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Letter

First chemosensor for selective detection and quantification of L-4-Hydroxyproline in collagen and other bio samples

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First chemosensor for selective detection and quantification of L-4-Hydroxyproline in collagen and other bio samples

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ABSTRACT: Amino pyridine-based rhodamine conjugate (APR) has been developed as a first chemosensor for selective detection and quantification of L-4-Hydroxyproline (Hyp). The "turn-on" fluorescence property of the chemosensor makes it unique for easy estimation of Hyp in collagen and biological samples.

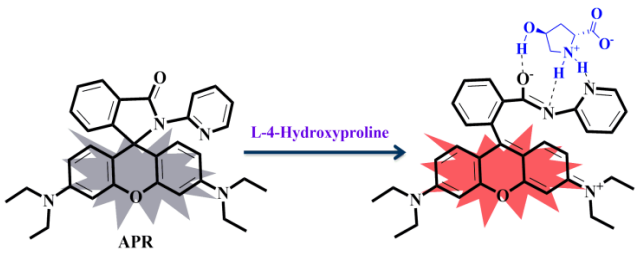
Hydroxyproline (**Hyp**) is a nonessential amino acid predominantly present in body protein - collagen. It is found to be present as much as 13.5% of mammalian collagen. However the skeletal system is the major reservoir of **Hyp** which contains more than 50% of body collagen. **Hyp** excretion through urine is the major indicator of collagen metabolism.[1-2] Urinary **Hyp** is largely derived from bone catabolism, reflecting bone collagen turnover, which in turn provides a good index of bone matrix turnover rate.[3-4]

Urinary **Hyp** levels are found to be increased majorly in patients with bone metastases. Detection of early bone metastases can be useful for appropriate staging of patients with breast cancer.[5-6] Other conditions in which **Hyp** excretion increase are Marfan's syndrome, Hurler's syndrome, rheumatoid arthritis,[7] Paget's disease,[8] osteomalacia,[9] osteolytic lesions,[10] metastatic prostatic carcinoma,[11] hyperthyroidism,[12] hyperparathyroidism, and dwarfism. Whereas reduced urinary **Hyp** levels signifies many deviant conditions, including severe protein-calorie malnutrition, muscular dystrophy and severe renal disease especially for children.[13-14]

Thus, selective detection and accurate quantification of **Hyp** in collagen/biological samples in general and urine or serum in particular holds significant promise for providing predictive and diagnostic information. There are few published methods like stable isotope labeling and LC-MS based,[15] colorimetric-based,[16] biochemical and morphometric based,[17] homogenization technique,[18] gas chromatography/ mass spectrometry,[19] enzymatic based[20] methods have been reported for the detection and estimation of **Hyp**. However, so far no published articles did emphasize any chemosensor which explains the detection and direct quantification of **Hyp** in collagen or in other biotic or abiotic sample. The choice of a chemosensor over other reported biosensors/detection techniques is due to its implementation in simple and in complex matrix, with much less time and cost, through simple one-step, direct detection method on biotic and abiotic samples. While comparing with other techniques our chemosensor is thus much advanced as it can easily detect **Hyp** selectively in a particular experiment and could be pertinent in high-throughput screening in biological samples (performance comparison is shown in table S1).[15-20]

In molecular recognition chemistry, the exploration of fluorescent probes[21-26] with enviable properties are significantly important due to their indispensable role in medicinal and biological applications.[27-29] The synthetic chemosensors containing optical-signaling chromophoric parts generally imply sensing of certain biomolecules selectively via covalent linking.

Scheme 1: "Turn-on" fluorescence changing of APR upon addition of Hyp.



In this work we prepared a simple chemosensor based on 2-amino pyridine appended rhodamine derivative (**APR**). It shows efficient and selective fluorescence signal for **Hyp** (*trans*-4-Hydroxy-L-proline) in aqueous medium (Scheme 1). Chemosensor **APR** was well characterized by ^{1}H NMR, ^{13}C NMR, IR, MS, and X-ray analysis (Figure S1-S5, Table S2, Supporting Information). To comprehend the interactive properties of **APR** towards **Hyp**, several studies were carried out by absorption, emission, ^{1}H-^{13}C NMR spectroscopy and the species of recognition was addressed by computations based on the density functional theory (DFT). The crystal structure (Figure 1) illustrates that the two planes of the spiro form of the probe **APR** remains vertical to each other.

From the crystal structure and DFT calculations it is evident that the pyridine 'N' atom always resides anti to the lactam 'O' atom in **APR**. But surprisingly, DFT calculation shows that during complexation with **Hyp** the pyridine 'N' and

lactam 'O' comes in *cis* fashion to stabilise the bound structure as shown in scheme 1.

