Vinyl-Triphenylamine Dyes, a New Family of Switchable Fluorescent Probes for Targeted Two-photon Cellular Imaging: from DNA to Protein Labeling.

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Supplementary information

Materials and methods

a- Chemicals and analyses

All chemicals were purchased from Sigma-Aldrich (USA) or Acros Organics (Belgium) and used as received. Human Serum Albumin (A3782, 99%, globulin free) and Bovin Serum Albumin (A7030, 98%, essentially fatty acid free and globulin free) were dissolved in 10 mM sodium cacodylate buffer pH 7.2 with a 5 mg/mL-1 concentration and stored at -20°C. The molar concentration of these solutions was then controlled by UV using the values DO = 0.667 (BSA) and 0.631 (HSA) for 1 g.L-1 and M = 66500 Da for both proteins.1 Warfarin (A2250) and Ibuprofen (I4883, >98%) were dissolved in anhydrous DMSO with a 50 mM concentration and stored at -20°C.
Preparative flash chromatographies were carried out with Merck silica gel (Si 60, 35-70 µm). 1H NMR spectra were recorded at 300 MHz on a Bruker Advance 300 spectrometer. Chemical shifts are reported in ppm downfield to TMS (δ = 0.00). High-performance liquid chromatography was carried out on a Waters Alliance equipped with a photodiode array detector using an XterraMS column with a linear gradient of acetonitrile versus water containing 0.05% of TFA ranging from 2 to 30% over 5 min then to 100% over 9 min at a flow rate of 1mL/min. Electrospray ionization mass spectrometry (ESI-MS) was performed at the Institut Curie and HRMS were performed at the Small Molecule Mass Spectrometry platform of IMAGIF (ICSN, Gif-sur-Yvette, France).

b- Fluorescence and Absorption Spectroscopies

UV-vis experiments were monitored on a Uvikon XL (Secomam, France) UV spectrophotometer. Fluorescence spectra were recorded on a FluoroMax-3 (Jobin Yvon), at room temperature. Measurements were performed with solutions of OD < 0.1 to avoid re-absorption of the emitted light, and data were corrected with a blank and from the variations of the detector with the emitted wavelength. All experiments were performed in 10 mM sodium cacodylate buffer pH 7.2. Fluorescence quantum yield were measured according to Williams comparative method using rhodamine 101 in ethanol as reference. Absorption and fluorescence spectra were recorded for five solutions of increasing concentrations with an absorbance comprised between 0.01 and 0.1 to avoid reansorption phenomenon. The fluorescence of the sample and the reference are then plotted versus the absorbance. The data should correlate linearly and after calculating the gradient of the curve, the fluorescence quantum yield is given by equation (1),

(1) $\Phi_x = \Phi_{ref} (Grad_x/Grad_{ref}) (\eta^2_x/\eta^2_{ref})$

where x stands for the sample, ref the reference, Φ the quantum yield and η the refractive index of the solvent.

The fluorimetric titrations were performed at a constant dye concentration of 0.25 µM with increasing concentrations of protein. The dyes were excited at 490 nm and the fluorescence recorded from 510 to 850 nm. The binding curves were obtained by plotting the fluorescence enhancement F/F_0 (F = integrated fluorescence area of the complex and F_0 = integrated fluorescence area of the free dye) versus the concentration in serum albumin.
To calculate the affinity constants, data were analyzed according to a one-site binding model described in equation (2) were K_d represents the dissociation constant of the complex.
(2) $F/F_0 = (F/F_0)_{max} \times [HSA] / (K_d + [HSA])$

c- Circular dichroism

The CD spectra were recorded on a Jasco J-710 spectrometer in a 1 cm quartz cuvette thermostated at 22°C. The DNA concentration was kept constant with increasing concentrations of dyes. Each spectrum is the accumulation of four scans performed with a 1 nm step and a 200 nm/min scanning speed.

d- Two-photon absorption cross-section measurements

Two photon induced fluorescence measurements were performed using an inverted microscope set-up coupled to a mode locked Ti-sapphire laser (Tsunami, Spectra Physics) delivering 100 fs pulses with a 76 MHz repetition rate over the spectral range covering 740 to 950 nm. The Ti-Saph excitation beam was focused in a cell filled with low concentration solutions using a 40x microscopic objective, the same objective being also used for the two-photon fluorescence signal collection. The signal is then sent either to a channel plate multiplier (Perkin Elmer MP-993-CL) or to a spectrometer coupled to a CCD camera (Andor DU401-BR-DD) for detailed study of the emission spectra. The laser beam was linearly polarized and a set of half-wave plate and polarizer was used to vary the fundamental beam intensity (700µW at the level of the sample). Excitation spectra were determined from measurements of the whole emitted light using a photomultiplier proceeded with filters cutting the fundamental beam (SemRock razor edge 785, stopline 785 and 808, FF735 and FF-01-750). The TPIF intensities of the samples were measured relative to a solution of fluorescein in water (pH= 13), the ratio of the fluorescent signals enabling further determination of the 2PA cross section (δ) assuming equal one- and two-photon fluorescence quantum yields.[2]

e- Cellular culture and confocal microscopy

Cells were grown on coverslips at 37°C in monolayer cultures in complete DMEM (Gibco, Cergy Pontoise, France) with 10% FCS and antibiotics (100 mg/ml streptomycin and 100 mg/ml penicillin) under conditions of 100% humidity, 95% air and 5% CO_2 for 24 hours. The cells were then incubated for two hours with the desired compounds (2 µM). After washing twice with PBS, the cells were fixed with 4% paraformaldehyde and washed with PBS twice. The coverslips were then mounted on microscope slides using Prolong Gold antifade reagent (Invitrogen).
The confocal (one- and two-photon excitation) imaging was performed using a confocal laser scanning microscope DMI 6000 with a SP5-AOBS unit (both Leica) equipped with a 63x (NA = 1.4) objective (oil immersion), an argon gas laser for one-photon excitation and a Chameleon Ti:Saph laser delivering pulses in the 100 to 200 fs range at an 80 MHz repetition rate with a tunability ranging from 705 to 980 nm for two-photon excitation.

