Mechanistic Studies on the Reaction of *o*-phthalaldehyde (OPTA) with Urea and its *N*-alkyl/aryl Derivatives

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Summary: Urea and its *N*-alkyl/aryl derivatives react with *o*-phthalaldehyde (OPTA) to yield blue to purple coloration along-with isoindoline compounds (VII a,b,c) in acidic media. The color is unstable and changes into various shades with the passage of the time. The assay of urea which entirely depends upon this color does not suggest its determination with OPTA present in biological and non-biological fluids. Moreover, it is found that compounds which enhance color stability have nothing to do with determination of urea. The structures of isoindolines (VII a,b,c) have been confirmed by ^{1}H-, ^{13}C-NMR and mass spectrometry techniques. The absolute authenticity comes from their (VII a,b,c) X-ray crystallography. The colors resulting from the said reactions fall in between 585-595 nm in UV/VIS spectra. As the use of OPTA for urea determination is known, hence, in this study, we are presenting chemistry for urea determination with OPTA.

Introduction

Urea and its derivatives are of widespread importance in pharmaceutical, biochemical and agricultural industry. Its determination is important in several fields, including clinical diagnosis [1], agriculture [2, 3], environmental monitoring [4] and food sciences [5]. Several methods have been reported [6] for the urea estimation and their merits and demerits are discussed elsewhere [7-10]. In one of the methods, *o*-phthalaldehyde has been used for the determination of urea and its *N*-alkyl/ aryl derivatives [2, 11] because it is an excellent flourogenic reagent which is capable of detecting picomolar quantities of amines, amino acids and proteins [12, 13]. The reaction product yields blue color on which the determination of the urea concentration depends. The nature of the color product remained mysterious and its exact determination appears to be uncertain which leads to wrong diagnosis of different fatal diseases. That's why, we have focused our attention for the isolation of products resulting from the reaction of *o*-phthalaldehyde with urea and its *N*-alkyl/aryl derivatives in order to characterize the nature of coloration and end products of the reaction.

Several scientists reported the reaction of urea with *o*-phthalaldehyde in acidic [1] and basic media [14]. In basic medium, compounds (I), (II) and (III) are reported (where X = O, S) (Fig. 1). They are colorless and cannot be used in a colorimetric determination of urea in biological fluids [5]. The mechanism of base catalyzed condensation of *o*-phthalaldehyde with urea and thiourea has been reported [14]. Moreover, amides also react with *o*-phthalaldehyde yielding isoindolines and phthalans (IV, V, Fig. 1) when the substituents (R, R') are bulky [15, 16]. Similarly, in acidic medium, urea gives I,3-dihydroxyindoline or 1-ureido-3-hydroxyphthalan [1]. It further reacts with *N*-(1-naphthyl)-ethylenediamine, producing an intense colored product absorbing at 505 nm. The method has been applied for the determination of urea in blood serum [17]. Jung and his co-workers [1, 18] have described urea determination method in which *o*-phthalaldehyde reacts with urea in the presence of *N*-(1-naphthyl) diamine dihydrochloride yielding colored product/s of unknown structure. Similar is the case with the work of Momose [2], Lequang *et al.* [11] and Nariensingh *et al.* [19] for the determination of urea by spectrophotometry [10, 19] and flow injection analysis [19]. Chromy *et al.* [17] modified the Jung method for urea determination. But the Jung's reagents were reported to interfere with a variety of sulpha drugs, at least some of which are commonly present in body fluids subject to urea analysis. Also α–ethylene-naphthyl amine is a known carcinogen and therefore product may contain at least traces thereof.

