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CN^- scavenger: a leap towards development of a CN^- antidote†

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Compound 4 removes cyanide from water and human serum in the form of compound 7 (cyano-derivative of 4) and experiments show its suitability as an antidote of CN^- poisoning.

Irrespective of the fact that they are integral components of fauna and flora, cations as well as anions are dangerous once their concentration exceeds a maximum limit and their selective removal from the system becomes an issue of medicinal and ecological importance.[1] Among the anions of biotic significance, cyanide is highly toxic when it exceeds its maximum permissible level of 1.9 μM.[2] It binds with Fe^{3+} of heme unit and retards the oxygen supply.[3] The cellular respiration is affected due to the binding of CN^- with cytochrome c which blocks the electron transport chain and hence the CN^- poisoning causes ultimate death.[4] Practically no effective antidotes of CN^- are available so far[5] which further aggravates the gravity of the issue. As per the reports available,[6] sensing of CN^- is achieved either *via* H-bonding or chemical reaction between the sensor and CN^-. However, these reports do not comment on the biological profiles (toxicity *etc.*) and probable metabolites of CN^- sensors and their use as antidotes of CN^-.

Being used for detection and binding of CN^- from living systems, molecules similar to trimethoprim[7]/its metabolite (**1–2**, Chart 1) were designed (**3–4**, Chart 1). Use of trimethoprim as antibacterial agent and also the biological acceptance of barbituric acid and pyrazole are the safeguards for non-toxicity of the new compounds to living systems. Compounds **3** and **4** were built in quantitative yield by Knoevenagel condensation between an equimolar mixture of pyrimidine/pyrazole and syringaldehyde on irradiation under microwaves (SI, Scheme 1).

Observation of a change in color of a solution of compound **3**/**4** (in dry ethanol) from light yellow to orange on addition of tap water, and no color change on addition of distilled water led to systematic investigations on these compounds. Various anions were added to the solution of compound **4** (10 μM in ethanol–distilled water, 1 : 1) but color change from light yellow to orange to red was observed only when minimum 0.1 μM solution of NaCN was added, and color change from light yellow to orange on addition of 1 μM solutions of Na_2CO_3 and $NaHCO_3$ (in ethanol–distilled water,1 : 1) (Fig. S19). It seems that CO_3^{2-}, HCO_3^- or may be CN^- present in tap water caused the color change when water was added to the ethanolic solution of compound **4**. A solution of compound **4** (10 μM in ethanol–distilled water, 1 : 1) exhibited UV-vis absorption bands at 219, 249 and 403 nm (Fig. 1a). On incremental addition of (0.01–1 equiv, 0.1–10 μM) tetrabutylammonium cyanide (TBACN)/NaCN to the above solution of compound **4**, absorption at 219 and 403 nm decreased with increase at 249 nm. A weak band at 302 nm and a strong band at 520 nm emerged with three isosbestic points at 276, 321 and 444 nm (Fig. 1a). Color of the solution changed from light yellow to orange to red within a second. The same change in color and absorption spectrum was observed when compound **4** (10 μM) was added to a solution of NaCN in ethanol–water. Job's plot indicated 1 : 1 stoichiometry of **4**·CN^- (Fig. S20) and K_a was 10^6 M^{-1}. UV-vis spectra of the above solution of **4** + CN^- did not undergo any change with time (Fig. S21) indicating that first order reaction (Fig. S22) between **4** and CN^- is very fast, completed in a fraction of a second, a time which is probably less than the poisoning time of CN^- (seconds to a few minutes). Further addition of CN^- (up to 100 equiv) to a solution of **4** + CN^- resulted in a decrease in absorption intensity at 520 nm (Fig. 1b). A solution of **4** + CN^- gives a new spot in TLC (thin layer chromatography) which after isolation showed a

