Spectrophotometric Determination of pK_a's of 1-Hydroxybenzotriazole and Oxime Derivatives in 95% Acetonitrile-Water

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Summary: 1-hydroxybenzotriazole derivatives are used with carbodiimide as additives to generate active esters during peptide bond formation. They are also used as additives during the peptide bond formation. Dissociation constants of the 1-hdroxybenzotriazole (HOBt) and its derivatives, 1-hydroxy-6-chlorobenzotriazole, 1-hydroxy-6-trifluoromethylbenzotriazole, 1-hydroxy-6-nitrobenzotriazole were determined spectrophotometrically in 95% acetonitrile-water. In addition, 7-aza-1-hydroxybenzotriazole (7-HOAt) and 4-aza-1-hydroxybenzotriazole (4-HOAt) were also studied. Recently, oxyma was reported as a good replacement for the benzotriazole derivatives. As alcoholic components of active esters, the oximes seem to be good leaving groups. Therefore it was expected, that the strongly acidic and nucleophilic oximes, which possess electron-withdrawing groups in the molecule, are suitable as additives during the peptide bond formation. The dissociation constant of some oximes, such as diethyl 2-(hydroxyimino)malonate, ethyl 2-cyano-2-(hydroxyimino)acetate (oxyma), hydroxycarbonimidoyl dicyanide and *N*-hydroxypicolinimidoyl cyanide in 95% acetonitrile-water are reported.

Introduction

The formation of the amide bond is the main goal in the synthesis of a huge array of organic compounds of biological interest [1-4] such as peptides, peptoids, oligocarbamates, oligoamides, β-lactams, polyenamides, benzodiazepines, diketo-piperazines and hydantoins. The ester group is another important functionality which can be prepared from the carboxylic acid and the corresponding alcohol using peptide coupling reagents [5].

The coupling technique can be carried out in solution or solid-phase by in situ activation of the carboxylic acid. The method used should be efficient and reliable, especially when long sequences of amino acid are incorporated in solid-phase peptide synthesis [6, 7]. The maintenance of configuration, especially in amino acids is also important. Thus the main challenge in the development of coupling reagents is the need for the combination of high yields and optical purity.

The activation of carboxylic acids using phosphonium and aminium salts (Fig. 1) is a fast and reliable process. These salts are prepared from a phosphonium or an iminium cation bonded generally to a XO- group, normally a hydroxylamine derivative. Carbodiimides which were used previously as coupling reagents, gained their importance again as useful reagents, by using them in the presence of several additives with an X-OH structure, again usually a hydroxylamine derivative [8].

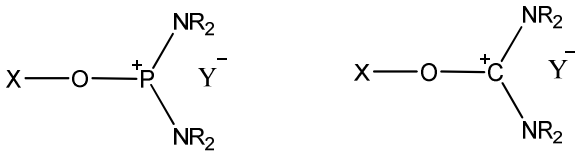

Fig. 1: Phosphonium and aminium salts.

Coupling reagents which are phosphonium or uranium salts derived from 1-hydroxy-benzotriazole (HOBt, **1a**, Fig. 2) are much used for peptide synthesis. In order to increase the coupling rates for difficult couplings, electronegative groups have been introduced into the benzene ring of HOBt, e.g. 1-hydroxy-6-chlorobenzotriazole **1b**, 1-hydroxy-6-trifluoromethylbenzotriazole **1c**, 1-hydroxy-6-nitrobenzotriazole **1d**. In addition, 7-aza-1-hydroxy-benzotriazole (7-HOAt, **2**) and 4-aza-1-hydroxy-benzotriazole (4-HOAt, **3**) were used instead of the HOBt [9].

With the aim of giving a clear picture about the reactivity of the different additives in peptide synthesis in term of racemization and leaving ability, we extended our kinetic investigation on the aminolysis of heterocycle carbamates [10]. The mechanism of the reaction of secondary alicyclic amines e.g. morpholine and piperidine with *N,N*-diethyl carbamate ester of HOBt and its derivatives were studied. Specific goals are to evaluate the effect of leaving groups in the carbamate derivatives and the basicity of the amine on the kinetic and

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mechanism. Our investigations were carried out using acetonitrile as solvent [11]. This prompted us to study the dissociation constants pK_a's of the leaving groups in 95 % acetonitrile-water. Some previous studies determined the pK_a value of several HOBt derivatives, but they were measured only in aqueous solution [12, 13].

