biological Activity and Computational Studies of Novel Azo-Compounds

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Summary: In the present protocol, we report the synthesis and characterization of some novel azo-compounds starting from 4-methoxyanniline and 4-aminophenazone, which were diazotised at low temperature. 4-nitrophenol, 2-aminobenzoic acid, benzamide, 4-aminobenzoic acid, resorcinol, *o*-bromonitrobenzene and 2-nitroaniline were used as active aromatic coupling compounds for the second step. The synthesized compounds were investigated for their potential antibacterial activities by using disc diffusion method against *Escherichia coli, Shigellasonnei, Streptococcus pyrogenes, Staphylococcus aureus* and *Neisseria gonorrhoeae* strains. They were also subjected to antioxidant activities by using DPPH method. Results revealed that the compounds of 4-methoxyaniline and 4-aminophenazone showed good antibacterial activity against all strains, where as some azo-compounds have moderate to good antioxidant activities. Furthermore, these compounds were studied by computational analysis.

Keywords: 4-methoxyaniline, 4-aminophenazone, Azo-compounds, Antibacterial and Antioxidant activities.

Introduction

Azo-dyes are the most significant and versatile class of synthetic organic compounds with enormous variety of applications [1]. Textile industry is a major consumer of these dyes. These can be obtained easily and economically by using a wide variety of different aromatic amines and more preferably activated aromatic coupling components. They have high dyeing and good fastness properties, and thus wide applications in areas such as dyeing of textile fibers, paper, plastics, leather and bio-medical studies [2]. The syntheses, spectroscopic and dyeing properties of these compounds were studied in last fifty years [3-4]. They show variety of interesting biological activities, such as antifungal [5], pesticidal [6] and antibacterial activities. Usually, azo-compounds are prepared by diazotization of the aromatic amine in mineral acid at about 0°C followed by coupling with nucleophiles [7]. According to statically data survey, one million tons of azo dyes are produced annually in the industrial sectors [8-9]. Together, the dye molecule is often described as a chromogen [10-11]. These compounds are more suitable for biocidal treatment of textile fibres due to their greater biological activities, because biocidal template forms a definite type of bonding with fibrous material [12]. They are important structures in the medicinal and pharmaceutical fields [13] as well. The pharmacological study of these compounds started from the effect of antibacterial action of Prontosil on streptococcal infections by Dog-magk [14]. It is evident from the literature [15-22] that these kinds of functionalities have great potential to be used as antibacterial as well as antioxidant agents. From this perspective, they have gained less attention.

Keeping in view the importance of this class of interesting compounds, we embarked a project to synthesize some highly interesting azo-dyes starting from 4-methoxyanniline and 4-aminophenazone. The synthesized derivatives were investigated for their potent antibacterial activities against the gram positive and gram negative bacteria. In addition, they were studied to check their potential against scavenging free radicals. The newly synthesized compounds have the great potential to be used as novel dyes as well as pharmacological active agents. The characterization of the above-mentioned derivatives was further supported by theoretical calculations.

Experimental

Chemistry

All the chemicals were purchased from Merck and used in chemical reactions without further purification. The melting points were measured by using standard melting point apparatus from Stuart and were uncorrected. The UV-Visible (ORI Germany UV4000 spectrophotometer) spectra were recorded in methanol with at concentration rate of 10^{-4} M. FTIR spectra were recorded in the region of 4000 cm^{-1} to 400cm^{-1} on a FTIR-ALPHA BRUKER IR spectrometer in KBr pellets. NMR spectra were measured with a Bruker DRX 300 instrument (^{1}H-

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NMR, 300 MHz). Accurate mass measurements were performed with the Fisons VG sector-field instrument (EI) and a FT-ICR mass spectrometer.

General procedure for the synthesis of azo-compounds **(1-7)**

The aniline derivatives (4-methoxyaniline and 4-aminophenazone) were added in a mixture of distilled water (15 ml) and conc. sulfuric acid (2 ml). The resulting mixture was warmed (up to ~ 50°C) to get clear solution. Sodium nitrite was dissolved in distilled water (10 ml) and the solution was added into the above solution. Both solutions were cooled to temperature below 5 °C by ice water bath. A cooled sodium nitrite solution was added drop wise into the solution of aniline derivatives with vigorous stirring. To this solution, separately prepared solutions of different active aromatic compounds (equimolar) were added slowly with parallel addition of sodium hydroxide (2 M). The different colored precipitates were formed by adjusting the pH at 7. These precipitates were filtered with the help of suction filtration and washed with distilled water to remove salt and extra acid from the product. The synthesized derivatives were purified by recrystallization in ethanol.