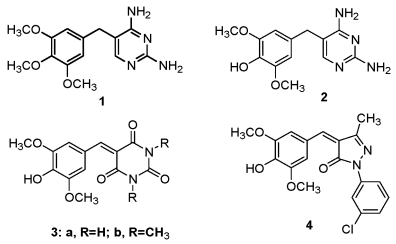

Chart 1

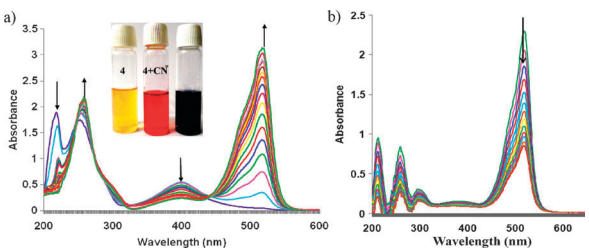

Fig. 1 (a) UV-vis absorption spectra of compound **4** (ethanol–water, 1 : 1 v/v) upon incremental addition of CN^- (up to 1 equiv). (b) Addition of CN^- (> 5 equiv) to solution of **4** + CN^- resulted in decrease in absorbance at 520 nm.

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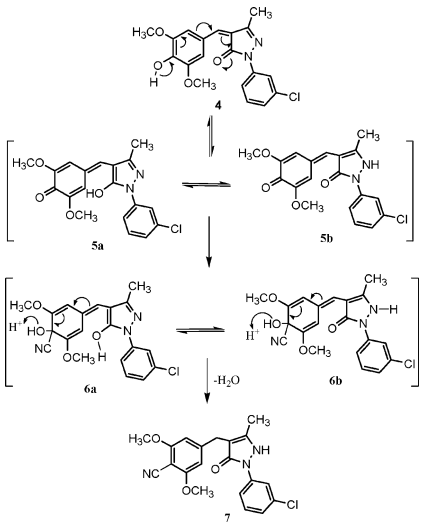


Scheme 1 Capturing of CN^- by compound **4**.

new absorption band at 590 nm (Fig. S23). It seems that initial deprotonation of phenolic H from compound **4** changed the conjugation of π-electrons to form species **5** (tautomeric forms, **5a** and **5b**) (Scheme 1). Now abstraction of H from OH/NH group on the pyrazole moiety changed **5** to compound **4** and a decrease in absorption band at 520 nm took place until equilibrium was attained between **4** and **5**. Reaction of CN^- at carbonyl carbon (1,2-addition[8]) of the α,β-unsaturated carbonyl system forms **6** which on protonation, loss of water and reduction ultimately gave compound **7**. Isolation of compound **7** from a solution of **4** + CN^- and an absorption band at 590 nm in its UV-vis spectrum (Fig. S23) supported the route of CN^- action as proposed in Scheme 1. NMR titrations also supported the mode of action of CN^- as the signal of OH (δ 6.24) disappeared on addition of CN^- (Fig. S24, S25). No response of compound **8** (Chart 2) to CN^- (Fig. S26) also indicated that action of CN^- started from the phenolic moiety of compound **4**. Moreover, addition of CN^- (1 equiv) to compound **9** (Chart 2) in water–ethanol resulted in an increase in absorption at 520 nm which is ~8% only of what was observed in the case of compound **4** (Fig. S27). This indicated the role of OCH_3 groups in the abstraction of phenolic H (probably H-bond of OH with O–CH_3 increases the acidity of phenolic OH).

To rule out if it is OH^-, generated from hydrolysis of NaCN, that is responsible for color change and absorption spectra of compound **4** in aqueous medium, all these experiments were also carried out in dry THF, dry ACN and dry $CHCl_3$ using TBACN (Fig. S28, S29). The same observations as in aqueous medium indicated the participation of CN^- only in changing the color of compound **4**.

To look into the behaviour of compound **4** towards CN^- in acidic and basic media, incremental addition of CN^- was made to the solution of compound **4** (10 μM, ethanol–distilled water, 1 : 1) at pH 2–7 (Fig. S30) (**4** is stable under aqueous, acidic conditions).