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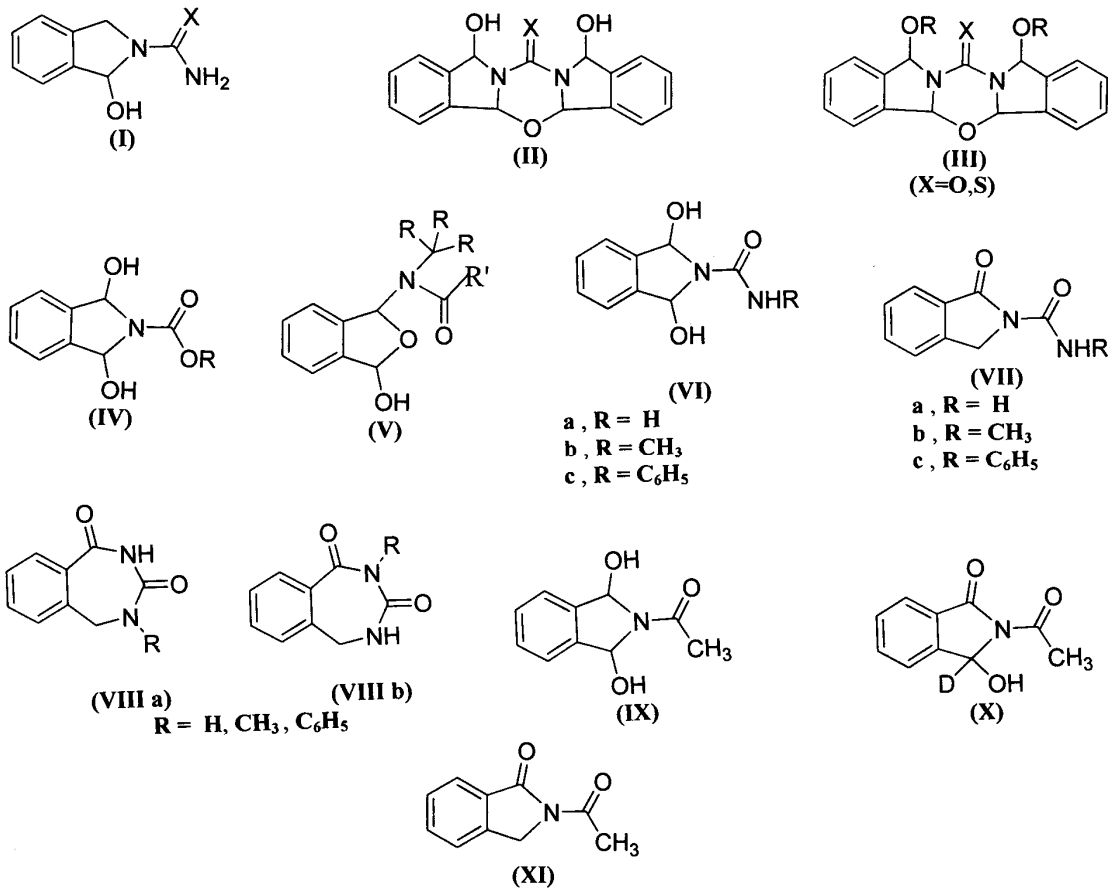


Fig. 1: Structures of Isoindolines (I-XI).

Denney [20] also gave urea determination method in acidic media, using *o*-phthalaldehyde and the color enhancing compounds. They reported chromogen/s of unknown structure, on which the entire analytical procedure is dependent. However, several disadvantages were associated with this method. Melvin [21] reported the reaction of urea and *o*-phthalaldehyde in acidic media using chromotropic acid resulting in highly intense colored product of unknown structure, whose concentration is linearly related to urea concentration. Later on, Melvin and Olga [22] described all previous methods as each of the foregoing methods for urea determination suffers from several disadvantages. They also reported the use of *o*-phthalaldehyde in acidic medium and long chain hydrocarbon amidobetaine, forming a highly colored substance of unknown structure.

Keeping in view the significance of urea determination in biological samples, we have intended to develop an accurate, specific and rapid chemical method for its determination. If the reaction of OPTA and nature of the chromogen, on which the entire analytical procedure is dependent is unknown, then how one can claim the authenticity of *o*-phthalaldehyde use in the determination of urea concentration? In the present work, we intend to disclose the products of *o*-phthaldehyde-urea reaction. Once they are known, then one can refine and establish the accurate method for the assay of urea in biological and non-biological samples.

Results and Discussion

The reaction of one molecule of each OPTA and urea with elimination of one water molecule yielded a compound of a molecular formula $C_9H_8N_2O_2$. This immediately suggested 1-oxoisoindoline-2- carboxamide (VII-a, Fig. 1) and

two isomers of 4,5-dihydro-1*H*-2,4-benzodiazepine-1,3(2*H*)-dione (VIII a-b, Fig. 1) as the structures of the product. The spectroscopic data was unable to make distinction between compounds (VII a) and (VIII a-b, Fig. 1). However, the X-Ray crystallography had confirmed the structure (VII a, Fig. 1) of the product [23]. The IR, ^{1}H- and ^{13}C-NMR spectra are entirely consistent with structure (VII-a, Fig. 1).

