SYNTHESIS, BIOLOGICAL EVALUATION, AND STRUCTURAL STUDIES OF 3-PHENYL[1,2,4]OXADIAZOLE-5-CARBOXYLIC ACID BENZO[1,3]DIOXOL 5-YLMETHYLENE-HYDRAZIDE

J. M. Santos-Filho[1 *], J. G. de Lima[2], L. F. C. C. Leite[3],
E. A. Ximenes[1], J. B. P. da Silva[1], P. C. Lima[2], I. R. Pitta[1]
[1]Departamentos de Engenharia Química, Antibióticos, Farmácia e Química Fundamental
Universidade Federal de Pernambuco, Recife, PE, 50740-521, Brasil
[2]Departamento de Química
Universidade Católica de Pernambuco, PE, 50.005-090, Brasil

ABSTRACT

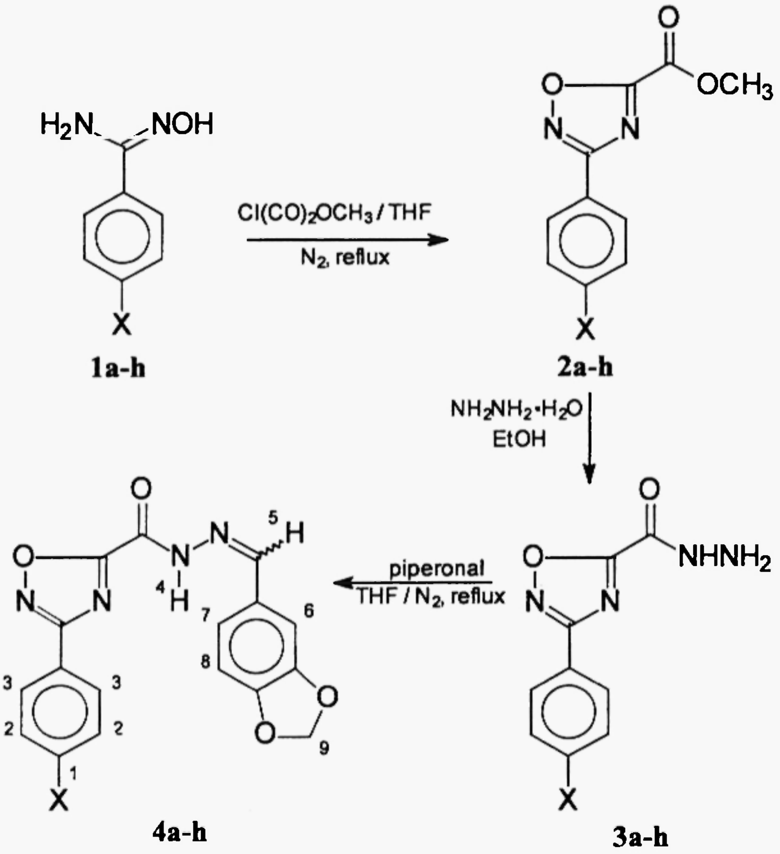
A series of 1,2,4-oxadiazole combined with carbohydrazides residues, designed as possible bioactive compounds, was obtained in an attempt to analyse the effects of this molecular hybridization on the biological properties of the resulting compounds. They were tested for their antimicrobial and antimalarial activity. *Ab initio* calculations were performed in order to strengthen experimental NMR ^{1}H structural data.

INTRODUCTION

Many compounds containing the oxadiazole ring are well known for their important biological activities. In recent years, several substances have been considered for different kinds of studies, and these have included this heterocyclic system, in an effort to find new drug candidates[1-5]. The bioisosteric approach is extensively used for this objective, especially to synthesize new oxadiazole-containing compounds with dual activity on cyclooxygenase and 5-lipoxygenase biosynthesis, in an attempt to produce analgelsic and antiinflammatory drugs without the undesirable side effects of the most commonly used NSAIDs[6-8]. Antimicrobial and hypoglicemic activities are also related with satisfactory performance[9-12]. Studies on bioisosteric replacements in the oxadiazole heterocycle have focused the rings of 1,3,4-oxadiazole and 1,2,4-oxadiazole, and the latter has been our main interest, because it has shown itself to be an important fragment for the success of molecular interaction with biological systems.

