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Laboratory note

Synthesis, pharmacological screening, quantum chemical and *in vitro* permeability studies of *N*-Mannich bases of benzimidazoles through bovine cornea

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Abstract

A novel series of *N*-Mannich bases of benzimidazole derivatives were synthesized and characterized by ^{1}H NMR, IR spectral studies and elemental analysis. The compounds were screened for analgesic and anti-inflammatory activity. 1-((Diethylamino)-methyl)-2-styryl benzimidazole **4** at 40 mg/kg was found to be equipotent to paracetamol. 1-((Piperidin-1-yl) methyl)-2-styryl-benzimidazole **6** at 40 mg/kg was found to be more potent than Diclofenac. Corneal permeability and quantum chemical calculations were performed to correlate the hydrogen bonding ability with permeability and activity. The energies of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) were correlated with pharmacological activity. The semi-empirical PM3 calculations (quantum chemical calculations) revealed that E_{LUMO} and energy gap ΔE were capable of accounting for the high *in vitro* bovine corneal permeability and activity of the compounds.

Keywords: Mannich base; Benzimidazole; Analgesic; Anti-inflammatory; Corneal permeability; Quantum chemical calculations

1. Introduction

The search for novel analgesic and anti-inflammatory agents devoid of side effects, such as irritant reactions on gastric mucosa, profound respiratory depression, nausea, constipation and physical dependence, continues to be an active area of research in medicinal chemistry. Among the various compounds developed as anti-inflammatory [1] and analgesic agents [2], the 2-substituted benzimidazoles [3,4] and *N*-Mannich bases of various heterocyclic compounds [5–8] were reported to exhibit anti-inflammatory [9] and analgesic properties. The aim of the present work is to incorporate both modules to the same molecule, with an anticipation of enhanced drug activity.

As clinical efficacy of these kinds of molecules depends upon tissue penetrations and retention, *in vitro* corneal permeability studies were performed by measurement of transcorneal flux in the isolated bovine cornea. Isolated tissues containing different endothelial and epithelial cell lines were subjected to drug permeation studies [10–12]. The cornea is one of the most widely used tissues for biological tissue permeability studies [13]. The permeation of non-polar compounds through cornea depends on their oil/water partition

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coefficients [14]. For instance, the influence of the substituent in a series of *p*-amino phenyl derivatives was correlated to their transport through rabbit cornea [15].

Electronic and steric effects have also been shown to control the pharmacological activities of drugs [16]. Quantum chemical parameters in quantitative structure–activity relationships (QSAR) are reported to yield promising results for correlation of biological activity [17,18]. At the molecular level, the reactivity of a molecule is dominated by the frontier molecular orbitals (FMO), namely the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO). The effects of electron withdrawing or donating substituents will be manifested in the HOMO and LUMO energies and in the molecular orbital coefficients. Consequently, these quantum chemical parameters can act as good indicators in QSAR studies. The energy of the lowest unoccupied molecular orbital (LUMO) was established as a good parameter [19] for hydrogen bond donor capacity. It is observed that the energy of LUMO, E_{LUMO}, correlates well with the experimental hydrogen bond acidity parameter $\Sigma\alpha_2H$ [20,21]. It should be also noted that when α_2H is high, E_{LUMO} is small.

In our present investigation, the benzimidazole derivatives of *N*-Mannich bases were synthesized (Fig. 1) and their analgesic and anti-inflammatory activities were studied. Corneal permeability of the synthesized compounds was assessed by measuring the transcorneal flux of the compounds through isolated bovine cornea. The resulting permeability data are compared with quantum chemical parameters, such as the energies of HOMO and LUMO [22]. Our study is important, since it provides information on the physical, chemical, and biological activities of *N*-Mannich bases of benzimidazoles.

2. Chemistry

Melting points were determined in open capillary tubes on a Thomas-Hoover melting point apparatus and are uncorrected. IR spectra were recorded by dispersing the compounds in KBr pellets on a Bomem FT-IR spectrophotometer M.B Serial II. ^{1}H NMR spectra were recorded on a 300 MHz Bruker DPX 200. The ^{1}H chemical shifts are reported as parts per million downfield from tetramethyl silane (Me_4Si). The purities of the compounds were checked by TLC on SiO_2 gel (HF_{254}, 200 mesh) coated glass plates using benzene:methanol (9:1 v/v for **2** and **3**) and chloroform:methanol (8:2 v/v for **1**, **4**, **5** and **6**) and visualized by iodine vapors. Microanalyses for C, H, and N were performed in a Heraeus CHN Rapid Analyzer, Division of Catalysis and Kinetics, Department of Chemistry, Indian Institute of Technology, Madras, India. UV measurements were made in a Shimadzu 1601 UV–Vis Spectrophotometer. ^{1}H NMR, IR and MS data were consistent with the assigned structures.