The product obtained from the reaction of OPTA and 1–methylurea is expected to be the same as in case of urea with the addition of methyl group at free NH_2 group. Elemental analysis indicated a formula of $C_{10}H_{10}N_2O_2.H_2O$ and it was also confirmed by its X-ray crystallography [24]. This formula corresponds to reaction of one molecule of each OPTA and 1-methylurea with the loss of a water molecule. Surprisingly, this formula contained one molecule of water as a water of crystallization. It remained associated with the molecule even after prolonged drying under vacuum. The IR spectrum suggested the presence of NH/OH and amide carbonyl group. *p-N,N*–Dimethylaminobenzaldehyde showed negative test indicating that there was no free NH_2 in the molecule (VII-b, Fig. 1). The IR, ^{1}H- and ^{13}C-NMR data of compound (VII-b, Fig. 1) suggested *N*-methyl-1-oxoisoindoline-2-carboxamide monohydrate which is analogous to compound (VII-a, Fig. 1). In compound (VII-b, Fig. 1), methyl protons appeared at δ 2.96 and water molecules at 2.10 ppm. The up-field shift of water molecule indicated that probably, it exists in the environment of high electron density. Moreover, it is sandwiched between two dimers of isoindoline rings as shown in its X-ray crystallographic picture (Fig. 2a-b) [24], where the electron density is expected to be higher. Further, the peak of water molecule disappeared with the addition of D_2O.

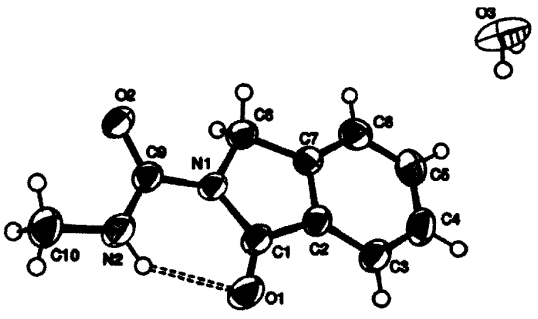


Fig. 2a: N-methyl-1-oxoisoindoline-2-carboxamide monohydrate.

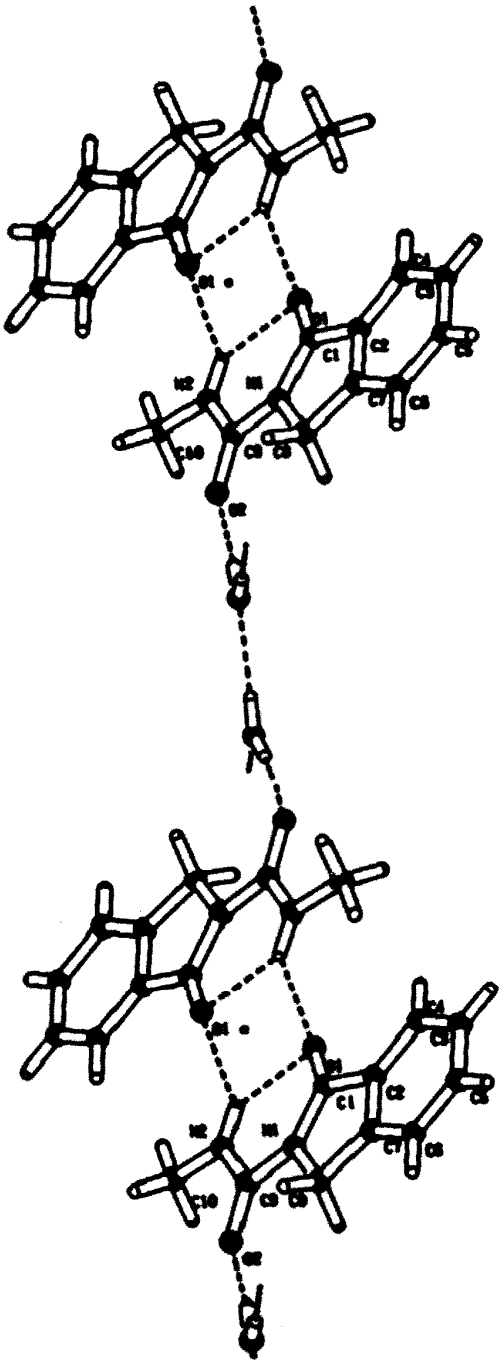


Fig. 2b: Sandwiched water molecules between crystals of VII-b.