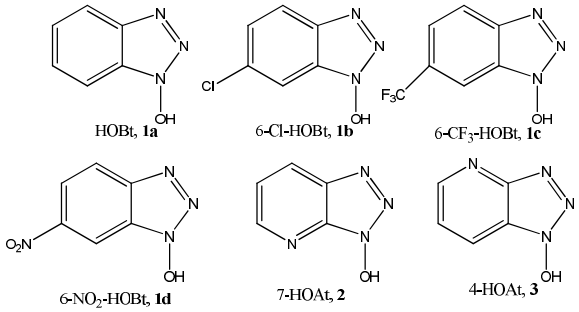


Fig. 2: 1-Hydroxybenzotriazole (HOBt, **1a**), 1-hydroxy-6-chlorobenzotriazole (6-Cl-HOBt, **1b**), 1-hydroxy-6-trifluoromethylbenzotriazole (6-CF_3-HOBt, **1c**), 1-hydroxy-6-nitrobenzotriazole (6-NO_2-HOBt, **1d**), 7-aza-1-hydroxybenzotriazole (7-HOAt, **2**) and 4-aza-1-hydroxybenzotriazole (4-HOAt, **3**).

Some oximes, such as diethyl 2-(hydroxyimino)malonate **4**, ethyl 2-cyano-2-(hydroxyimino)acetate (oxyma) **5** [14], hydroxycarbonimidoyl dicyanide **6** and *N*-hydroxypicolinimidoyl cyanide **7** (Fig. 3) reported as alcoholic components of active esters seem to be good leaving groups. It was expected, therefore, that strongly acidic and nucleophilic oximes, which possess electron-withdrawing groups in the molecule, might be suitable as additives. One of the oximes studied, has been tested by our research group as an additive for the use in the carbodiimide approach for the formation of peptide bonds [15]. Recently, a third generation of uronium type coupling reagents derived from oxyma were also studied [16]. In addition, a new family of sulfonate ester coupling reagents derived from oxyma was reported [17]. Some previous studies determined the pK_a value of several oxime derivatives in aqueous solution [14], but it has not been determined in 95% acetonitrile-water so far.

In order to study the reactivity of different coupling reagents derived from the oximes **4-7**, kinetic investigations studying their leaving group ability are completed [18]. Our investigations were carried out using acetonitrile as solvent. This provoked us to study the dissociation constants of these leaving groups in 95% acetonitrile-water.

Acetonitrile is one of the most important dipolar aprotic solvents; it is used frequently as a solvent for mechanistic studies. Acetonitrile acts as a weaker base and as a much weaker acid than water. It has a relatively high dielectric constant ($\varepsilon = 36$) and a small autoprotolysis constant pK_s = 33.6. Accordingly, acetonitrile acts as a strongly differentiating solvent with modest solvating power for many polar ionic solutes [19].

The acid dissociation constants are important parameters to indicate the extent of ionization of molecules in solution at different pH values. The acidity constants of organic reagents play an important role in many analytical procedures. The acid-base properties affect the properties of organic acids and bases.

The spectrophotometric method depends upon the direct determination of the ratio of molecular species (neutral molecule) to ionized species [20-22]. For this reason, the spectrum of the non-ionized species is obtained, using a buffer solution whose pH is so chosen that the compound to be measured is present completely as this species. This spectrum is compared with that of pure ionized species similarly isolated at another suitable pH. A wavelength is chosen at which the greatest difference between the absorbance of the two species is observed. This is termed the "analytical wavelength". By using this at various pH values, intermediate between those at which the spectra of the two species were obtained, the ratio of ionized to non-ionized species is calculated. This is possible because a series of two component mixture is formed in which the ratio of the two species depends on the pH at which the solution is optically measured.

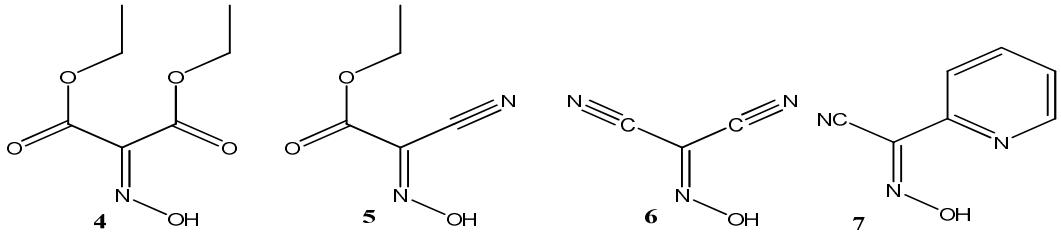


Fig.3: Diethyl 2-(hydroxyimino)malonate **4**, ethyl 2-cyano-2-(hydroxyimino)acetate (oxyma) **5**, hydroxycarbonimidoyl dicyanide **6**, *N*-hydroxypicolinimidoyl cyanide **7**.