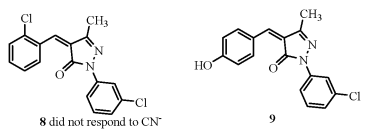


Chart 2

Similar change in the color of the solution and absorption spectra of compound **4** was observed as in neutral medium indicating its suitability for working under acidic conditions (usual pH of major part of digestive system).[9] A solution of compound **4** in basic medium (pH 8, pH range of intestinal part of digestive system)[9] showed absorption bands at 219, 249, 369 and 520 nm. The absorption band at 520 nm was probably due to abstraction of phenolic H as in the presence of CN^-. However, addition of CN^- to the basic solution of compound **4** intensified the absorption band at 520 nm (Fig. S31, S32) which means this compound is detecting CN^- in alkaline medium also. Compound **7** was isolated from both experiments (in acidic and basic media).

The UV-vis spectrum of compound **4** in presence of other anions like F^-, ^-OAc, $H_2PO_4^-$, Cl^-, Br^- except CO_3^{2-} and HCO_3^- did not undergo any change indicating the selectivity of compound **4** for CN^- over other anions (Fig. S19, S33, S34). Addition of CO_3^{2-} (up to 10 equiv, 100 μM, no change on further addition of CO_3^{2-}) to a solution of **4** (10 μM) in ethanol–water resulted in an increase in absorbance at 520 nm. It is apparent from Fig. 1 and Fig. SI35 that increase in absorbance of **4** at 520 nm is much higher when CN^- (1 equiv only) was added in comparison to the addition of CO_3^{2-} (1/10 equiv). This might be due to early attainment of equilibrium between **4** and **5** (Scheme 1) during the addition of CO_3^{2-} while in presence of CN^-, equilibrium is more to the forward direction. This is also supported by the increased absorbance at 520 nm on addition of CN^- to the solution of **4** + CO_3^{2-} and **4** + HCO_3^- (Fig. S35, S36). K_a for **4**·CO_3^{2-} and **4**·HCO_3^- was 0.3 × 10^6 M^{-1} and 1.6 × 10^4 M^{-1}, respectively (Fig. S37 and S38) which were considerably less than that of **4**·CN^- indicating better interactions of **4** with CN^-. Based on this observation, the UV-vis spectrum of compound **4** in tap water–ethanol (1 : 1) was recorded. Irrespective of the initial color change of compound **4** in tap water and the appearance of a weak absorption band at 520 nm, addition of CN^- further intensified this band indicating that CO_3^{2-} and HCO_3^- present in natural water (here ground water through tube-well) did not interfere much in the detection/capturing of CN^- by compound **4** (Fig. S139a).

Practicability of compound **4** was evident from its competitive binding with CN^- in presence of other anions. Solution of compound **4** did not undergo color change even in presence of 100 equiv of various anions. But addition of CN^- to these solutions made the color change from light yellow to orange. The UV-vis spectrum of a solution of compound **4** + anions (other than CN^-) exhibited characteristic absorption bands of compound **4** but an

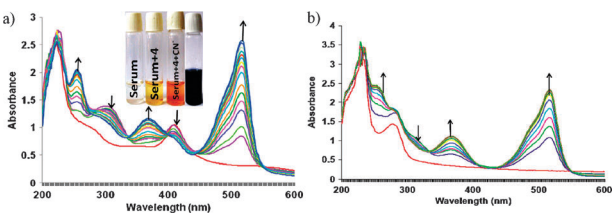


Fig. 2 (a) UV-vis spectrum of proteinaceous human serum (lower red trace), on addition of compound **4** (second lower purple trace) and further addition of CN^-. (b) UV-vis spectrum of non-proteinaceous human serum (lower red trace), additon of compound **4** (second lower purple) and further addition of CN^-.

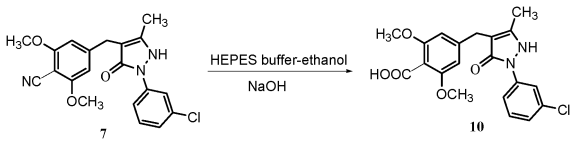
intense new band at 520 nm appeared on addition of CN^- indicating competitive binding of **4** with CN^- (Fig. S40).