The formula $C_{15}H_{12}N_2O_2$ is consistent with the reaction of one molecule of each OPTA and 1-phenylurea with the loss of a water molecule. It suggested 1-oxo-*N*-phenylisoindoline-2-carboxamide (VII-c, Fig. 1) as the structure of the product. The structure was confirmed by X-ray crystallography [25], ^{1}H- and ^{13}C-NMR spectra. The entire spectroscopic data is in agreement with structure (VII-c, Fig. 1). Hence, structure (VII-c Fig. 1), is analogous to structures (VII-a, Fig. 1) and (VII-b, Fig. 1).

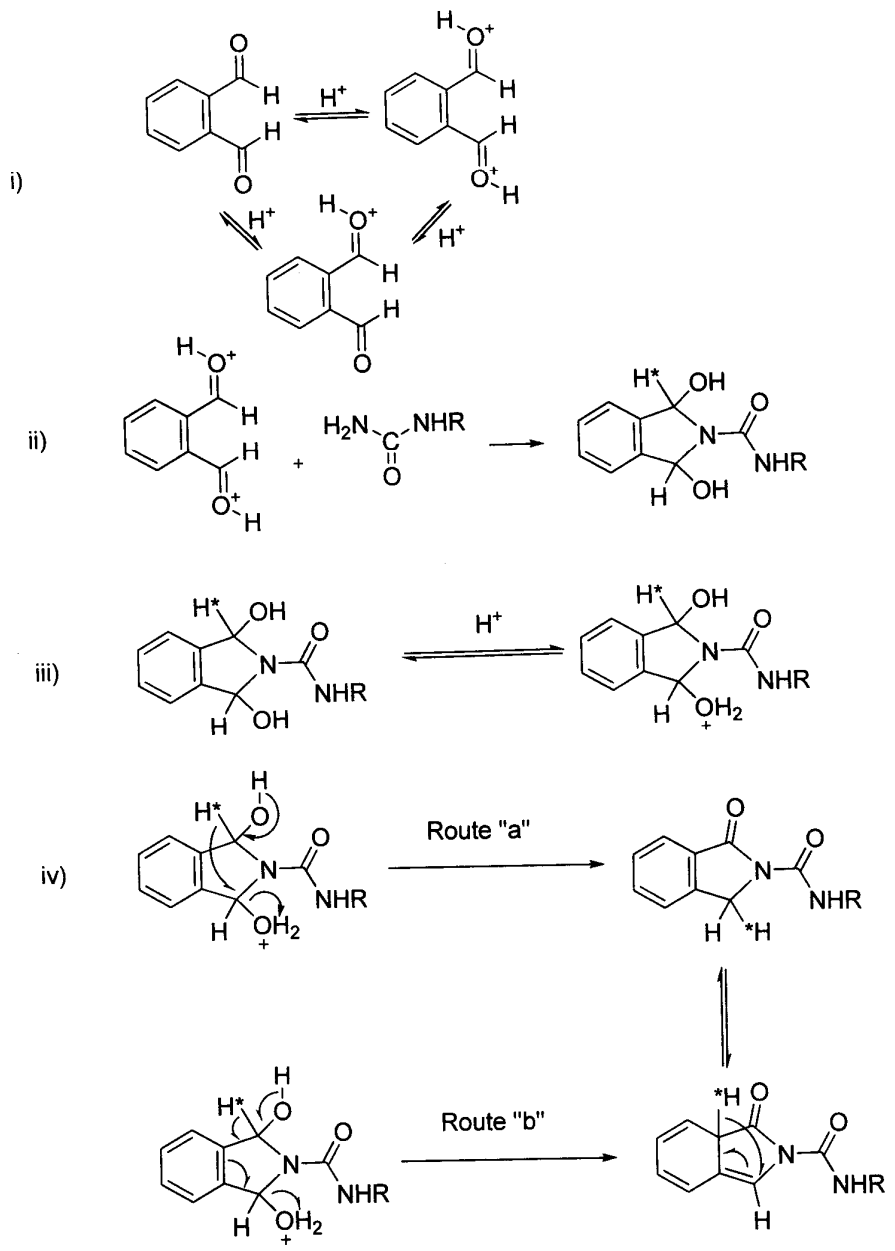
Recently, Wan *et al.* [26] proposed the same products (VII a-c, Fig. 1) obtained by different reaction conditions in the presence of ionic liquid and catalysts. They elucidated the structure with the help of different spectroscopic techniques (ESI-MS fragmentation, ^{1}H- and ^{13}C-NMR) whereas; in our case, the structures have been confirmed by single crystal X-ray analysis.