On the other hand it has been known that aliphatic moieties are very potent pharmacophoric groups, furnishing compounds with a large range of biological applications. The acyclic molecular fragment that has attracted more and more attention is the carbohydrazide, reported as essential for positive results for biological activity in some derivatives containing this group[13-15].

Analysing this information, we planned the synthesis of a new series of molecules bringing into their structures a ring of 1,2,4-oxadiazole linked to an carbohydrazide fragment **4a-h** for studying their biological applications. For compounds **4a-h**, the bioisosteric replacing groups chosen (i.e. H, CH_3, F, Cl, Br, NO_2, OCH_3, OH) were elected in an attempt to introduce an important variation in σ_p-Hammet values[16] (ranging from -0.37 for OH to 0.78 for NO_2), which can set up some electronic contribution of the *para*-substituent on the phenyl ring to the possible activity. In this paper, we report the synthesis, and characterization of the proposed compounds, as well as their preliminary antimicrobial and antimalarial evaluation. Finally, *ab initio* Hartree-Fock[17] calculations using the 3-21G**[18] basis set were made using the Gaussian 94 program[19] in order to access structural and electronic information for some predicted configurational and conformational isomers. The synthetic route applied three steps until the derivatives 4a-h had been obtained according to figure 1.



X: a =H, b =Me, c =OMe, d =NO_2, e =Br, f=F, g =Cl, h =OH

Figure 1. Synthetic pathways

CHEMISTRY

The methodology applied for the synthesis of compounds **4a-h** was modified in an attempt to obtain the final products in better yields than those reported for similar syntheses[8]. Traditional methodology used for obtaining carbohydrazide residues was not adequate in this case, generating final products with a high level of impurity and low yield of the main compound. With the change in the solvent used and the introduction of inert atmosphere into the methodology, it became easier to purify the final products using chromatographic methods and yields were greatly increased.

Compounds **4a-h** were initially obtained as a mixture of (*E*)-(*Z*) isomers, due to the two possible configurations around the N=C bond in carbohydrazide derivatives. In order to assure the determination of the diastereomeric ratio, essential to understanding the biological profile, we analysed the ^{1}H NMR spectra of these derivatives, which revealed the presence of two sets of signals referring to the protons (H-5) and (H-9). The imino hydrogen (H-5) signal of the major diastereomer is downfielded by 0.43-0.47 ppm when compared to the minor one, and this result is commonly used to characterize the isomeric mixture, based on results previously published by Karabatsos[23,24]. However, a 300 MHz spectrometer is now able to detect other protons associated with this phenomenon. In this case, we have identified two signals related to the methylenedioxy protons (H-9) in the spectrum, which are separated by 0.08-0.09 ppm, and the more intense signal, associated with the main diastereomer, is in a down field, exactly like the (H-5) protons signals. Another important observation is the duplication of the aromatic hydrogens (H-6, -7, -8). The integration of the signals produced by (H-5) and (H-9) has conducted to the (*E*)/(*Z*) ratio.