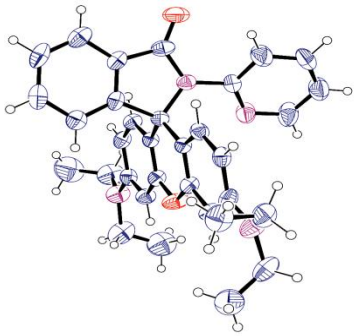


Figure 1. ORTEP diagram of **APR**. Ellipsoids are drawn at 40% probability level.

EXPERIMENTAL METHODS

All procedural information can be found in the Supporting Information.

RESULTS AND DISCUSSION

More interestingly, when we tried to put pyrrole or 1,8-napthyridine in place of pyridine of **APR**, **Hyp** doesn't respond with any of those particular derivatives except **APR** (Figure S6).

The interaction of **APR** with **Hyp** was established by absorption and fluorescence titrations, carried out in aqueous medium at neutral pH.

From the UV-vis absorption spectra of the probe **APR** in aqueous medium at neutral pH, it has been revealed that the gradual accumulation of **Hyp** significantly enhance the absorbance of **APR** at 558 nm (Figure 2a), accompanied by a color change from colorless to deep pink. The binding constant (K_a) of the **APR- Hyp** complex from UV-vis titration was calculated $4.09 \times 10^5\ M^{-1}$ (Figure S7).

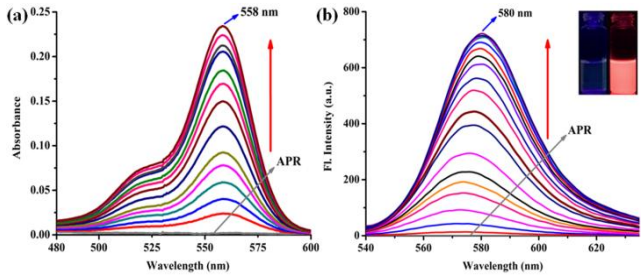


Figure 2. (a) UV-vis absorption spectra of **APR** (1 μM) upon gradual addition of **Hyp** upto 1.2 equiv. in H_2O-CH_3CN (10:1, v/v) at neutral pH. (b) Fluorescence emission spectra (λ_{ex} = 510 nm) of **APR** (1 μM) toward **Hyp** at varied concentrations (0, 0.01, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.60, 0.70, 0.80, 0.90, 1.0, 1.10, 1.20 μM) in H_2O-CH_3CN (10:1, v/v) at neutral pH.

On addition of **Hyp**, fluorescence intensity of **APR** (Φ_f = 0.008) increases extensively at 580 nm (Figure 2b) with a 72-fold increase in fluorescence (Φ'_f = 0.57, Table S3). The corresponding association constant (K_a) of **APR-Hyp** complex from the fluorescence titration was determined $4.12 \times 10^5\ M^{-1}$ (Figure S8) by non-linear fitting analysis of the titration curves.[30-31] It was found that **APR** has a detection limit of 0.15 nM for **Hyp** (Figure S9). The 1:1 stoichiometry of **APR** to **Hyp** was determined from Job's plot (Figure S10). The above spectroscopic observations indicate the probe which exists in a spiro cyclic form being non-fluorescent and colorless, undergoes ring opening of the corresponding spirolactam via irreversible coordination upon addition of **Hyp** to the probe.

While doing experiment with rhodamine derivatives, maintaining pH of the solution is very important. Before going to our experiment we used different metal ions (Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Zn^{2+}, Pb^{2+}),[32] for complexation studies with **APR**. However, probe **APR** doesn't show any color or fluorescence with none of these metal ions at neutral pH. While moving to our case we checked that the pH of **APR** and **Hyp** in aqueous solution is 7.3 and 6.93, respectively. Still we took both of these in PBS buffer (pH 7.4) and after two hours we record that the final pH of each solution is 7.4. **APR** is non-fluorescent at pH range 5-13 but while adding **Hyp** the probe shows deep pink color from pH 1 to 8. To understand the above phenomena we performed the detailed pH titration as shown in Figure S11.

The change in color and fluorescence of **APR** upon addition of various amino acids (**Hyp**, L-proline, glycine, alanine, serine, homoserine, threonine, tyrosine, arginine, glutamine and histidine) has been observed in necked eye and under a UV lamp as well (Figure 3a). As in presence of **Hyp**, a change of intense color/fluorescence of **APR** was observed while addition of L-proline shows very weak fluorescence and others do not respond. The above observation shows consistency with the fluorescence titration experiments where no such binding of **APR** with other amino acids was found (Figure 3b and Figure S12). This result reveals the binding efficiency and sensing selectivity of **APR** towards **Hyp**.