A reasonable mechanism for the formation of isoindoline ring is suggested in Scheme I. Mono or diprotonation of oxygen of aldehyde group stepwise or concerted makes the carbons good electrophile. The unsubstituted nitrogens of ureas attack the carbons of aldehyde and yield an intermediate (VI, Fig. 1), which is not possible by the attack of substituted nitrogens, either due to stearic effect, or ring nitrogen will contain positive charge which is adjacent to carbonyl group. This carbonyl group further destabilizes the ring. The intermediate (VI, Fig. 1) is not isolable and under the reaction conditions it is further protonated and 1, 4-hydride shift takes place via routes "a" or "b" yielding compounds (VII-a,b,c, Fig. 1). The driving force for this rearrangement is the elimination of water molecule.

Jung *et al.* [1] have assumed the formation of 1, 3–dihydroxyindoline in the reaction of OPTA with urea in acidic media. Similarly, Reynolds *et al.* [14] have reported the formation of 1, 3–dihdroxyisoindoline in reaction of OPTA with urea and thiourea in basic media, which in case of thiourea, on standing forms dimer. The 1, 4 hydride shift has been reported within η-5-naphthalneyl complex $\{Mn(\eta5\text{-}C_{10}H_9)(CO)_3\}$ [27] and in homoallylic alcohol [28] via acid catalyzed shift to yield a saturated ketone. Same kind of 1, 4 –hydride shift has been reported by Gwynn and Skillern [29] in the preparation of σ-methylene-2-oxobornanol. Moreover, 1, 2-hydride shift is well known in literature. Most recently, Wan *et al.* [26] reported the same kind of rearrangements in the reaction of *o*-phthalaldehyde with ureas in the ionic liquids $[bmim]BF_4$. Contrary to their work [26], our reaction conditions are quite different and intermediate isolated (XI, Fig. 1) is not possible in the highly protic solvent (C_2H_5OH + HCl).