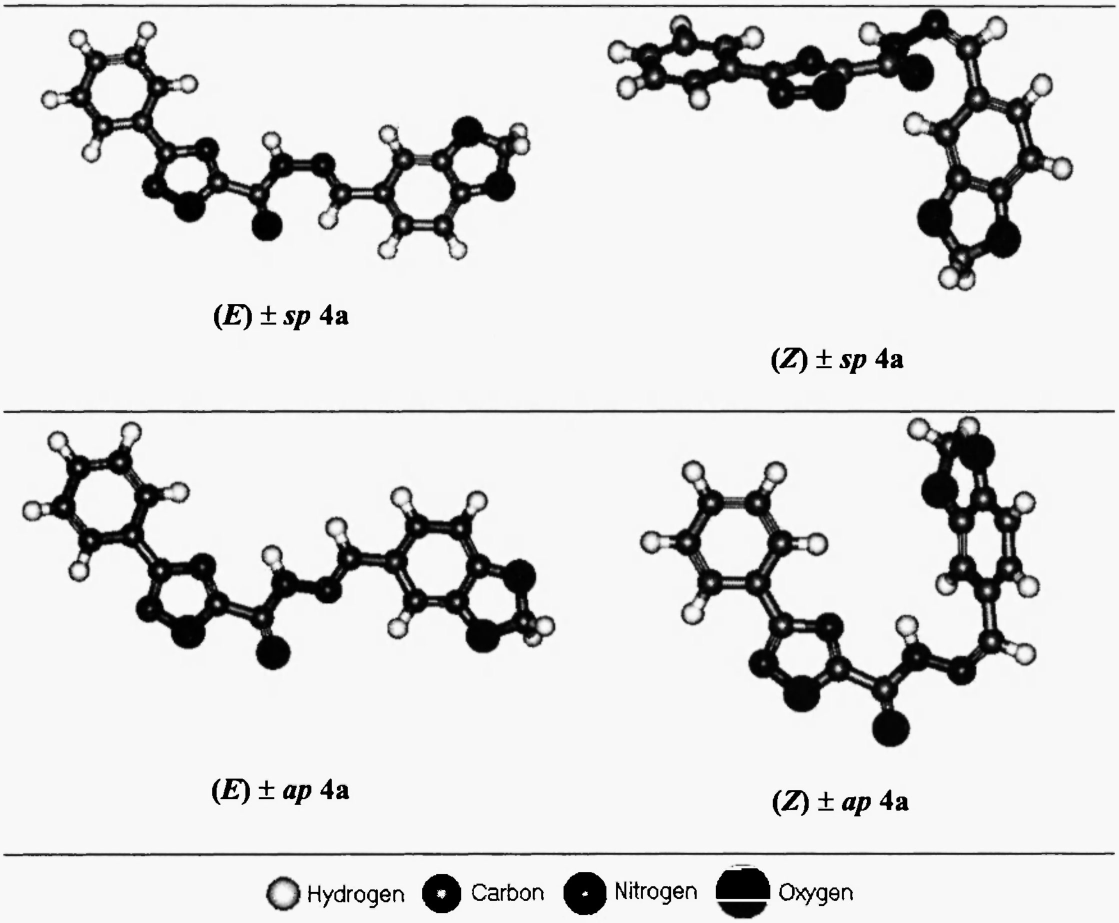
MOLECULAR MODELING

A simple inspection of the structure of the compounds **4a-h** shows the possibility of geometric isomerism at imine C=N bond, i.e., the (*Z*)- and the (*E*)-diastereomers. Additionally, we have investigated the conformational behavior of the carbohydrazide framework, considering two minimum-energy conformers with *syn* and *anti* arrangements, corresponding to the CNNC dihedral angle equal to 0 (±*sp*) and 180 degrees (±*ap*). In order to evaluate these four minimal structures we decided to choose the compound **4a** as a prototype system and to perform *ab initio* calculations at Hartree-Fock level[17] using the 3-21G** basis set and the Gaussian 94 program.

The HF/3-21G** relative energy (ΔE) were calculated for the four minimal structures of **4a**. We can observe that the structures associated with ±*sp* (*E*) and ±*ap* (*E*) are the most stable as demonstrated with the ΔE values (kcal/mol) (*E*) ± sp = 0, (*E*) ± ap = 2.74, (*Z*) ± sp = 10.41, (*Z*) ± ap = 6.72. In both cases the (*E*) isomers are planar leading to a resonance effect that stabilizes them, whereas the less stable (*Z*) isomers have non-planar configurations (figure 2).