Dissociation constants of compounds were determined by several methods. Chromatographic, potentiometric and electrophoretic methods have been usually used. In addition, method based on spectrometry was frequently used due to the help of the improving computer programs [23]. In most of these methods a physical property of the analyte is measured as a function of pH of the solution and the resulting data are used for the determination of dissociation constant. The use of acetonitrile-water mixture requires the measurement of pH in this media.

In our study, we have determined the dissociation constant pK_a value of the different N-hydroxy derivatives **1-7** in 95% (v/v) acetonitrile-water at 25 ± 0.1 °C, using spectrophotometric measurements. The results are necessary for our mechanistic investigation involving the additives **1-7** [11, 18].

Results and Discussion

Fig. 4 and 5 show sample of the electronic spectra of the 4-aza-1-hydroxybenzotriazole (4-HOAt, **3**) and the ethyl 2-cyano-2-(hydroxyimino)acetate (oxyma) **5** respectively in 95% acetonitrile-water mixtures at various pH values at 200 to 600 nm.

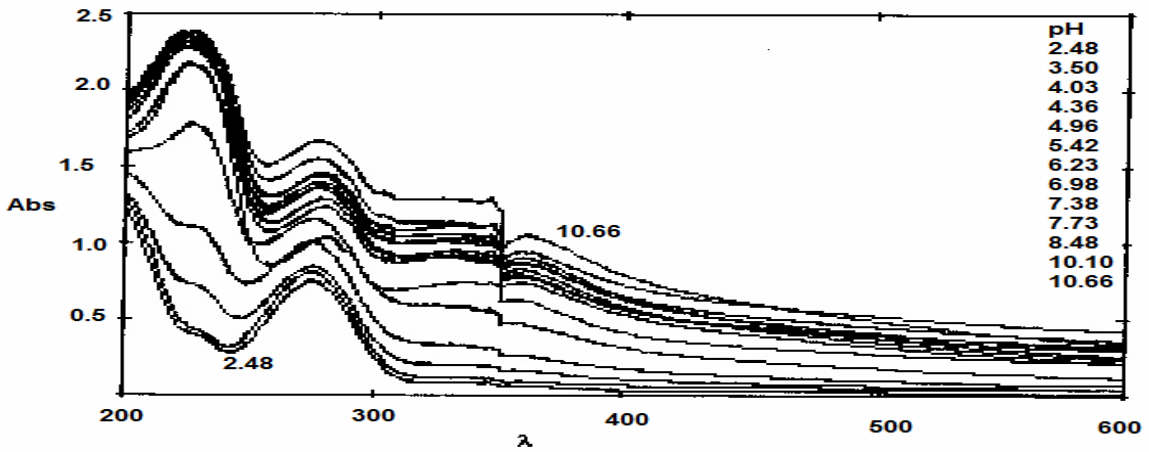


Fig. 4: Plots of experimental absorbance values of 4-aza-1-hydroxybenzotriazole (4-HOAt, **3**) as a function of pH in 95% (v/v) acetonitrile-water at 25 °C.

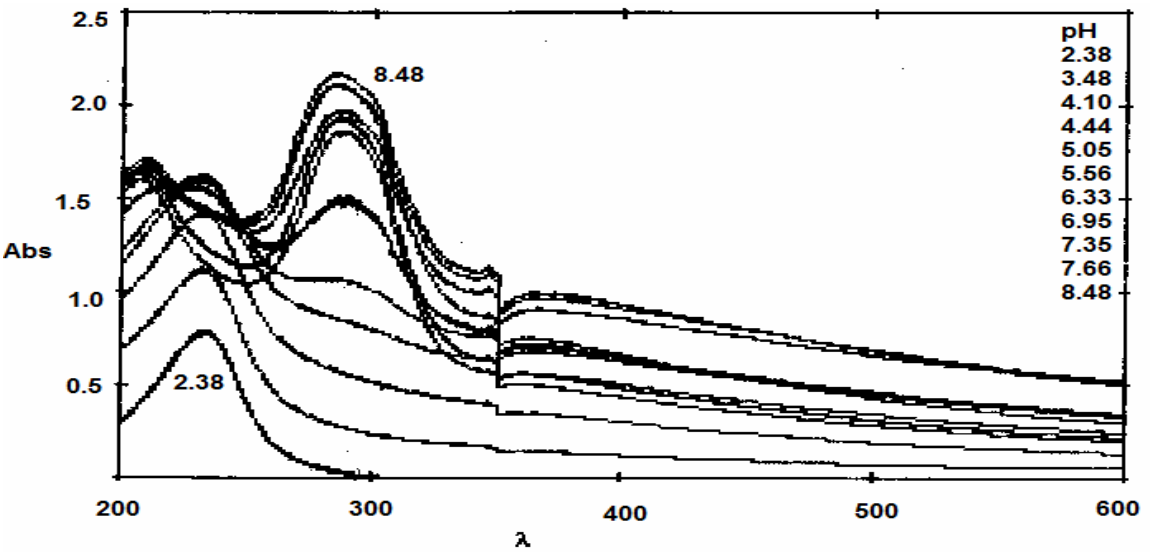


Fig. 5: Plots of experimental absorbance values of ethyl 2-cyano-2-(hydroxyimino)acetate (oxyma) **5** as a function of pH in 95% (v/v) acetonitrile-water at 25 °C.