2-[(Z)-(4-methoxyphenyl)diazenyl]-4-nitrophenol **(1)**

Yield: 38%, λ_{max}: 359 nm**,** m.p.121-122 °C, FTIR (KBr, cm^{-1}): 1598 (N=N), 1505 (NO_2), 3658 (O-H stretching), 1104 (OCH_3), 1456 (C=C of aromatic ring), 756 (C-H of aromatic ring); ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 11.96 (s, 1H, Ar-OH), 8.10 (s, 1H, Ar-H), 7.97 (d, *J* = 9.0 Hz, 1H, Ar-H), 7.86-7.65 (m, 2 H, Ar-H), 7.16 (d, *J* = 9.0 Hz, 1H, Ar-H), 6.49-6.34 (m, 2 H, Ar-H), 3.85 (s, 3H, OCH_3); accurate mass (EI-MS) of $[M]^{+\cdot}$: calcd. for $C_{13}H_{10}BrN_3O_3$ 334.99 ; found 334.92.

(Z)-1-(4-bromo-3-nitrophenyl)-2-(4-methoxyphenyl) diazene **(2)**

Yield: 51%, λ_{max}: 358 nm, m.p. 48-50 °C, FTIR (KBr, cm^{-1}): 1577 (N=N), 1351 (NO_2), 1577 (C=C of aromatic ring), 1146 (OCH_3 group), 756 (C-H of aromatic ring); ^{1}H-NMR (300 MHz, DMSO-d_6): δ =7.89-8.49 (m, 3 H, Ar-H), 7.19-7.89 (m, 4 H, Ar-H), 3.75 (s, 3 H, OCH_3); accurate mass (EI-MS) of $[M]^{+\cdot}$: calcd. for $C_{13}H_{11}N_3O_4$ 273.07 ; found 273.01.

4-amino-5-[(E)-(4-methoxyphenyl)diazenyl]benzoic acid **(3)**

Yield: 54%, λ_{max}: 364 nm, m.p. 108-110 °C, FTIR (KBr, cm^{-1}): 1599 (N=N), 1107 (OCH_3), 1505 (C=C of aromatic ring), 1668 (C=O of COOH), 3410 (NH_2 group), 750 (C-H of aromatic ring); ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.01-8.19 (m, 2 H, Ar-H), 7.19-7.87 (m, 5 H, Ar-H), 6.35 (b, 1 H, NH_2), 3.78 (s, 3H, OCH_3); accurate mass (EI-MS) of $[M]^{+\cdot}$: calcd. for $C_{14}H_{13}N_3O_3$ 271.10; found 271.03.

4-[(E)-(2-amino-3-nitrophenyl)diazenyl]-1,5-dimethyl-2-phenyl1,2-dihydro-3H-pyrazol-3-one **(4)**

Yield: 72%, λ_{max}: 359 nm, m.p. 102-103 °C, FTIR (KBr, cm^{-1}): 1652 (N=N), 1555 (NO_2), 1652 (C=C of aromatic), 2920 (C-H of CH_3), 746 (C-H of aromatic), 3230 (NH_2); ^{1}H-NMR (300 MHz, DMSO-d_6), δ =11.0 (s, 1H, COOH), 7.01-7.89 (m, 3 H, Ar-H), 2.90 (s, 6H, CH_3); accurate mass (EI-MS) of $[M]^{+\cdot}$: calcd. for $C_{17}H_{16}N_4O_3$ 324.12; found 324.10.

4-amino-3-[(E)-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl]benzoic acid **(5)**

Yield: 82%, λ_{max}: 368 nm**,** m.p. 181-182 °C, FTIR (KBr, cm^{-1}): 1601 (N=N), 2969 (C-H of CH_3), 1683 (C=O of COOH), 1660 (C=C of aromatic), 3210 (NH_2 group), 3110 (O-H of COOH); ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.21 (s, 1H, Ar-H), 7.99 (d, J = 6.0 Hz, 1H, Ar-H), 7.63-7.49 (m, 4 H, Ar-H), 7.21-7.12 (m, 2 H, Ar-H), 3.38 (s, 6H, CH_3); accurate mass (EI-MS) of $[M]^{+\cdot}$: calcd. for $C_{18}H_{17}N_5O_2$ 335.14; found 335.11.