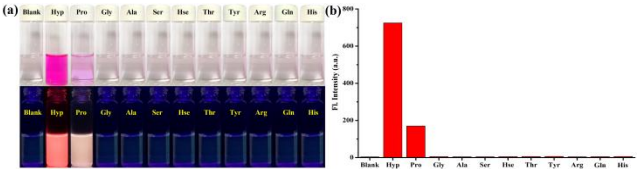


Figure 3. (a) The visible color (top) and fluorescence changes (bottom) of **APR** (1 μM) in aqueous medium upon addition of 1.2 equiv. of various amino acids (λ_{ex}= 510 nm). (b) Histogram representing competitive fluorescence spectra of **APR** (1 μM) upon addition of 1.2 equiv. of guests at 580 nm (λ_{ex}= 510 nm) in H_2O-CH_3CN (10:1, v/v) at neutral pH. [From left to right: **APR**, **APR** with- **Hyp**, L-proline, glycine, alanine, serine, homoserine, threonine, tyrosine, arginine, glutamine, histidine]

Binding of **APR** and **Hyp** was further studied by density functional theory (DFT) calculations using the 6-31G+(d,p) method basis set implemented at Gaussian 09 program. CPCM solvent model included the solvent effects. Geometry optimization resulted in conformational changes at the spirolactam position of **APR** while **Hyp** takes part to accommodate a probe molecule. The pyridine 'N' and lactam 'O' of **APR** comes in *cis* fashion to stabilise the bound structure and the energy is minimized by 18.34 kcal from the probe to get the stable complex structure (Figure 4). This theoretical study strongly correlates the experimental findings.

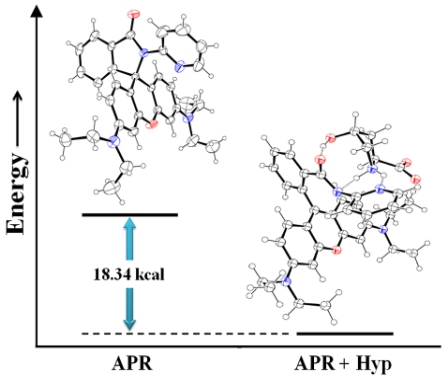


Figure 4. Energy diagram showing the energy differences between **APR** and **APR-Hyp** complex.

To explore the interactions of the chemosensor **APR** with **Hyp**, ^{1}H NMR titration was carried out in DMSO-d_6 (Figure S13). Pyridine protons of **APR** exhibits gradual downfield shift upon addition of **Hyp**. This is owing to the electron density of the pyridine ring of **APR** decreases upon binding with **Hyp** through hydrogen bonding. Xanthene protons also shifted downfield upon spirolactam ring opening, indicating the strong coordination of probe **APR** with **Hyp**. In ^{13}C NMR titration the spiro cycle carbon peak of **APR** at 66 ppm was shifted to 148 ppm after complexation with **Hyp** along with a downfield shift of the aromatic region of **APR** (Figure S14). This coordination led to the spiro cycle opening and changes to the absorption and emission spectra. From IR titration it has also been revealed that the amide carbonyl peak of **APR** at 1695.31 cm^{-1} shifted to 1657.70 cm^{-1} upon addition of 1 equiv. of **Hyp** which signifies the involvement of amide carbonyl 'O' in the formation of **APR-Hyp** complex.(Figure S15).

In addition, the specific and selective recognition of **Hyp** by the chemosensor **APR** was also examined in biological samples. **APR** doesn't show any cytotoxicity (Figure S16)[33] to the tested cell (A549 human cancer cell line) and could be a choice of probe to detect **Hyp** in any bio sample.

The concentration of **Hyp** was quantified from commercially available normal collagen and from collagen-rich chicken and goat ligament (Table 1). In order to detect **Hyp** present in collagen or other complex sample, treatment of the sample is needed to get **Hyp** in its free form. In this work we treated dried collagen sample with 6(M) HCl and get homogeneous solution of **Hyp** after acid hydrolysis (details in Supporting Information).[16] **Hyp** was detected and quantified from these samples by **APR** (1 µM) with the virtue of its selective and direct recognition properties. All estimation was done in triplicate. **Hyp** concentrations were calculated by comparison with **APR-Hyp** standard fluorescence curve (Figure 5). The amount of collagen in ligaments or collagen-rich tissues is already been standardized.[34] The amount of **Hyp**, in turn, is almost constant in collagen.