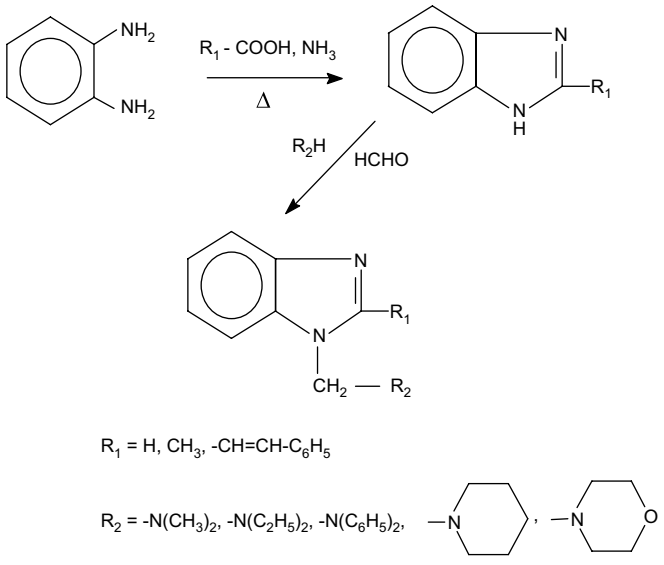

Fig. 1. Protocol for the synthetic compounds.

3. Biological investigation

The synthesized compounds were evaluated for analgesic and anti-inflammatory activities. Statistical analysis (student's *t*-test) was performed for the activities to ascertain their significance. The dose was fixed between the minimal effective dose and maximal non-lethal dose. The test compounds were administered in the form of a suspension (1% carboxy methyl cellulose as vehicle).

3.1. Acute toxicity studies

Acute toxicity tests [23] were performed on the compounds to determine LD_{50} values by Karber's arithmetical method. Each group consisted of six Wistar albino mice of either sex. The synthesized compounds were administered at doses of 100, 200, 400, 800 or 1000 mg/kg orally by gavage. The animals were observed for 24 h for mortality. The data are presented in Table 1.

3.2. Analgesic activity

Analgesic activity [23] was determined by the acetic acid-induced writhing method using Wistar albino mice of either

Table 1
Analgesic activity and LD_{50} of the synthesized compounds

Compound	Dose (mg/kg)	% Protection	LD_{50} (mg/kg)
1	100	25.87**	>1000
	200	31.4**	
2	100	11.35*	>1000
	200	43.8**	
3	100	8.5*	>1000
	200	16.1*	
4	20	32.2**	175
	40	47.49**	
5	20	20.58**	200
	40	27.45**	
6	20	29.56**	180
	40	43.80**	
Paracetamol	100	47.76	–

* $p < 0.05$ and ** $p < 0.001$ compared to control.

sex selected by random sampling techniques. A dose level of 100 mg/kg paracetamol (oral) served as the standard drug for comparison. The test compounds were administered orally at two doses by gavage 30 min prior to administration of the writhing agent (0.6% v/v aqueous acetic acid − 1 mL/100 g). The writhings produced in the animal were observed for 30 min and percentage protection was calculated for analgesic activity. Results are presented in Table 1.

3.3. Anti-inflammatory activity

Anti-inflammatory activity [24] was determined by the formalin-induced paw edema method in Wistar albino rats by using plethysmography. Diclofenac sodium at an oral dose of 50 mg/kg served as the standard drug for comparison. The test compounds were administered orally 30 min prior to administration of formalin (0.1 mL of 1% w/v) in the plantar region of the paw. The paw volumes were measured at 30, 60, 90 and 120 min after formalin administration. The results are presented in Table 2.

3.4. In vitro permeability studies

Bovine corneas were obtained within 1 h of sacrifice. Integrity of the corneal tissue was checked by microscopic observation. The eye was proptosed, a small transverse incision was made about 5 mm from the limbus, and the cornea with the scleral ring was carefully excised. Forceps were used to delicately remove first the lens and then the iris, with the cornea being left as a transparent film. The fresh cornea [13,25] was placed between the donor and receptor cell of a side-by-side perfusion apparatus, which maintained the corneal curvature. The donor contained 7 mL of a predetermined concentration of drug solution buffered by 0.01 M methanolic phosphate buffer solution (pH 7). Samples of 1 mL were taken from the receptor side at 15 min intervals for 3 h. The sample volume was immediately replaced with an equal amount of 0.01 M methanolic phosphate buffer solution, pre-equilibrated at 37 °C. The amount of the drug diffused was determined by measurement of its absorption in a UV−vis spectrophotometer.