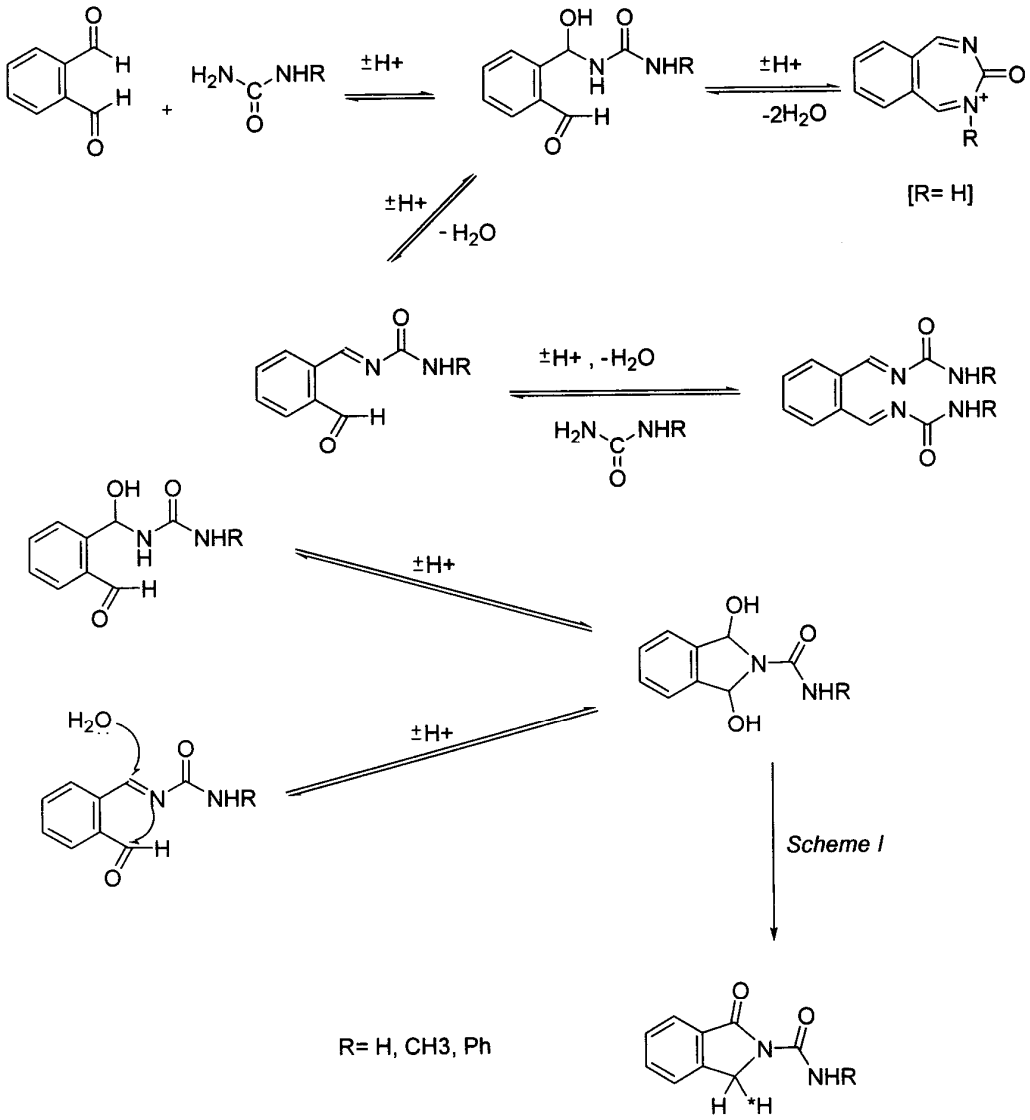
Color Reactions

Urea, 1- methyl urea and 1- phenylurea in 2M HCl gave blue to purple coloration when their solutions were heated with *o*-phthalaldehyde for 30 min. at 40 °C. The color changed from red → green → purple → blue and finally became light yellow with the passage of time. The purple color in case of urea appeared to be more stable than in case of its *N*-alkyl/aryl derivatives. Moreover, it was reported [1-2], that the stability of the color was enhanced with the addition of *N*-(1-naphthyl)-ethylene diamine. However, the assay of urea in clinical laboratories entirely depends upon the formation of this purple color. Contrarily, we have observed that the reaction of *o*-phthalaldehyde with this amine also yielded purple color without the addition of urea. The color absorbed at λ_{max} 495-505 nm, while the color of *o*-phthaldehyde-urea reaction peak appeared at 585nm. When a mixture of OPTA, urea and amine containing 2M HCl was heated the absorption of color falls in between λ_{max} 495-505 nm. It means, the amine reacts faster than urea molecule and a very slight absorption occur at 585nm. It indicates that after the complete consumption of amine, then excess amount of OPTA and urea if present reacts to give absorption peak at λ_{max} 585 nm. In another experiment, a mixture containing an excess amount of OPTA, urea and a lesser amount of amine was heated; again purple color developed which absorbed at λ_{max} 495–505 nm and 585 nm. It clearly indicated that first absorption is due to OPTA–amine reaction and the last one is assigned to OPTA–urea reaction. It means the addition of *N*-(1-naphthyl)-ethylene diamine prevents the OPTA–urea reaction and itself involves leading to purple color which absorbed at 505 nm and exact determination seems to be impossible. Moreover, use of excess amine has completely snubbed the OPTA-urea reaction because NH_2 group of amine is stronger nucleophile than NH_2 group of urea. Similarly, the addition of chromogenic agents like chromotropic



Scheme-1: Reaction of *o*-phthalaldehyde with urea and its *N*-alkyl/ aryl derivatives in ethanol.