4-[(E)-(2,4-dihydroxyphenyl)diazenyl]-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one **(6)**

Yield: 47%, λ_{max}: 369 nm**,** m.p. 159-162 °C, FTIR (KBr, cm^{-1}): 1606 (N=N), 1606 (C=C of aromatic ring), 2931 (C-H of CH_3), 3580 (O-H stretching), 772 (C-H of aromatic ring); ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 6.92-7.40 (m, 8 H, Ar-H), 5.37 (s, 2H, OH), 3.20 (s, 6H, CH_3); accurate mass (EI-MS) of $[M]^{+\cdot}$: calcd. for $C_{17}H_{16}N_6O_3$ 352.13; found 352.10.

4-[(E)-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl]benzamide **(7)**

Yield: 55%, λ_{max}: 369 nm**,** m.p. 210-212 °C, FTIR (KBr, cm^{-1}): 1600 (N=N), 2967 (C-H of CH_3), 1644 (NH_2 of amide group), 3165 (C=O of amide group), 1489 (C=C of aromatic ring), 767 (C-H of aromatic ring); ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 7.50 (s, 1H, $CONH_2$), 6.90-7.45 (m, 8 H, Ar-H), 2.98 (s, 6H, CH_3); accurate mass (EI-MS) of $[M]^{+\cdot}$: calcd. for $C_{18}H_{17}N_5O_3$ 351.13; found 352.11.

Biological Activity

Bacterial Strains

The following strains were used for antibacterial activity.

1- *Escherichia coli* (ATCC 35318)
2- *Escherichia coli* (ATCC 25922)
3- *Shigellasonnei*(ATCC 25931)
4- *Staphylococcus aureus* (ATCC 38541)
5- *Streptococcus pyrogenes* (ATCC 19615)
6- *Staphylococcus aureus* (ATCC 25923)
7- *Neisseria gonorrhoeae* (ATCC 49226)

All bacterial strains were maintained on nutrient agar medium at ±37°C.

Antibacterial Activity

Antibacterial activity was determined by disc diffusion method. Disc diffusion method is a versatile and famous method for determining the antibacterial activity. Nutrient agar media was prepared, sterilized and poured into sterile petri dishes under sterile environment. The plates were inoculated by 15 µl suspension of bacterial growth culture. Stock solutions (10 µg/1.0 µl for all the compounds) were prepared. Drug solution was used for soaking filter discs. These filter discs were placed with the help of sterilized forceps on these inoculated plates. These plates were then incubated at 37 ^{o}C for whole night. The results were obtained after 24 h by measuring the inhibition zone diameter values of each compound with the help of scale.

Antioxidant Activity by DPPH Method

The antioxidant activity of compounds (**1-7**) was determined by DPPH method [23].The 1 mg/1.0 ml solutions of all synthesized azo-compounds were prepared. 50 µl solution of each compound was added into the 2 ml of freshly prepared 0.2 mM DPPH solution in methanol. All the above prepared solutions were incubated for 20 min at 37 °C. Their absorbance was taken at 517 nm with the help of double beam spectrophotometer (Humas). Ascorbic acid (1 mM) was taken as a positive control. Percentage scavenging activity of the synthesized azo- compounds was calculated by using the following formula.

$$\text{Percent Scavenging} = \left(\frac{A_D - A_C}{A_D}\right) \times 100 \Rightarrow (1)$$

where

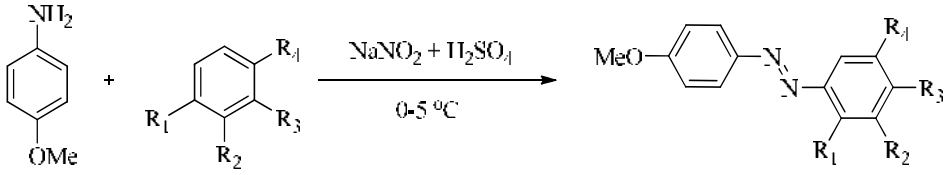
A_D = Absorbance of DPPH solution
A_C= Absorbance of sample solution

Computational Procedure

All computations were performed using Gaussian 09 program package. All systems were optimized using density functional theory. B3LYP functional and 6-31g (d,p) basis set were used.