The apparent corneal permeability coefficient (P_{app}p cm/s) was determined according to the following formula:

$$P_{app} = \frac{\Delta Q}{\Delta t A C_0}$$

where Q is the total amount permeated at time t; $\Delta Q/\Delta t$ (the slope of the linear portion of the graph) is the steady flux of the compound (**1**−**8**) to the receiver's side (μg/min); A is the corneal surface area (1 cm^2) and C_0 is the initial donor side drug concentration C (μg/mL). The lag time of permeation (t_L) is obtained from the above plot of concentration versus time (Figs. 2 and 3). This is the time required to establish a steady concentration gradient within the membrane separating the donor from the receptor compartment [13]. The data obtained are presented in Table 4.

Table 2
Anti-inflammatory activity (formalin-induced paw edema method)

Compound	Dose (mg/kg)	% Reduction of edema			
		30 min	60 min	90 min	120 min
1	200	48	51	64	53
2	200	28	59	63	61
3	200	32	54	60	56
4	40	20	50	51	56
5	40	52	59	74	70
6	40	48	56	59	62
Diclofenac	50	48	65	64	65

Significance level = $p < 0.001$ compared to control.

4. Results and discussion

4.1. Pharmacological screening

The synthesized compounds were evaluated for analgesic and anti-inflammatory effects against chemically induced nociception [26] and formalin-induced acute paw edema methods. The results of the evaluations for analgesic and anti-inflammatory effects are shown in Tables 1 and 2, respectively. The data indicate that the compounds exhibited dose dependent analgesic properties. One of the compounds, 1-((diethylamino)-methyl)-2-styryl-benzimidazole **4**, was found to be equipotent to paracetamol, at a dose of 40 mg/kg.

The decreases in paw volume noted after administration of the compounds (**1**−**9**) indicate that these compounds possess highly significant anti-inflammatory properties. Among the compounds 1-((piperidino-1-yl)-methyl)-2-styryl-benzimidazole **6** was found to be more potent than Diclofenac, at a dose of 40 mg/kg. According to previous reports, the anti-inflammatory activity of substituted benzimidazoles may be due to 5-lipoxygenase [4] and/or due to inhibition of immigration of leukocytes and the exudation of proteins into the site of injury [27].

The molecular orbital calculations revealed that E_{LUMO} and the energy gap ΔE are capable of accounting for the high

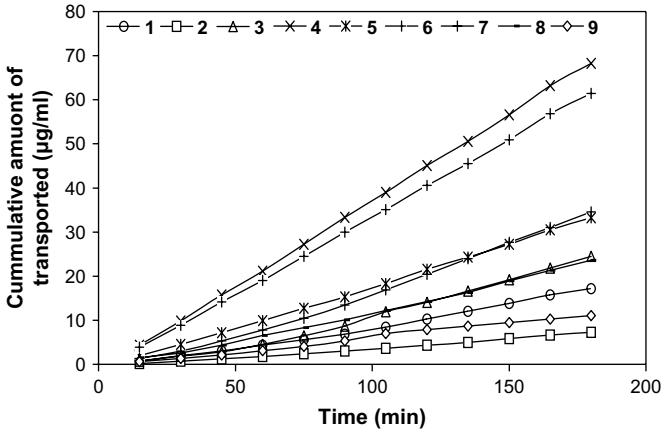

Fig. 2. Cumulative release of the synthesized compounds across bovine cornea.

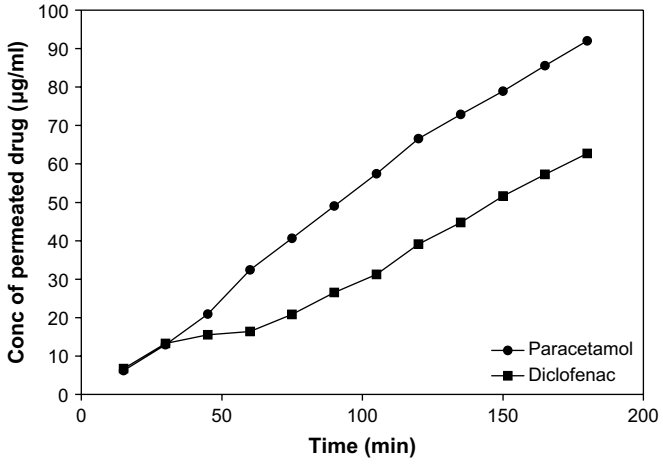

Fig. 3. Cumulative amount of standard drugs permeated through isolated bovine cornea.

permeability and pharmacological activity of the *N*-Mannich bases of 2-styryl-benzimidazole derivatives.