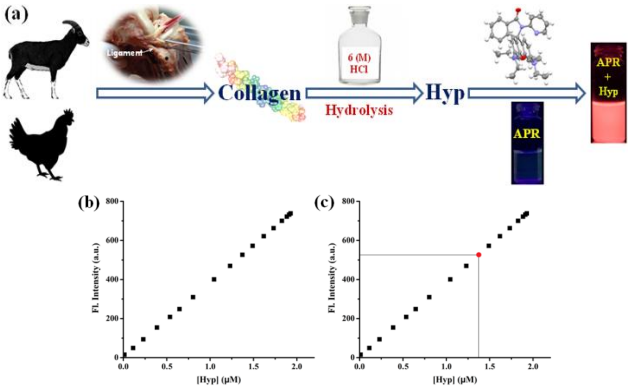


Figure 5. (a) Schematic diagram of collagen extraction from knee ligament of Goat and Chicken and quantification of **Hyp**. (b) Standard fluorescence curve obtained for the estimation of **Hyp**. (c) Estimation of unknown concentration (1.38 µM) of **Hyp** (red point) in collagen from the standard fluorescence curve.

Table 1. Determination of Hyp in collagen

Entry	Collagen (% Dry Weight)	Weight taken (mg)	**Hyp** (µM)	% of **Hyp** present
Commercially available collagen	99	10	1.38	13.8
Goat Ligament	70.8[b]	10	1.05	10.5
Chicken Ligament	65.3[b]	10	0.85	8.5

[a] An average of three replicate measurements with standard deviation.
[b] % Dry weight of collagen from each ligament as estimated by Adam P. Rumian et.al.[34]

In consequence, we also estimated the amount of **Hyp** in human urine and serum samples (Table 2). Collected serum and urine samples were processed following the published report.[35] The final extracted parts were subjected to fluorimetric analysis to quantify the amount of **Hyp** present therein.

Table 2. Determination of Hyp in biological samples

Entry	Conc. of **APR**	Samples	Amount of sample taken (µL)	**Hyp** present (µg/ml)[a]
Urine	1 µM	U1	100	19.5 ± 0.01
		U2	100	56.2 ± 0.03
		U3	100	95.6 ± 0.04
Serum		S1	100	1.32 ± 0.03
		S2	100	1.56 ± 0.04
		S3	100	1.78 ± 0.02

[a]An average of three triplicate measurements with standard deviation.

Assay of **Hyp** was further validated from multiple samples of human urine and blood using **APR**. Each sample has been assayed in triplicate. The increased fluorescence signals have also been statistically validated after calculating the Z' value (Table S4).[36] The Z' score from all tested samples (more than 0.9) indicates an optimized and validated assay of **Hyp**.

Thus occurrence of **Hyp** in various tissues and urine or serum samples need to be quantified in order to detect the metabolic dysfunction or various carcinomas. Hence the present research offers an indispensable chemosensor which can selectively quantify **Hyp** from various biotic or abiotic samples in nanomolar range from aqueous solutions.

CONCLUSIONS

In summary, the selective detection and quantification of **Hyp** in collagen-rich tissues have successfully been accomplished by a simple rhodamine based chemosensor **APR**. The response of **APR** towards **Hyp** evinces high sensitivity and selectivity both in the colorimetric and fluorimetric detection method at neutral pH with very low concentration. The above method was successfully applied to estimate **Hyp** in normal collagen and in chicken or goat ligament along with human urine and serum samples through ''turn-on'' fluorescence signal. Moreover, this method can also be applied to quantify **Hyp** in disease collagen tissues like cystic fibrosis or even in urine and blood serum samples of patients with metabolic dysfunction or various carcinomas. The potentiality of the chemosensor **APR** could be treated as a simple, time consuming, cost-effective and very useful tool for estimating **Hyp** in biological samples.

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, full characterization and binding studies have been included in Supporting Information.

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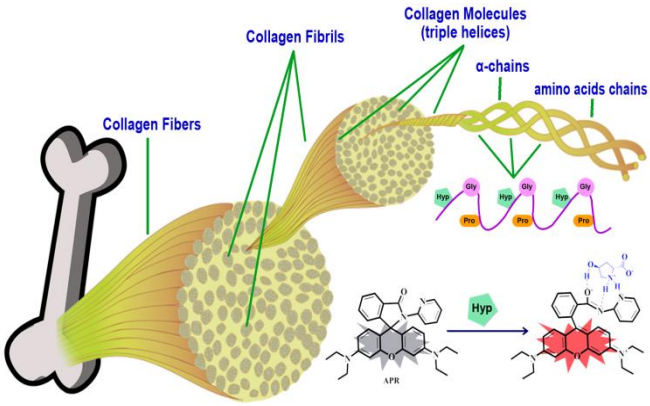
Collagen Molecules
(triple helices)
Collagen Fibrils
α-chains
amino acids chains
Collagen Fibers
Hyp
Gly
Pro
Hyp
APR