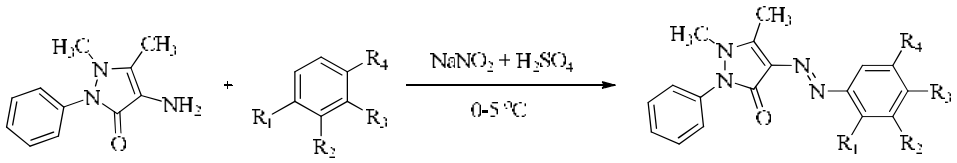
Results and Discussion

Azo-compounds **(1-7)** were prepared by diazotization of 4-methoxyaniline and 4-aminophenazone followed by treating with active aromatic compounds at temperature below 5°C [7]. The aromatic compounds used were 4-nitrophenol, 2-aminobenzoic acid, benzamide, 4-aminobenzoic acid, resorcinol, *o*-bromonitrobenzene and 2-nitroaniline as coupling partners (Scheme I and II). The synthesized compounds were purified through recrystalisation and characterized by usual spectroscopic techniques (FTIR, UV and ^{1}H-NMR etc.) and mass spectrometry. For example, the azo functionality was proved by the presence of stretching frequency around 1600 cm^{-1}, and λ_{max} stands about 358 nm almost for each new compound. These results were further supported by measuring ^{1}H-NMR spectrum in deuterated dimethylsuloxide (DMSO-d_6) at 300 MHz. For instance, in case of compound **1** the spectrum shows the presence of only one downfield singlet at 8.10 ppm for the solo proton adjacent to nitro group. Similarly, the proton NMR spectrum manifests a downfield doublet at 7.97 ppm for other aromatic proton neighboring to the nitro group. Moreover, a multiplet ranging from 7.86 to 7.65 ppm for aromatic protons, and two singlets at 3.85 and 11.96 for methoxy and hydroxyl groups respectively have been observed too. The ^{1}H-NMR spectrum unequivocally confirms the structure of compound **1**. The molecular masses of compounds (**1-7**) were corroborated by Electron Ionization mass spectrometry. Their mass spectra showed molecular ion peaks as base peaks. So all the spectral data are in good agreement with the structures of the presented compounds.



1: R_1 = OH R_2 = H R_3 = H R_4 = NO_2
2: R_1 = Br R_2 = NO_2 R_3 = H R_4 = H
3: R_1 = NH_2 R_2 = COOH R_3 = H R_4 = H

Scheme: I



4: R_1 = NH_2 R_2 = NO_2 R_3 = H R_4 = H
5: R_1 = NH_2 R_2 = H R_3 = H R_4 = COOH
6: R_1 = OH R_2 = H R_3 = OH R_4 = H
7: R_1 = H R_2 = $CONH_2$ R_3 = H R_4 = H

Scheme: II

Biological Activity

Antibacterial activity

Azo-compounds (**1-7**) were evaluated for their antibacterial activity against seven aforementioned microbes. Compounds (**1-7**) showed medium to good antibacterial activity against all strains (Table-1 and Fig 1). Compound **4** showed good results against *E. coli* (ATCC 35318) while most of the compounds exhibited comparable activity against *S. aureus* (ATCC 38541) to that of standard drug (Cefpodoxime). All compounds were remarkably active against *S. sonnei* (ATCC 25931) and *E. coli* (ATCC 25922). Almost low activity of test compounds has been observed against *S. pyogenes* (ATCC 19615). Test compounds were found significantly active against *S. aureus* (ATCC 25923) and less active against *N. gonorrhoeae* (ATCC 49226).

DPPH Scavenging Activity

Compounds (**1-7**) were also analyzed for their antioxidant potential. DPPH method was used to determine the free radical scavenging activities of the newly prepared compounds by adapting literature methodology [23]. Solution of test compounds (50 µl each) was added to 2 ml of 0.2 mM ethanolic solution of DPPH. After incubation for 20 min at temperature = 37 °C, absorbance of the mixtures was noted at λ = 517 nm. Ascorbic acid (1 mM) was used as positive control. Free radical scavenging (% age) of the samples was calculated by the formula given below.

$$\% \text{ Scavenging} = \left(\frac{Ao - AT}{Ao}\right) \times 100$$

where; A_o = Absorbance of DPPH solution, A_T = Absorbance of sample solution

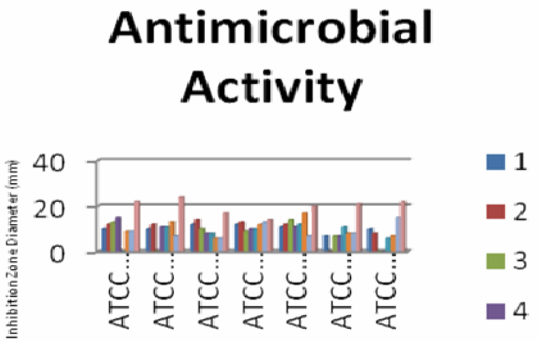


Fig. 1: Antibacterial activity of synthesized compounds against seven different ATCC bacterial strains. Cefpodoxime was used as e reference drug