4.2. Quantum chemical analyses

The quantum chemical parameters of the synthesized compounds (**1**–**9**) were determined using semi-empirical PM3 calculations. Results are summarized in Table 3. E_{LUMO} is significantly low (−0.71 to −0.83 eV) in the 2-styryl-benzimidazole derivatives (**4**, **5**, **6** and **9**), while in the remaining derivatives E_{LUMO} is high in the range −0.15 to −0.05 eV. The theoretical results predict that the hydrogen bond donor ability is highest in 1-morpholino-methyl-2-styryl-benzimidazole **5**, which has the lowest E_{LUMO} of −0.83 eV. The 2-styryl-benzimidazoles (**4**, **5**, and **6**), having low E_{LUMO} values, show high permeability. On the other hand, the derivatives of *N*-Mannich bases of benzimidazole, which have high E_{LUMO} values, show low permeability.

The relative permeability within the 2-styryl derivatives and non-styryl derivatives could not be predicted with precision due to the different factors operating in the experimental systems. However, the present study clearly indicates that it is able to explain the high permeability observed in the compounds (**4**, **5** and **6**). The presence of a hydrophobic phenyl nucleus in these derivatives lowers E_{LUMO} due to the electronic effects originating from the π-conjugation. We also observed that the HOMO energy is higher in the styryl derivatives. Similar results were observed using the semi-empirical AM1 method.

According to the FMO concept [22], the HOMO and LUMO of a molecule play important roles in intermolecular interactions. Extending the concept to binding in drug–receptor systems, the major contribution to binding involves the interaction between the HOMO of the drug with the LUMO of the receptor and that between LUMO of the drug with the HOMO of the receptor. The extents of these stabilizing interactions are inversely related to the energy gap between the interacting orbitals. Higher HOMO energy and lower LUMO energy in the drug molecule result in larger stabilizing interactions and, hence, binding with the receptor. It is remarkable that compound **6**, having the lowest energy gap of ΔE of 7.776 eV, exhibits the highest anti-inflammatory activity.

Our investigation revealed that the analgesic and anti-inflammatory activities of benzimidazole derivatives are related to different substituent effects. Electron withdrawing substituents increase the activity. According to the observed influence of HOMO orbitals on the analgesic and anti-inflammatory activities, it can be concluded that the receptor sites seem to have an electron accepting property.

4.3. In vitro permeability studies using isolated bovine corneas

The efficacies of the compounds (**1**–**9**) for corneal penetration were assessed by measuring the transcorneal flux (Fig. 1) of the drug through isolated bovine cornea. Results indicate that among the compounds, **4** exhibits maximum permeability with a permeability coefficient of 17.44×10^{-2} cm/s and **2** exhibits the least permeability, with a permeability coefficient of 1.92×10^{-2} cm/s. Benzimidazole **7**, when compared with styryl benzimidazole **9**, shows high permeability (permeability coefficients of 9.21×10^{-2} and 3.03×10^{-2} cm/s, respectively). No significant differences were observed between the permeability coefficient of 2-methyl benzimidazole **8** (6.56×10^{-2} cm/s) and that of 1-diphenyl amino-methyl-2-methyl benzimidazole **3** (6.83×10^{-2} cm/s). From the permeability data, it can be concluded that when an *N*-Mannich base was incorporated with styryl benzimidazole, there was a rapid increase in the permeability coefficient (Table 4). The lag