acid, mercapto ethanol and *N*-acetyl-L-cystiene have nothing to do with the stabilization of color and don't contribute in the determination of urea present in biological samples. Concisely, *o*-phthalaldehyde condenses with urea in 2M HCl, resulting in the formation of Schiff-base (–C=N–) as shown in Scheme II. The stability of the Schiff-base depends upon the strength of the acid. The variations in colors are observed due to use of higher acid strength as well as on prolong heating. Below 2M HCl, the full chromogenic value does not develop. Finally, the solution becomes light yellow yielding isoindoline



Scheme-2: Reaction of *o*-phthalaldehyde with urea and its *N*-alkyl/ aryl derivatives in *Benzene.*

compounds. They remain soluble in strong acid media and precipitate on dilution with water. Concurrent formation of non-chromogenic compounds and chromogens (Schiff-base) don't suggest the assay of urea present in biological and non-biological fluids. The colors absorption resulting from the reaction of urea, 1-methylurea and 1-phenylurea fall in the range of λ_{max} 585-595 nm in UV/VIS spectra while, the color produced by the reaction of OPTA-amine absorbed in between λ_{max} 496-505 nm. The highly intense colors of Schiff-bases resulting from the reactions of various aromatic aldehydes with ureas [7, 8], thioureas [7, 8], amines [30, 31] and their stability have been discussed.

Several attempts have been made to isolate chromogen/s but all in vain. It might be due to the reason that during isolation process, there is continuous variation in the colored solution and finally becomes colorless yielding either starting

materials or isolindoline compounds. It has been reported that the reaction of OPTA with ammonia resulting in carbinolamine. It undergoes side reaction to form an imine (–C=N–), which is shifted more in favour of the cyclic species. Carbinolamine is also involved in isoindoline ring formation [32]. Hence, his work is in agreement with our proposed mechanism (scheme I, II). The colored species is either a single species or a mixture of chromogen/s. That is why; the colors resulting from reaction of OPTA with ureas impart several shades and when the color is deprotonated with alkali, it becomes colorless.

Experimental

All chemicals used in the experiments were reagent grade procured from Merck, Germany and Fluka, Switzerland. Melting points were recorded on a Gallenkemp apparatus and were uncorrected, while UV/VIS spectra was carried out on Spectro UV-VIS Double beam, UVD-3500 Labomed, Inc. IR spectra were recorded on a Perkin-Elmer 1600 FT spectrophotometer. Mass Spectra of the compounds were carried out at MAT-312; ^{1}H- and ^{13}C-NMR spectra were taken on Bruker DPX 300 (300 MHz), whereas elemental analysis was done on CHNS analyser Carlo Erba 1106. Nonius and Bruker Kappa APEXII CCD diffractometers were used for X-ray crystallography.

General Procedure

A mixture of *o*-phthalaldehyde (200 mmol) and respective urea (200 mmol) in 100 ml of ethanol (95%) containing a few drops of conc. HCl were refluxed for 6-12 hrs. A blue-purple color developed initially which gets fade away with time. The solvent was taken off and the flask contents were allowed to stand at room temperature. A white solid (VIIa, Fig. 1) was separated from the colored solution. It was washed with ethanol and dried. Its TLC (Al_2O_3 plate, pH 6.5) in ethyl acetate and chloroform showed a single spot.

1: 1-oxoisoindoline -2- carboxamide (VII-a)

The crystals suitable for X-ray diffraction were grown from a mixture of acetone-ethanol (1:1) by slow evaporation at room temperature. The compound (VIIa) is soluble in DMSO, DMF, acetone, ethyl acetate and $CHCl_3$. M.p. 220 °C; m/z 176; IR (nujol, ν_{max}, cm^{-1}): 3222 and 3368 (NH st.), 1668 and 1712 (C=O st.) 1586 and 1550 (C=C st.) 1150 (C-O st.). δ ^{1}H ($CDCl_3$): 4.77 (s, $-CH_2-$), 7.52-7.81 (aromatic ring protons, 4H) and 7.93 ppm (s, broad NH). δ ^{13}C ($CDCl_3$): 48.18, 123.92, 123.95, 128.35, 130.65, 133.61, 141.62, 152.51 and 168.48 ppm. In DEPT spectra, $-CH_2-$ has inverted its position and all quaternary carbons (133.61, 141.62, 152.51 and 168.48) disappeared. $C_9H_8N_2O_2$ calculated C, 61.36; H, 4.58; N, 15.90; O, 18.16 found: C, 61.38; H, 4.60; N, 15.87; O, 18.15.