Table-1: Antibacterial activity of synthesized compounds **(1-7).**

Sample codes	*Escherichia coli*	*Staphylococcus aureus*	*Staphylococcus aureus*	*Shigellasonnei*	*Escherichia coli*	*Streptococcus pyrogenes*	*Neisseria gonorrhoeae*
				Mean (IZD) mm			
1	10±0.2	10±0.5	12±0.8	12±0.4	11±0.2	7±0.8	10±0.5
2	12±0.2	12±0.6	14±0.2	13±0.7	12±0.2	NIL	8±0.6
3	13±0.2	NIL	10±0.1	9±0.5	14±0.7	7±0.6	NIL
4	15±0.3	11±0.4	8±0.2	10±0.3	11±0.3	7±0.5	NIL
5	NIL	11±0.4	8±0.3	10±0.7	12±0.1	11±0.2	6±0.6
6	9±0.6	13±0.5	6±0.2	12±0.6	17±0.6	8±0.7	7±0.2
7	9±0.4	7±0.3	6±0.5	13±0.5	7±0.7	8±0.2	15±0.6
Ref	22±0.3	24±0.4	17±0.6	14±02	20±0.2	21±0.1	22±0.4

Reference: Cefpodoxime

Table-2: %age scavenging of different synthesized compounds (1-7) at concentrations (1 µg/µl).

Comp. Code	Observation	Activity	Difference	%age scavenging
1	0.629	good	0.762	54.78
2	1.050	weak	0.341	24.51
3	1.115	weak	0.276	19.84
4	1.171	weak	0.220	15.81
5	1.304	weak	0.087	6.25
6	0.797	Good	0.594	42.70
7	1.358	weak	0.033	2.37
Ascorbic acid	0.201	+ive Control	1.19	85.55

The decrease in absorbance by the test samples is due to the pairing up the free electron of DPPH which correspond to its ability as antioxidant. Compounds **1** and **6** showed better antioxidant activity as compared to other compounds. Compound **7** showed much less antioxidant activity. The results of antioxidant activities are summarized in Table-2 and Fig. 2.

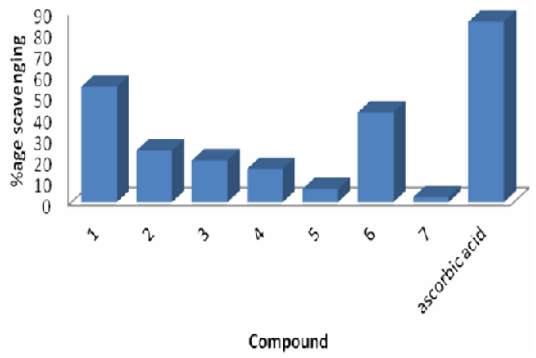


Fig. 2: Antioxidant activity of synthesized compounds.

Computational Part

Molecular Electrostatic Potential (MEP)

Molecular electrostatic potential (MEP) is a useful tool to study the structure–reactivity relationship of the molecules. It is related to electronic density and used to predict the active sites for electrophilic and nucleophilic attacks in the substrate. It is also helpful to study the hydrogen bonding interactions of biological recognition systems [24, 25]. MEP provides a visual understanding of the relative polarity of the molecule. In this study, MEP is calculated to predict reactive sites for electrophilic and nucleophilic attack of the investigated compounds, MEP studies was carried out by B3LYP using 6-31G(d,p) basis set. A visual representation of the chemically active sites such as the negative (red) regions of the MEP are related to electrophilic reactivity and the positive (blue) regions to nucleophilic reactivity as shown in Fig. 3. The electrophilic attack is illustrated by red (negative) regions whereas nucleophilic reactivity is shown by the blue (positive) regions and the green region covers the parts of the molecule where electrostatic potentials are close to zero.

The region for electrophilic attack (red) is localized on the oxygen atoms having double bond with carbon, whereas the nucleophilic attack is localized on major part of molecule. The frontier molecular orbital picture can offer a reasonable qualitative prediction of the excitation properties. The HOMO energy characterizes the electron donating ability and the LUMO energy characterizes the electron accepting ability, while HOMO-LUMO energy gap is very important molecular descriptor to study reactivity and stability of molecule.