Table 3
Quantum chemical parameters of the synthesized compounds

Compound	E_{HOMO} (eV)	E_{LUMO} (eV)	ΔE	Molecular weight	Molar volume $(A^0)^3$
1-((Dimethylamino)-methyl)-benzimidazole **1**	−8.897	−0.11	+8.787	175.2	196.1
1-((Diethylamino)-methyl)-benzimidazole **2**	−8.869	−0.089	+8.780	203.3	236.0
1-((Diphenylamino)-methyl)-2-methyl-benzimidazole **3**	−8.680	−0.146	+8.534	313.4	342.0
1-((Diethylamino)-methyl)-2-styryl-benzimidazole **4**	−8.626	−0.798	+7.828	305.4	344.5
1-((Morpholino)-methyl)-2-styryl-benzimidazole **5**	−8.656	−0.829	+7.827	333.4	361.4
1-((Piperidin-1yl)-methyl)-2-styryl-benzimidazole **6**	−8.482	−0.706	+7.776	317.4	355.8
Benzimidazole **7**	−8.889	−0.070	+8.819	118.1	112.0
2-(Methyl)-benzimidazole **8**	−8.822	−0.054	+8.768	132.2	144.5
2-(Styryl)-benzimidazole **9**	−8.593	−0.776	+7.817	220.3	212.6

Table 4
Permeability coefficients and lag times (t_L) of *N*-Mannich bases of benzimidazole and standard drugs Diclofenac and paracetamol

Compound	$P_{app} \times 10^{-2}$ (cm/s)	t_L (min)
1-((Dimethylamino)-methyl)-benzimidazole **1**	5.29	15.31
1-((Diethylamino)-methyl)-benzimidazole **2**	1.92	16.79
1-((Diphenylamino)-methyl)-2-methyl-benzimidazole **3**	6.83	23.49
1-((Diethylamino)-methyl)-2-styryl-benzimidazole **4**	17.44	4.90
1-((Morpholino)-methyl) 2-styryl-benzimidazole **5**	8.88	7.33
1-((Piperidin-1yl)-methyl)-2-styryl-benzimidazole **6**	16.08	4.51
Benzimidazole **7**	9.21	18.81
2-(Methyl)-benzimidazole **8**	6.56	11.68
2-(Styryl)-benzimidazole **9**	3.03	9.57
Paracetamol **10**	23.1	1.55
Diclofenac **11**	21.3	3.36

P_{app} = permeability coefficient, t_L = lag time.

times of the compounds (**1**–**11**) are inversely related to the permeability coefficients. The permeability coefficients of the standard drugs paracetmol and Diclofenac are shown in Fig. 3. The present permeability data on the compounds studied do not correlate with the molecular size given by molecular weight and molar volume (Table 3).

5. Experimental protocols

5.1. General procedure for the synthesis of N-Mannich bases of benzimidazole

Formaldehyde (0.05 mol) was added slowly to 0.05 mol of benzimidazole/2-substituted benzimidazole and 0.05 mol of secondary amine (dimethyl amine, diethyl amine, diphenylamine, piperidine and morpholine) in 15 mL of ethanol, with continuous stirring for 45 min, and refrigerated overnight. The product was filtered, recrystallized using absolute alcohol, and vacuum dried.

Microanalytical CHN results were within ±0.4% of theoretical values.

5.1.1. 1-((Dimethylamino)-methyl)-benzimidazole *1*

Yield = 78%, m.p 156–158 °C, $R_f = 0.78$. ^{1}H NMR ($CDCl_3$) δ: 7.26–8.06 (m, 4H; 4,5,6,7H), 5.17–5.27 (s, 1H; 2H), 4.86–5.03 (s, 2H; $-CH_2-$), 2.24–2.42 (s, 6H; $-[CH_3]_2$). IR (KBr) cm^{-1}: 3115 (Ar C–H), 1587, 1496 (Aliph C–H), 1364, 1346 (Ar C–N), 1244, 1202 (Aliph C–N). Anal. Calcd for $C_{10}H_{13}N_3$: C, 68.57; H, 7.43; N, 24.01. Found: C, 68.04; H, 7.84; N, 24.20. M^+ 175, 129, 117, 105, 91.

5.1.2. 1-((Diethylamino)-methyl)-benzimidazole *2*

Yield = 77%, m.p 122–124 °C, $R_f = 0.53$. ^{1}H NMR ($CDCl_3$) δ: 7.24–8.23 (m, 4H; 4,5,6,7H), 5.88–6.64 (s, 1H; 2H), 4.87–5.04 (s, 2H; $-CH_2-$), 2.49–2.77 (q, 4H; $-[CH_2]_2$), 0.80–1.45 (t, 6H; $-[CH_3]_2$). IR (KBr) cm^{-1}: 3100 (Ar C–H), 1459 (Aliph C–H), 1362, 1326 (Ar C–N), 1028 (Aliph C–N). Anal. Calcd for $C_{12}H_{17}N_3$: C, 69.42; H, 8.81; N, 21.77. Found: C, 70.31; H, 8.51; N, 20.27. M^+ 193, 129, 117, 105, 91.