Table-1: pK_a values of the dissociation constant of compounds 1-3 in 95% acetonitrile-water at 25 °C.

Series 1	HOBt 1a	6-Cl HOBt 1b	6-CF_3-HOBt 1c	6-NO_2-HOBt 1d	7-HOAt 2	4-HOAt 3
Half height method	5.74	4.74	4.38	3.59	4.53	4.00
L. As. method*	5.81	4.48	4.17	3.73	4.76	4.01
Colleter method	5.41	4.63	4.27	3.52	4.66	4.25
Mean values	5.65	4.62	4.27	3.61	4.65	4.086

* L. As.: Limiting absorption method

Table-2: pK_a values of the dissociation constant of compounds 4-7 in 95% acetonitrile-water at 25 °C.

Series 2	Diester 4	Cyanoester 5	Dicyano 6	Pyridinocyano 7
Half height method	5.76	4.36	4.04	4.80
L. As. method*	5.42	4.2297	4.14	5.04
Colleter method	5.60	4.136	4.18	5.03
Mean values	5.59	4.242	4.12	4.96

* L. As.: Limiting absorption method

Tables-1 and 2 collect the values of the dissociation constant (pK_a) of compounds **1a-1d, 2-7** in 95% acetonitrile-water at 25 °C which are calculated using three methods: the half height method [24], the modified limiting absorption method and the Colleter method [25].

The variation of the Absorbance versus pH for the two series are represented in Fig. 6 and 8 and plots of the modifying limiting absorption methods are collected in Fig. 7 and 9.

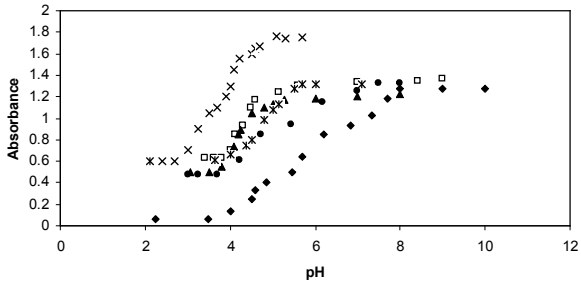


Fig. 6: Variation of Absorbance vs pH for compounds **1a** (♦);**1b** (□); **1c** (▲); **1d** (×); **2** (*); **3** (●) in 95% acetonitrile-water at 25°C.

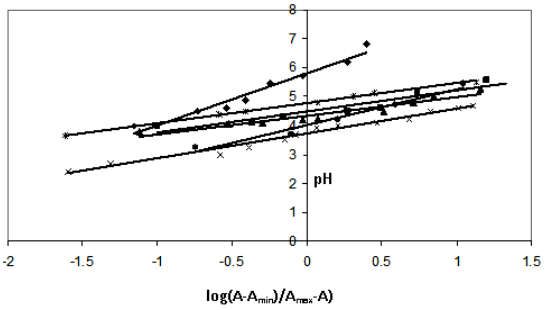


Fig. 7: Variation of pH vs log(A-A_{min})/ (A_{max}-A) for compounds **1a** (♦);**1b** (□); **1c** (▲); **1d** (×); **2** (*); **3** (●) in 95% acetonitrile-water at 25°C.

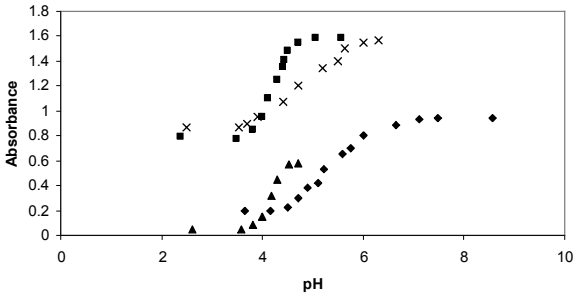


Fig. 8: Variation of Absorbance vs pH for compounds **4** (♦); **5** (▪); **6** (▲); **7** (×) in 95% acetonitrile-water at 25 °C.