If the lowest energy structure associated with ±*sp* (*E*) isomer is taken as a point of reference, then relative energies of the (*Z*) isormers are above at least 6.7 kcal.mol^{-1}. This may partially justify the ^{1}H NMR spectra in this series where (*Z*) isomers do not exceed 14% in the isomeric mixture as discussed above. With respect to the values given above, we can also observe that the ±*sp* (*E*) isomer is predicted to be more stable than the ±*ap* (*E*) isomer in at least 2.7 kcal.mol^{-1}. The distance between the oxygen atom of the carbonyl group and the H5 proton (2.13 Å) configure the formation of a non-standardized hydrogen bond between C=O···H–C in ±*sp* (*E*) isomer[25]. It is important to stress that the proximity of H-5 to the C=O oxygen atom can explain the major separation between the two signals of this proton observed in 'H NMR, because in the ±*sp* (*E*) isomer the proton H-5 is deshielded by the oxygen, laying around 0.43-

0.47 ppm in a down field compared with the same proton in the (*Z*) isomers, where the proton and the oxygen atom are far one from another as shown in figure 2. The two singlets of protons H-9 have a difference of around 0.08-0.09 ppm. The duplication of aromatic protons (H-6, -7, -8) has not been observed in analogous compounds when the ^{1}H NMR spectra are recorded at 200 MHz. This intensity of field was not enough to resolve these protons for which the chemical environment is almost the same. However, at 300 MHz this phenomenon was detected, especially for the protons in the methylenedioxy moiety, H-9, which are very important for the characterization of this series of compounds.


(*E*) ± *sp* 4a

(*Z*) ± *sp* 4a

(*E*) ± *ap* 4a

(*Z*) ± *ap* 4a

Figure 2. Representation of the minimal energy conformations of the (*E*) (*Z*) 3-phenyl-[1,2,4]oxadiazole-5-benzo[1,3]dioxol-5-ylmethylene-carbohydrazide derivative **4a**.

BIOLOGICAL ACTIVITY

The antimicrobial activity of compounds **4a-h** was determined *in vitro* against nine different strains of pathological microorganisms: Gram-positive bacteria (*Staphylococcus aureus*, *Bacilus cereus*, *Mycobacterium smegmatis*, *Mycobacterium phlei*, and *Mycobacterium fortuitum*), Gram-negative bacteria (*Escherichia coli*, and *Pseudomonas aeruginosa*), and yeasts (*Candida albicans* and *Candida krusei*). The compounds under study were evaluated using the disk method proposed by Kirby and Bauer[20] for a preliminary determination of their activity. Bacteria were tested using a Muller-Hinton agar medium which is the reference medium for antimicrobial tests. Each disk, previously saturated with 20µL of a solution of compounds 4a-h in THF in a concentration of 2560µg/mL and dried, was placed on the agar surface. A disk containing only the solvent and another one saturated with 20µL of the aqueous solution of standard antibiotic ciprofloxacin chlorohydrate in concentration of 1280µg/mL were also placed on the agar surface. Plates were incubated for about 18h at 37^0C and activity was evaluated by the presence or absence of inhibition zones and by comparison of the diameters of the halos with that shown by the standard drug. Yeasts were tested using the same technique, but they were grown in a Sabouraud broth medium and Petri's plate as prepared with Sabouraud agar. The incubation temperature was 30^0C for 18h. Cycloheximide was used as the standard antifungal drug at a concentration of 1280µg/mL.