5.1.3. 1-((Diphenylamino) methyl)-2-methyl-benzimidazole *3*

Yield = 72%, m.p 165–167 °C, $R_f = 0.47$. ^{1}H NMR ($CDCl_3$) δ: 6.84–7.45 (m, 4H; 4,5,6,7H), 6.55–6.84 (m, 10H; Ar–H), 3.16–3.74 (s, 2H; $-CH_2-$), 2.48–2.81 (s, 3H; 2-CH_3). IR (KBr) cm^{-1}: 3041 (Ar C–H), 1457, 1450 (Aliph C–H), 1395 (Ar C–N), 1319 (Aliph C–N). Anal. Calcd for $C_{21}H_{19}N_3$: C, 80.50; H, 6.07; N, 13.43. Found: C, 80.56; H, 6.16; N, 13.21. M^+ 313, 252, 129, 117, 105, 91, 77.

5.1.4. 1-((Diethylamino) methyl)-2-styryl-benzimidazole *4*

Yield = 81%, m.p 181–183 °C, $R_f = 0.65$. ^{1}H NMR ($CDCl_3$) δ: 7.07–8.10 (m, 9H; Ar–H), 6.38–6.69 (d, 2H; vinyl-H), 3.59–3.86 (s, 2H; $-CH_2-$), 2.84–3.11 (q, 4H; $-[CH_2]_2$), 0.91–1.57 (t, 6H; $-[CH_3]_2$). IR (KBr) cm^{-1}: 3102 (Ar C–H), 3022 (Vinyl C–H str), 1682 (C=C), 1448 (Aliph C–H), 1395 (Ar C–N), 1203, 1159 (Aliph C–N), 979 (Alkene C–H). Anal. Calcd for $C_{20}H_{23}N_3$: C, 78.68; H, 7.54; N, 13.77. Found: C, 78.07; H, 7.65; N, 13.62. M^+ 305, 240, 129, 117, 105, 91, 77.

5.1.5. 1-((Morpholino) methyl)-2-styryl-benzimidazole *5*

Yield = 82%, m.p 131–133 °C, $R_f = 0.46$. ^{1}H NMR ($CDCl_3$), δ: 7.04–6.75 (m, 9H; Ar–H), 4.45–4.83 (s, 2H; $-CH_2-$), 3.60–4.02 (t, 4H; 2″,6″H), 2.85–3.56 (t, 4H; 3″,5″H). IR (KBr) cm^{-1}: 3080 (Ar C–H), 3027 (Vinyl C–H), 1642 (C=C), 1496, 1448 (Aliph C–H), 1389, 1374 (Ar C–N), 1102, 1075 (Aliph C–N), 976, 963 (Alkene C–H), 776, 732, 718 (Ar C–H). Anal. Calcd for $C_{20}H_{21}N_3O$: C, 75.22; H, 6.58; N, 13.17. Found: C, 78.98; H, 7.74; N, 12.88. M^+ 319, 240, 129, 117, 105, 91, 77.

5.1.6. 1-((Piperidin-1-yl) methyl)-2-styryl-benzimidazole *6*

Yield = 78%, m.p 142–144 °C, $R_f = 0.71$. ^{1}H NMR ($CDCl_3$) δ: 7.01–8.12 (m, 9H; Ar–H), 6.35–6.60 (d, 2H; vinyl-H), 4.50–4.91 (s, 2H; $-CH_2-$), 2.81–3.30 (t, 4H; 2″, 6″H), 1.37–1.93 (m, 6H; 3″,4″,5″H). IR (KBr) cm^{-1}: 3402 (Ar C–H), 2948 (Vinyl C–H), 1687 (C=C), 1448 (Aliph C–H), 1390 (Ar C–N), 1238, 1027 (Aliph C–N), 980, 971 (Alkene C–H). Anal. Calcd for $C_{21}H_{23}N_3$: C, 79.48; H, 7.25; N, 13.26. Found: C, 75.16; H, 7.08; N, 12.92. M^+ 317, 252, 129, 117, 105, 91, 77.

5.2. Quantum chemical analyses

Quantum chemical computations were performed for compounds **1**–**9** by all valence electron semi-empirical molecular orbital method PM3 [28,29], using MOPAC 2000 software. The molecules were subjected to complete structural optimization using the PRECISE option and the energy minimization was carried out until the gradient norm dropped to 0.05 or lower. Molar volume was computed at the PM3 optimized geometry using MOLDRAW software (release 1.0, version E). The data are presented in Table 3.

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