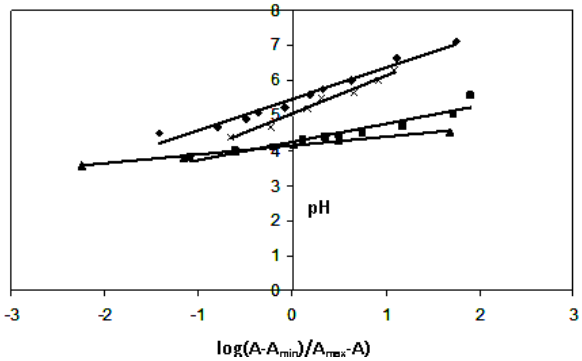
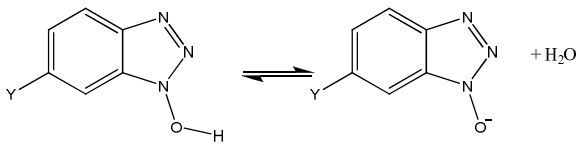


Fig. 9: Variation of pH vs log(A-A_{min})/ (A_{max}-A) for Compounds **4** (♦); **5** (•); **6** (▲); **7** (×) in 95% acetonitrile-water at 25 °C.

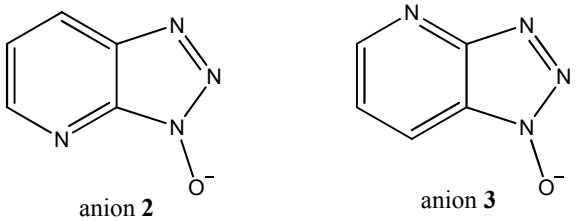
The mechanism of the reaction of secondary alicyclic amines e.g. morpholine and piperidine with *N,N*-diethyl carbamate ester of HOBt and its derivatives was studied lately [11].

The rate enhancement found with 6-NO_2-HOBt **1d** relative to 4-HOAt **2** relative to 6-CF_3-HOBt, **1c,** relative to 6-Cl-HOBt **1b,** to 7-HOAt **3** and to HOBt analogs **1a** is in harmony with the order of pK_a values of the liberated leaving groups [11]. The pK_a values of the liberated leaving groups shown in Table-1 follows the trend: 6-NO_2-HOBt > 4-HOAt > 6-CF_3-HOBt > 6-Cl HOBt ~ 7-HOAt > HOBt. Accordingly, the rate enhancement is consistent with increasing the stability of the leaving group anion liberated (Fig. 10, 11).



1a, Y = H
1b, Y = Cl
1c, Y = CF_3
1d, Y = NO_2

Fig. 10: The leaving group anions librated from the 1-hydroxybenzotriazole derivatives.



anion **2** anion **3**

Fig. 11: The leaving group anions librated from 7-aza-1-hydroxybenzotriazole (7-HOAt, **2)** and 4-aza-1-hydroxybenzotriazole (4-HOAt, **3**).

The stability of compounds **1a-1d** arises from the effect of substituent at position 6. The electronic effect of the substituent may be quantified by the use of a Hammett equation [26]. Good linear correlation between log pK_a versus σ-Hammett constants is found with ρ value equals -0.27 (Fig. 12). The sign and magnitude of ρ indicated that the substituents affect the ionization as well as the stability of **1a-1d** anions by polar effect. The smaller pK_a value of compound **2** compared to that of **1a** (HOBt) and of **3** compared to that of **1a-c** is attributed to the greater stability of their anions by the electron withdrawing effect of the aza group. This indicates that the aza group has a more powerfully electron withdrawing effect more than 6-CF_3, 6-Cl, 6-H groups. However, the larger pK_a value of compound **2** compared to that of compound **3**, both containing an aza group at position 7 and 4 respectively, is presumably attributed to the possible hydrogen bonding which inhibit the ionization of the former compound (Fig. 13). Furthermore, this indicates that hydrogen bonding effect overcomes the proximity effect of the 7-aza group.

In order to study the reactivity of different coupling reagents derived from the oximes **4-7**, kinetic investigations studying their leaving group ability was performed. The mechanistic study of the nucleophilic aromatic substitution, through the nucleophilic attack of an amine involving the departure of different the leaving groups was carried out [18]. This reaction involves the leaving group anions corresponding to the N-hydroxy derivatives **4-7** [15]. The rate enhancement was found to be in agreement with the pK_a values determined in Table-2. Table-2 shows that the pK_a values follow the trend **4** > **7** > **5**> **6**. The change in pK_a of compounds is due to the stability of the developed anion.