Conclusion

In this research work, a series of azo-compounds were prepared from 4-methoxyaniline and 4-aminophenazone. Their characterization was checked along with biological activities including antibacterial and antioxidant activities. Results reveal that these compounds have moderate to excellent antibacterial activities against all seven strains in comparison to reference drug (Cefpodoxime). Thus these synthesized azo-compounds can be used as potent drugs for bacterial infections as well as efficient dyes in textile industry.

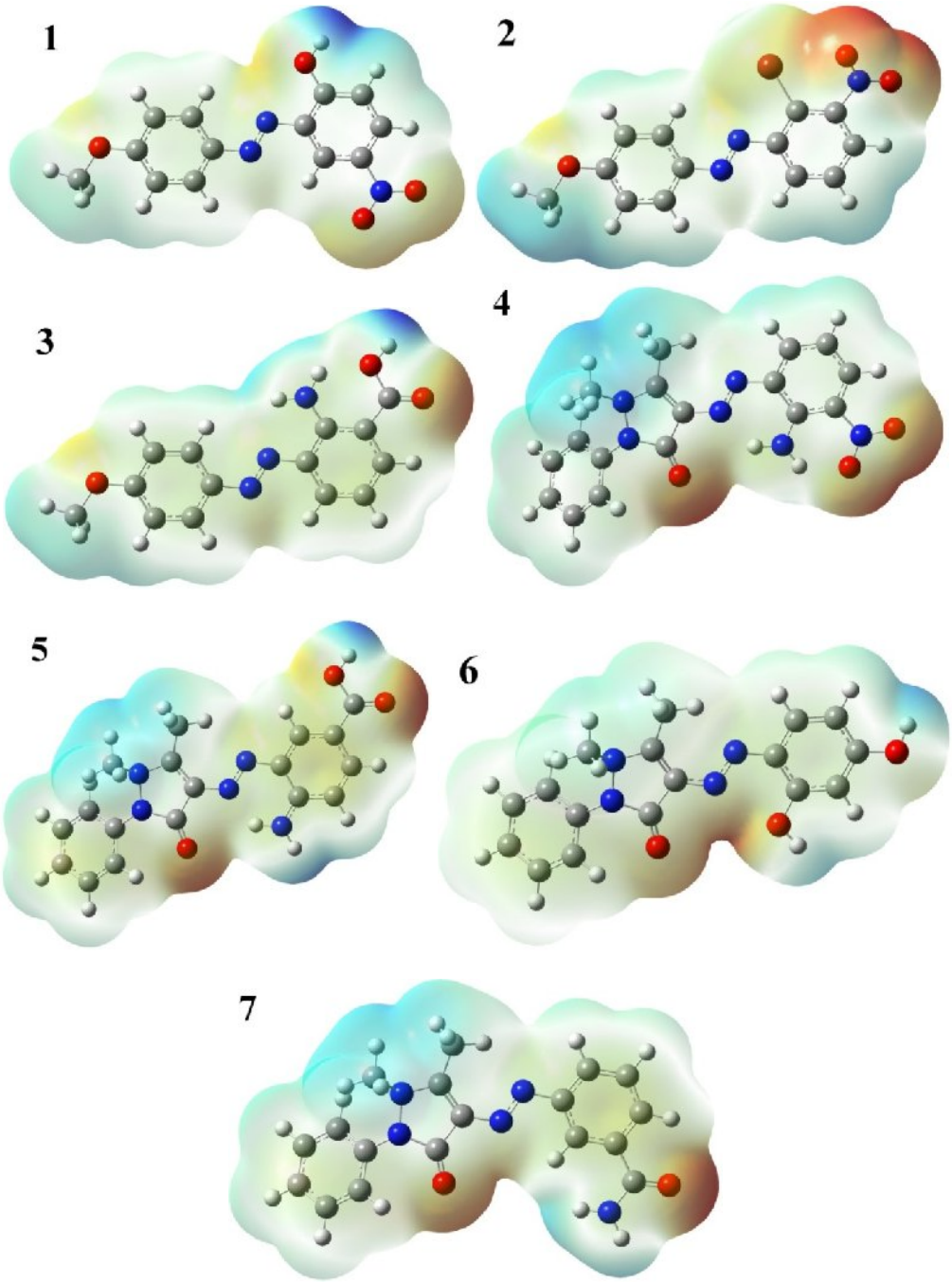


Fig. 3: MEP surface map of compounds **(1-7)**

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