2: N-methyl-1-oxoisoindoline-2-carboxamide monohydrate (VII-b)

Crystals were grown from a mixture of methanol-acetone (1:1) by slow evaporation at room temperature. It is soluble in DMSO, DMF, acetone, ethyl acetate, $CHCl_3$ and CCl_4. M.p 140 °C; m/z 190 = (208 – H_2O); IR (nujol, ν_{max}, cm^{-1}): 3300 (NH/OH st.), 1714 and 1680 (C=O st.), 1600 and 1547 (C=C st.), 1150 (C-O st.). δ ^{1}H ($CDCl_3$): 2.10 (H_2O, it disappeared on addition of D_2O), 2.96 (s, $-CH_3$), 4.82 (s $-CH_2-$), 7.45-7.87 (aromatic ring protons, 4H) and 8.45 ppm (broad s, NH). δ ^{13}C ($CDCl_3$): 26.45, 48.57, 123.33, 124.71, 128.48, 131.22, 133.61, 141.61, 153.68 and 169.29 ppm. In DEPT spectra $-CH_2-$ has inverted its position and all quaternary carbons (133.61, 141.61, 153.68 and 169.29) disappeared. $C_{10}H_{10}N_2O_2.H_2O$ calculated C, 57.69; H, 5.76; N, 13.46; O, 23.07 found: C, 57.72; H, 5.77; N, 13.49; O, 23.02.

3: 1-oxo-N-phenylisoindoline-2-carboxamide (VII-c)

Crystals were grown on slow evaporation of acetone solution at room temperature. It melted at 168 °C. It is soluble in DMSO, DMF, acetone, ethyl acetate, $CHCl_3$ and CCl_4. M.p, 168 °C; m/z 252; IR (nujol, ν_{max}, cm^{-1}): 3425 (*N*-H st.), 1714 and 1680 (C=O st.), 1600 and 155.8 (C=C st.), 1150 (C-O st.). δ ^{1}H ($CDCl_3$): 4.92 (s $-CH_2-$), 7.10-7.93 (aromatic ring protons, 9H) and 10.72 ppm (s, NH). δ ^{13}C ($CDCl_3$): 48.59, 120.06, 123.37, 124.08, 124.86, 128.65, 129.03, 130.94, 133.93, 137.50 141.07, 150.25 and 169.44 ppm. In DEPT spectra, $-CH_2-$ has inverted its position and all quaternary carbons (133.93, 137.50, 141.07, 150.25 and 169.44) disappeared. Anal. Calcd for $C_{15}H_{12}N_2O_2$, calculated C, 71.42; H, 4.79; N, 11.10; O, 12.68 found: C, 71.45; H, 4.81; N, 11.07; O, 12.65.

4: UV/VIS Studies

A mixture of equimolar volume of OPTA and urea containing a few drops of 2M HCl was warmed at 40°C for 30 min. The blue color developed which gave at absorption peak at λ_{max} 585 nm in UV/Visible spectra. Similarly, 1-methyl urea and 1-phenyl urea reflected absorbances at λ_{max} 595 and 590 nm, respectively.

0.1 M ethanolic solution of each OPTA and N–(1–naphthyl)–ethylenediamine dihydrochloride showed absorption peaks at λ_{max} 310 and 455 nm, respectively in UV/Visible spectra using water as reference.

Similarly, equimolar volumes of OPTA, urea and *N*-(1-naphthyl)-ethylenediamine dihydrochloride were mixed together containing a few drops of 2M HCl, warmed at 40°C for 30 min. Consequently, the blue color developed and absorbed at λ_{max} 495-505 nm.

No peak was observed at 585nm which we had assigned to the blue color produced by OPTA-urea reaction. When excess OPTA was used in the same experiment, the peak at λ_{max} 585 nm also appeared in addition to peak at λ_{max} 495-505 nm. Similarly, with excess *N*-(1-naphthyl)-ethylenediamine dihydrochloride only absorption peak emerged at λ_{max} 495-505 nm and no peak at λ_{max} 585 nm was seen.

Acknowledgement

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