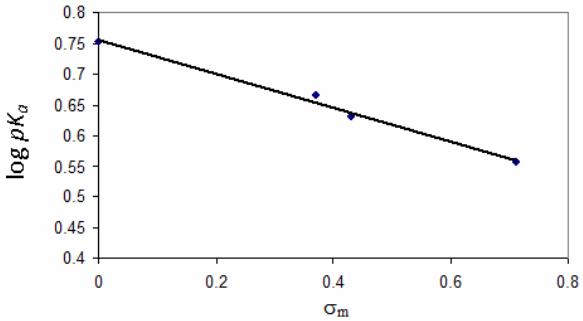


Fig. 12: Linear correlation between log pK_a versus σ-Hammett constants for compounds **1a, 1-b, 1c, 1d** with ρ value equals -0.27. The correlation coefficient is 0.993.

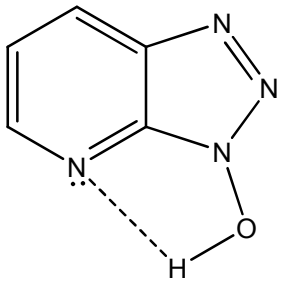


Fig. 13: Possible hydrogen bonding of 7-aza-1-hydroxybenzotriazole (7-HOAt, **2**).

The high acidity of **6** compared to **5** and **7** is due to the presence of two CN groups which favor the stability of its corresponding anion by the resonating structures (Fig. 14-16) and in turn increases the acidity of the OH. The presence of only one CN group and the 2-pyridyl ring increases the stability of the corresponding anion more than the anion containing two COOEt groups (Fig. 17). This is in agreement with the electron withdrawing effect of CN, pyridyl and COOEt groups.

Experimental

Materials

Acetonitrile (Merck) HPLC grade was used, further purification was carried out through distillation from K_2CO_3 anhydrous. Ethyl 2-cyano-

2-(hydroxyimino)acetate (Oxyma) was obtained from commercial sources (Aldrich). Melting points were determined with a Mel-Temp apparatus and are uncorrected. Ultraviolet (UV) data were measured spectrophotometrically using a SHIMADZU (UV-160A) UV-Visible recording spectrophotometer in conjunction with a Shimadzu thermo bath (TB-85).

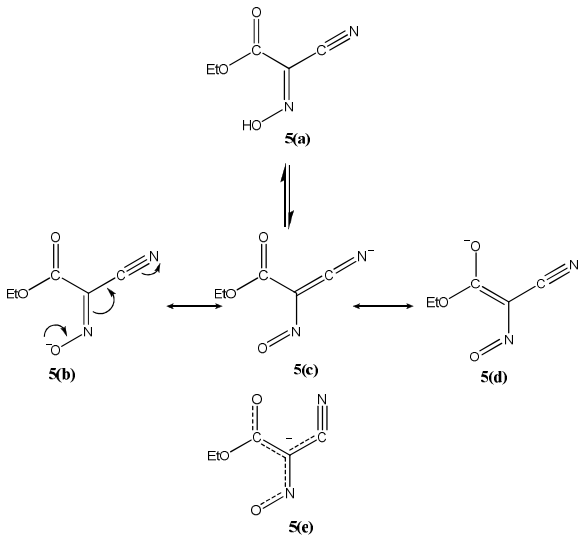


Fig. 14: Resonating structures of ethyl 2-cyano-2-(hydroxyimino)acetate (oxyma) **5**.

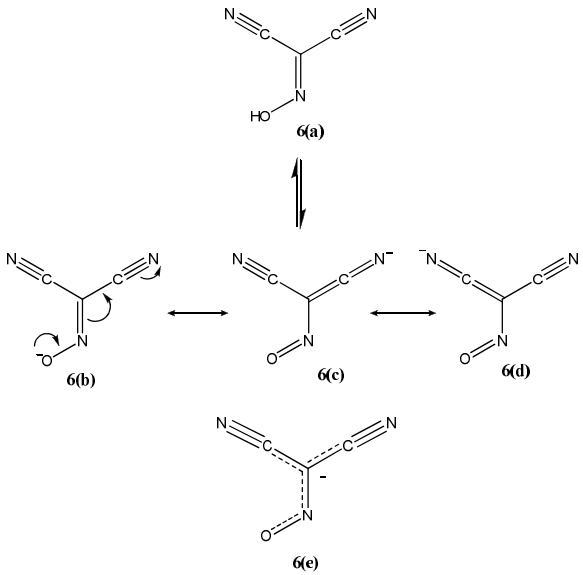


Fig. 15: Resonating structures of hydroxycarbonimidoyl dicyanide **6**.