As the concentration of Fe^{3+} (0.01 mM) in the human body[10] is ~5 times the lethal concentration of CN^- (>1.9 μM), it was worth checking the behaviour of **4** towards CN^- in presence of Fe^{3+}. Addition of 15 equiv of Fe^{3+} (w.r.t. CN^-) to a solution of **4** + CN^- did not affect the color of the solution and the absorption spectrum as well but further addition of Fe^{3+} changed the color of the solution from orange to light yellow and an absorption band at 520 nm disappeared (Fig. S139b). Therefore, irrespective of the strong affinity of CN^- for Fe^{3+}, CN^- preferred compound **4** in the presence of even 15 equiv of Fe^{3+}. Moreover, addition of Mg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} did not disturb the color and the absorption spectrum of a solution of **4** + CN^-. Compound **4** did not respond to the biological cations like Fe^{3+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}. Solutions of compounds **3a** and **3b** in the same solvent (ethanol–water, 1 : 1) also gave similar responses to CN^- (SI, Fig. S41–50).

To check further the working of compound **4** under biological conditions and the affinity of CN^- for compound **4** *vs.* biological Fe^{3+} (Fe^{3+} in porphyrin), human serum (diluted in water) was used as medium for performing the experiments.

Proteinaceous blood sample (sample I)[11] showed absorption bands at 215 nm and 412 nm (Fig. 2a). Addition of 20 μL of compound **4** (10 μM) to the above solution of sample I turned the colorless solution light yellow. The UV-vis spectrum of this solution exhibited usual absorption bands of compound **4** and a new band at 520 nm (Fig. 2a). To rule out if the absorption band at 520 nm is due to the interactions of compound **4** with some proteins in the blood sample, a non-proteinaceous sample (sample II)[11] of blood serum was prepared. Sample II showed absorption bands at 227 nm and 271 nm (Fig. 2b). Addition of 20 μL of compound **4** (10 μM) to a solution of sample II turned the colorless solution light yellow and the UV-vis spectrum of this solution also exhibited an absorption band at 520 nm (Fig. 2b). The absorption band at 520 nm seems to be due to the presence of $CN^-/CO_3^{2-}/HCO_3^-$ in blood itself and this band gets intensified on incremental addition (1 μM to 5 μM) of CN^- along with color change of the solution from light yellow to orange to red (Fig. 2a,b). Addition of CN^- to a solution of sample I + **4** also led to an increase in the absorption intensity at 250 nm and 360 nm with concomitant decrease at 228 and 299 nm (Fig. 2a). Alternatively, addition of compound **4** (10 μM) to a solution of sample I + CN^- (10 μM CN^-) resulted in change in color and the appearance of an absorption band at 520 nm. Compound **7** was also isolated from this solution showing the scavenging of CN^- from blood serum by compound **4**.

It was ensured that treatment of CN^- containing water sample and human serum with compound **4** completely removes CN^- from the medium. The aqueous part, after extraction of solution of **4** + CN^- (10 μM compound **4** and 10 μM NaCN) with ethyl acetate, did not show color change on addition of compound **4** which otherwise could have been there if CN^- had been present in the aqueous part. The aqueous part, left after extraction of solution of NaCN (in water–ethanol) with ethyl acetate, gave a color change on addition of compound **4** indicating that CN^- was not extracted with ethyl acetate only but it is compound **7** which is carrying cyanide from the aqueous solution (Fig. S51). The same observations of complete removal of CN^- by compound **4** from



Scheme 2 Hydrolysis of compound **7** to compound **10**.

human serum were recorded. Non-toxicity of compound **4** was apparent from its higher LC_{50} value at various cell lines of human cancer (data not shown here) as well as its similarity to trimethoprim.[7b,12] Under reaction conditions of **4** and CN^-, **3**/**4** did not react with cysteine (analogue of GSH).[13] Hydrolysis of compound **7** under basic conditions (usual pH in lower part of digestive system) provided compound **10** (Scheme 2) indicating the conversion of CN group to COOH and hence the safe removal of CN^- at the end.

Therefore, a simple synthesis which could be scaled up to procure tons of compounds provided compounds **3** and **4** in which the appreciable acidity of phenolic OH helped in the detection and binding of CN^- in presence of other anions and even 15 equiv of Fe^{3+} also (biological target of CN^-). All the experiments carried out here with compound **4** indicated the suitability of this compound as an antidote of CN^-. Study in living systems is underway.

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