The *in vitro* antimalarial assay[21] used for evaluation of the compounds was an adaptation of the parasite lactate dehydrogenase (pLDH) based assay originally developed by Makler *et al.*[22] The assay was set up in 96 well microplates. The antimalarial action of each compound was tested at at least three different concentrations (i.e. 4760, 1587 and 528,8 ng/mL) on two *Plasmodium falciparum* clones: a chloroquine sensitive D6-Sierra Leon clone and a chloroquine resistant W2-Indochine clone. The antimararial drugs chloroquine and artemisinin were used as the positive controls and DMSO was tested as the negative control for each assay. The level of pLDH enzyme produced by the parasite depends on the activity of each compound and was measured in spectrophotometer at 650 nm by the reduction of nitroblue tetrazolium to the blue formazan salt. Results are analysed using the Log-Logit method on specific software, furnishing the IC_{50} data values. Compounds **4a-h** are inactive against tested microorganisms and also for the antimalarial screen at the three experimental concentrations.

EXPERIMENTAL

Melting points were determined in a glass capillary tube with a QUIMIS 340 apparatus. Infrared spectra (IR) were recorded on a Bruker IFS66 spectrometer using KBr pellets. Electronic impact mass spectra (MS) were obtained using a Finnigan GCQ Mat quadripole Ion-Trap type instrument operating at 70eV, and samples were introduced by using a direct inlet system. Proton magnetic resonance spectra were determined in DMSO-d_6, using tetramethylsilane as an internal standard with a Varian Unit Plus 300MHz spectrometer. Splitting patterns are as follows: s, singlet; d, doublet; t, triplet; dd, double doublet; m, multiplet; b, broad. Elemental analysis (C, H, N) data were obtained using a Carlo Erba Elementary Analyser Model EA1110. The analytical data for C, H, N were within ± 0.4% of calculated values. The progress of all reactions was monitored by TLC performed on 2.0cmx6.0cm plates prepared with silica gel 60 (PF-254 with gypsum, Merck) of a thickness of 0.25mm. The developed chromatograms were visualized under ultraviolet light at 254-265nm. For column chromatography, Merck silica gel 60 (230-400 mesh) was used.

General procedure for the preparation of substituted *methyl 3-phenyl-[1,2,4]oxadiazole-5-carboxilates* **2a-h**[8]

A solution of 0.01mol of benzamidoxime derivatives **1a-h**, obtained as described by Leite *et al.*[8], and 1.63mL (0.01mol) of methyl oxalyl chloride in 50mL of dry THF was refluxed in an inert atmosphere for 4.5h. The solvent was then completely removed and the methyl esters **2a-h** were recrystallized from ethanol and characterized in terms of their physical properties.

General procedure for the preparation of substituted *3-phenyl-[1,2,4]oxadiazole-5-carbohydrazide* **3a-h**

To a solution of 0.01mol of methyl ester derivatives **2a-h** in 10mL of ethanol were added 0.57mL (0.01mol) of 55% hydrazine monohydrate. The mixture was stirred in a cold ice bath for 2h, when TLC analysis indicated the end of the reaction. The solid material was filtrated out, washed with cold water and ethanol, affording the hydrazide derivatives **3a-h** in almost quantitative yields[8].

General procedure for the synthesis of *3-phenyl[1,2,4]oxadiazole-5-benzo[1,3]dioxol-5-ylmethylene-carbohydrazide* derivatives **4a-h**

A mixture of 0.01mol of derivative **3a-h** and 0.01mol of piperonal in 50mL of THF was refluxed in an inert atmosphere for 4h. Using a not very polar solvent on synthesis, we obtained better yields than those found for analogous compounds. Then, all solvent was evaporated out and compounds were purified in a flash chromatographic column before being recrystallized from dioxane/water system.