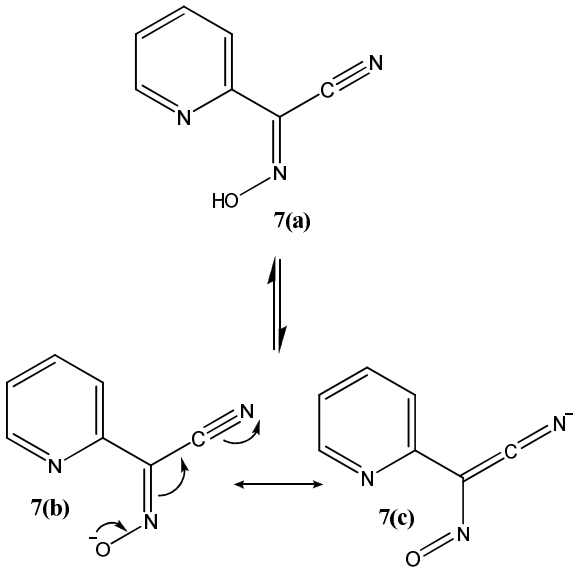


Fig. 16: Resonating structures of *N*-hydroxypicolinimidoyl cyanide **7**.

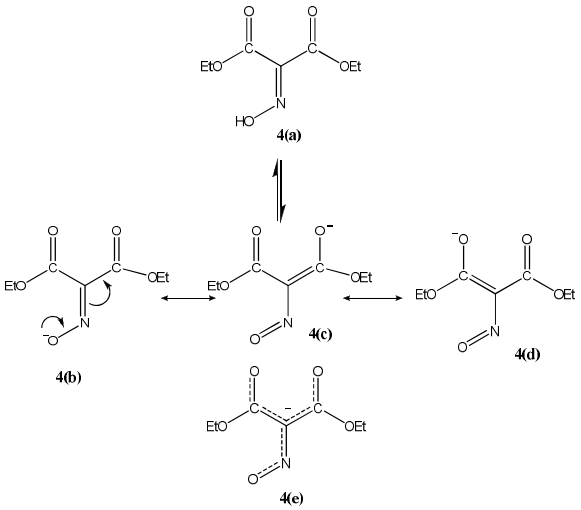


Fig. 17: Resonating structures of diethyl 2-(hydroxyimino)malonate **4**.

General procedure for the preparation of HOBt derivatives **1-3** [9, 27].

5mmol of 1-chloro-2-nitrobenzene [28], 1,4-dichloro-2-nitrobenzene [9], 1-chloro-2-nitro-4-(trifluoromethyl)-benzene [9, 27], 1-chloro-2,4-dinitrobenzene [29], 3-chloro-2-nitropyridine [30] or 2-chloro-3-nitropyridine [30, 31] was added to (15 mmol) hydrazine hydrate dissoved in 30 mL ethanol. The reaction mixture was refluxed for 24 h. After removing the solvent in vacuum, the residue was dissolved in 10% aqueous Na_2CO_3 solution. The solution was washed with ether to

remove the starting material and acidified with concentrated HCl to precipitate the product. The crude product was filtered and dried. The resulting product was recrystallized from ethanol.

1-hydroxybenzotriazole (HOBt) **1**a was obtained as white powder, mp 153-154°C [Lit. 155-158 °C] [28].

1-hydroxy-6-chlorobenzotriazole (6-Cl-HOBt) **1b**, obtained as white powder, mp 195-196°C [Lit. 197-198 °C] [9]**.**

1-hydroxy-6-trifluoromethylbenzotriazole (6-CF_3-HOBt) **1c,** obtained as white powder, mp 145-146 °C [Lit. 146-147 °C] [9, 27]**.**

1-hydroxy-6-nitrobenzotriazole (6-NO_2-HOBt) **1d**, obtained as yellow crystals, mp. 190-191 °C [Lit. 191-192 °C] [29]**.**

7-aza-1-hydroxybenzotriazole (7-HOAt) **2**, obtained as white powder, mp 209-210 °C [Lit. 210-211 °C] [30]**.**

4-aza-1-hydroxybenzotriazole (4-HOAt) **3**, obtained as pale grey crystals, mp 210-211 °C [Lit. 210-212 °C] [30, 31]**.**

Synthesis of Diethyl 2-(hydroxyimino)malonate **4** [29]

A solution of 16g (100 mmol) of diethyl malonate in 17.5 mL (300 mmol) of glacial acetic acid was stirred vigorously at 0-5°C, while addition of a solution of 20.7 g of $NaNO_2$ (300 mmol) in 250 mL of water was added drop-wise during 3-4 h. The ice bath was removed and the mixture was stirred vigorously for 20 h more. The nitrosation was carried out in three necked flask with appropriate fitting and a small vent to permit escape of nitric oxide. The reaction mixture was extracted with 400 mL CH_2Cl_2 and then three 100 mL portions of CH_2Cl_2. The combined CH_2Cl_2 extracts were dried over anhydrous Na_2SO_4. The organic solvent was removed under vacuum and the resulting oily product was disolved once again in 400 mL of CH_2Cl_2 and stirred with anhydrous K_2CO_3 (32 g) for 15 min. The reaction mixture was filtered and and the organic solvent was concentrated until 200mL, ether was added until it became cloudy and then kept in the refrigerator overnight to afford white crystals in yield 12.28 g (65%), mp. 114-115 °C [Lit. 116-118 °C] [32].

Potasium Salt of Hydroxycarbonimidoyl Dicyanide **6** [33]

To a solution of malononitrile (9.06 g, 138 mmol) in acetic acid (20 mL) and water (50 mL), $NaNO_2$ (14.2 g, 206 mmol) was added slowly at 0-5°C for 20-30 min. Then the reaction mixture was stirred at the same temperature for 45 min. After quenching the reaction with 2N HCl (100 mL), the reaction was extracted three times with ether (3 × 100mL). The extracts were dried over anhydrous Na_2SO_4 and the ether was removed under vacuum to give an oily residue. The oily product was added slowly to a cold solution of KOH (8.0 g) in MeOH (100mL) and then the reaction mixture was stirred at 0-5 °C for 20 min. Excess ether was added to afford the potassium salt as yellow crystals in yield 15.3 g (83.3 %), mp. 195-196 °C.

Synthesis of N-hydroxypicolinimidoyl Cyanide **9** [34]

To a solution of 2.24 g (19 mmol) of 2-pyridylacetonitrile in 4.5 mL of glacial acetic acid, after cooling, a solution of sodium nitrite (4.5 g, 65 mmol) in 5 mL water was added dropwise. After 12 h standing the precipitate was filtered off, washed with water, dried and then recrystallized from ethanol to afford the product in yield 1.82 g (65.1 %), mp. 220-222 °C [Lit. mp. 220-222 °C] [34].

pH Measurements and pK_a Calculations

The dissociation constants pK_a's of compounds **1a-1d, 2-7** were determined spectrophotometrically in 95% (v/v) acetonitrile-water at 25 ± 0.1°C at wavelength ranged between 260 to 329 nm for compounds **1a-1d, 2, 3** and between 234 to 303 nm for compounds **4-7**, depending on absorption of different compounds. The Ultraviolet (UV) data were recorded on a SHIMADZU (UV-160A) UV-Visible recording spectrophotometer. Temperature control (± 0.1°C) was attained by circulating water through cell compartments.

Final concentrations of substrates, 10^{-4} mol dm^{-3} **1a-1d, 2-7** were prepared in 95% (v/v) acetonitrile-water; 1 mL of substrate (10^{-3} M) in acetonitrile, 8.5 mL acetonitrile and 0.5 mL buffer solution. The buffer solution was prepared by adding a given volume of 0.2M sodium hydroxide into 100 mL mixture of phosphoric acid (0.04 M), acetic acid (0.04 M) and boric acid (0.04 M) to obtain pH range between 2-12. The pH values of the different

solutions were checked before and after adding the substrate to the buffer solution with a JENWAY 3305 pH-meter.

The pK_a values of compounds **1a-1d, 2-7** were obtained using the half height method [24]. A plot of the variation of absorbance against the pH values at one or more characteristic wavelength gave sigmoid curve. The vertical line at which there is no variation in pH with absorbance gave the pK_a values for the compounds under investigation.

The modified limiting absorption method is another method to calculate the pK_a values, (Equation 1).

$$pKa = \log \frac{A-A_{min}}{A_{max}-A} + a\,pH \quad (1)$$

where A is the absorbance of the solution at a given pH value. A_{min} is the minimal absorption (the absorption at the end of the ionization process). A_{max} is the maximum absorption and is the number of protons released. A plot of pH versus $\log \frac{A-A\,min}{A\,max-A}$ gave a linear relationship where pK_a values were calculated from the intercept with pH.

The third method used for pK_a calculations of compounds **1-7** is the Colleter method [25]. Three different values of pH from half height curve at their corresponding absorbance were used in the following equations 2 and 3.

$$Ka = \frac{[H^+]_2 - M\,[H^+]_3}{M-1} \quad (2)$$

where $[H^+]$ value was calculated from the corresponding pH value and

$$M = \frac{Abs_3 - Abs_1}{Abs_2 - Abs_1} \cdot \frac{[H^+]_1 - [H^+]_2}{[H^+]_1 - [H^+]_3} \quad (3)$$

Abs is the absorbance at a given pH where $[H^+]_1 > [H^+]_2 > [H^+]_3$ and $Abs_3 > Abs_2 > Abs_1$.

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