3-Phenyl[1,2,4]oxadiazole-5-benzo[1,3]dioxol-5-ylmethylene-carbohydrazide **4a**, yield =98; Mp (°C) = 183-185; IR (υ cm^{-1}) = 3311 (N-H), 1706 (C=O), 1593 (C=N), 3068 (C-H); MS (m/z %) = 336 M$^+$(100), 190(12), 189(4), 162(13), 147(89), 134(40), 104(9), 103(4); ^{1}H NMR (δ ppm) = 7.66-7.62 (m, H-1, H-2), 8.1(dd, H-3), 12.62(s, H-4), 8.54(s, H-5 *E*), (H-5 *Z*, masked by aromatic protons), 7.32 (d, H-6, J=1.5Hz), 7.23(dd, H-7, J=8.1, 1.5Hz), 7.01 (d, H-8, J=8.1Hz), 6.11(s, H-9, *E*), 6.02(s, H-9, *Z*); ratio (*E*/*Z*) = 86/14.

3-(4-Methyl-phenyl[1,2,4]oxadiazole-5-benzo[1,3]dioxol-5-ylmethylene-carbohydrazide **4b**, yield = 91; Mp (°C) = 218-220; IR (υ cm^{-1}) = 3313 (N-H), 1705 (C=O), 1591 (C=N), 3045 (C-H); MS (m/z %) = 350 M$^+$(100), 203(12), 190(9), 162(11), 147(68), 134(32), 117(5), 104(6); ^{1}H NMR (δ ppm) = 2.41 (s, H-1), 7.42 (d, H-2, J=8.1Hz), 7.99(d, H-3, J=8.4Hz), 12.6(s, H-4), 8.53(s, H-5 *E*), 8.09(s, H-5 *Z*), 7.31 (d, H-6, J=1.5Hz), 7.22(dd, H-7, J=8.1, 1.5Hz), 7.01 (d, H-8, J=8.1Hz), 6.1(s, H-9, *E*), 6.02(s, H-9, *Z*); ratio (*E*/*Z*) = 88/12.

3-(4-Methoxi-phenyl[1,2,4]oxadiazole-5-benzo[1,3]dioxol-5-ylmethylene-carbohydrazide **4c**, yield = 98; Mp (°C) = 184-186; IR (υ cm^{-1}) = 3312 (N-H), 1708 (C=O), 1592 (C=N), 3009 (C-H); MS (m/z %) = 366 M$^+$(100), 219(57), 190(9), 162(12), 147(54), 134(33), 133(22), 104(6); ^{1}H NMR (δ ppm) = 3.86 (s, H-1), 7.15 (d, H-2, J=9Hz), 8.04(d, H-3, J=9Hz), 12.59(s, H-4), 8.53(s, H-5 *E*), 8.08(s, H-5 *Z*), 7.32 (d, H-6, J=1.5Hz), 7.22(dd, H-7, J=8.1, 1.5Hz), 7.01 (d, H-8, J=7.8Hz), 6.11(s, H-9, *E*), 6.02(s, H-9, *Z*); ratio (*E*/*Z*) = 86/14.

3-(4-Nitro-phenyl[1,2,4]oxadiazole-5-benzo[1,3]dioxol-5-ylmethylene-carbohydrazide **4d**, yield = 94; Mp (°C) = 258-260; IR (υ cm^{-1}) = 3304 (N-H), 1712 (C=O), 1591 (C=N), 3095 (C-H); MS (m/z %) = 381 M$^+$(74), 190(22), 162(12), 148(12), 147(100), 134(38), 104(7); ^{1}H NMR (δ ppm) = 8.36 (d, H-2, J=9Hz), 8.46(d, H-3, J=9Hz), 12.6(s, H-4), 8.55(s, H-5 *E*), 8.08(s, H-5 *Z*), 7.31 (d, H-6, J=1.2Hz), 7.24(dd, H-7, J=8.1, 1.5Hz), 7.01 (d, H-8, J=8.1Hz), 6.11(s, H-9, *E*), 6.02(s, H-9, *Z*); ratio (*E*/*Z*) = 91/9.

3-(4-Bromo-phenyl[1,2,4]oxadiazole-5-benzo[1,3]dioxol-5-ylmethylene-carbohydrazide **4e**, yield = 81; Mp (°C) = 241-243; IR (υ cm^{-1}) = 3318 (N-H), 1708 (C=O), 1596 (C=N), 3023 (C-H); MS (m/z %) = 415 M$^+$(24), 417(6), 267(4), 190(17), 162(14), 147(100), 134(39); ^{1}H NMR (δ ppm) = 7.84 (d, H-2, J=8.4Hz), 8.03(d, H-3, J=8.4Hz), 12.64(s, H-4), 8.53(s, H-5 *E*), 8.09(s, H-5 *Z*), 7.32 (d, H-6, J=1.5Hz), 7.22(dd, H-7, J=8.1, 1.5Hz), 7.01 (d, H-8, J=8.1Hz), 6.11(s, H-9, *E*), 6.02(s, H-9, *Z*); ratio (*E*/*Z*) = 87/13.

3-(4-Fluoro-phenyl[1,2,4]oxadiazole-5-benzo[1,3]dioxol-5-ylmethylene-carbohydrazide **4f**, yield = 94; Mp (°C) = 195-197; IR (υ cm^{-1}) = 3306 (N-H), 1704 (C=O), 1592 (C=N), 3045 (C-H); MS (m/z %) = 354 M^+(100), 207(3), 190(12), 162(17), 147(95), 134(36), 121(6), 104(5); ^1H NMR (δ ppm) = 7.46 (m, H-2), 8.17(m, H-3), 12.63(s, H-4), 8.53(s, H-5 *E*), 8.1(s, H-5 *Z*), 7.32 (d, H-6, J=1.5Hz), 7.23(dd, H-7, J=8.1, 1.5Hz), 7.02 (d, H-8, J=7.8Hz), 6.11(s, H-9, *E*), 6.03(s, H-9, *Z*); ratio (*E*/*Z*) = 86/14.

3-(4-Chloro-phenyl[1,2,4]oxadiazole-5-benzo[1,3]dioxol-5-ylmethylene-carbohydrazide **4g**, yield = 86; Mp (°C) = 232-234; IR (υ cm^{-1}) = 3307 (N-H), 1709 (C=O), 1596 (C=N), 3023 (C-H); MS (m/z %) = 370 M^+(90), 372(24), 223(4), 225(2), 190(14), 162(14), 147(100), 134(38); ^1H NMR (δ ppm) = 7.7 (d, H-2, J=8.7Hz), 8.11(d, H-3, J=8.7Hz), 12.64(s, H-4), 8.53(s, H-5 *E*), (H-5 *Z*, masked by aromatic protons), 7.32 (d, H-6, J=1.5Hz), 7.23(dd, H-7, J=8.1, 1.5Hz), 7.02 (d, H-8, J=7.8Hz), 6.11(s, H-9, *E*), 6.03(s, H-9, *Z*); ratio (*E*/*Z*) = 88/12.

3-(4-Hydroxi-phenyl[1,2,4]oxadiazole-5-benzo[1,3]dioxol-5-ylmethylene-carbohydrazide **4h**, yield = 93; Mp (°C) = 231-233; IR (υ cm^{-1}) = 3260 (N-H), 1679 (C=O), 1598 (C=N), 3068 (C-H); MS (m/z %) = 352 M^+(7), 351(100), 205(31), 190(7), 162(14), 147(57), 135(11), 134(79); ^1H NMR (δ ppm) = 10.25(s, H-1), 6.96 (d, H-2, J=8.7Hz), 7.93(d, H-3, J=8.4Hz), 12.65(s, H-4), 8.51(s, H-5 *E*), 8.07(s, H-5 *Z*), 7.32 (d, H-6, J=1.5Hz), 7.22(dd, H-7, J=8, 1.5Hz), 7.01 (d, H-8, J=8.1Hz), 6.1(s, H-9, *E*), 6.02(s, H-9, *Z*); ratio (*E*/*Z*) = 91/9.

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