Synthesis

Detailed synthesis of the neutral precursors of TP-2PySf and TN-3Py can be found in our previous papers, (Allain *et al. ChemBiochem* **2007** and Bordeau *et al. J. Am. Chem. Soc.* **2009**).
TP-2Rho and TP-3Rho were synthesized according to the previously reported procedure.[3]

Synthesis of N,N-Bis[4-(N-sulfobutyl-4-pyridinioethenyl)phenyl]-aniline (TP-2PySf)

A large excess of 1,4-butanesultone (1 mL) was added to a solution of neutral TP-2Py (50 mg, 0.11 mmol) in *o*-dichlorobenzene (3 ml). The solution was stirred at 120°C in the dark overnight. The mixture was cooled and product was then precipitated by addition of diethyl ether and filtered to afford TP-2Py sf in a 55% yield (45 mg).
MS (ESI^+) : m/z 384.8 $[M+2Na]^{2+}$, 746.5 $[M+Na]^+$
HRMS (ESI^+) calcd for $C_{40}H_{42}N_3O_6S_2$ 724.2515, found 724.2488
^1H NMR (300 MHz, DMSO-d_6) δ 8.90 (d, J = 6.5,4H), 8.20 (d, J = 6.5, 4H), 7.98 (d, J = 16.0, 2H), 7.70 (d, J = 8.5, 4H), 7.43 (t, J = 8.0, 2H), 7.38 (d, J = 16.0, 2H), 7.28 (d, J = 8.0, 1H), 7.17 (d, J = 8.0, 2H), 7.11 (d, J = 8.5, 4H), 4.55-4.45 (m, 4H), 2.10-2.00 (m, 4H), 1.60-1.50 (m, 8H).
HPLC t_r = 7.87 min (94 %)

Synthesis of Tris[6-(4-pyridylethenyl)naphth-2-yl]amine (TN-3Py)

A large excess of methyl iodide (1 mL) was added to a solution of neutral TN-3Py (50 mg, 0.090 mmol) in a 1:1 methanol:dichloromethane mixture. The reaction was stirred at reflux overnight. Solvent was concentrated under vacuum and the product was precipitated by addition of diethyl ether to afford 83 mg of TN-3Py as a dark red powder in a 94% yield.
MS (ESI$^+$): m/z 250.0 $[M-3I^-]^{3+}$, 438.2 $[M-2I^-]^{2+}$, 1003.4 $[M-I^-]^+$.
HRMS (ESI$^+$) calcd for $C_{54}H_{45}N_4I$ 876.2689, found 876.2644
^{1}H NMR (300 MHz, DMSO-d_6) δ 8.87 (d, J = 6.7, 6H), 8.24 (d, J = 6.7, 6H), 8.20 (s, 3H), 8.17 (d, J = 15.0, 2H), 8.02 (d, J = 9.0, 3H), 7.95 – 7.85 (m, 6H), 7.67 (s, 3H), 7.64 (d, J = 15.0, 2H), 7.44 (dd, J = 9.0, J = 2.0, 2H), 4.27 (s, 9H).
HPLC t_r = 7.35 min

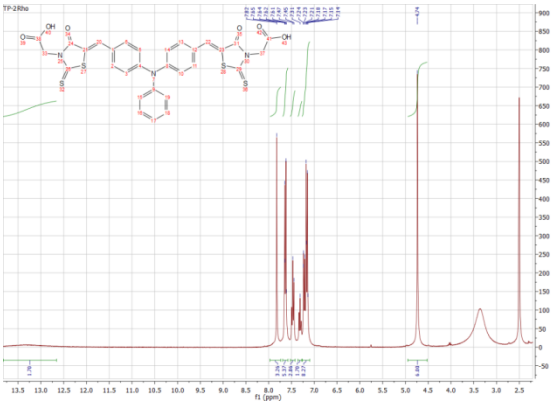
2,2',2''-((5Z,5'Z,5''Z)-5,5',5''-((nitrilotris(benzene-4,1-diyl))tris(methanylylidene))tris(4-oxo-2-thioxothiazolidin-3-yl-5-ylidene))triacetic acid (TP-3Rho)

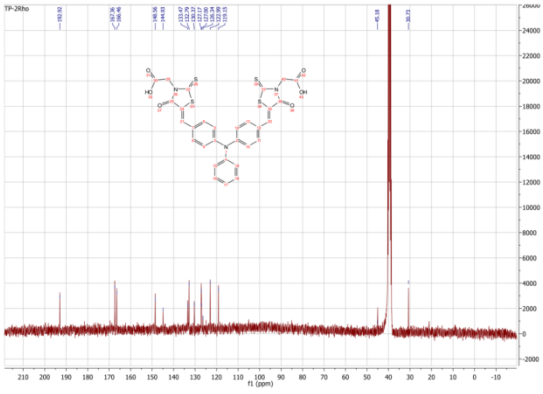
^{1}H NMR (300 MHz, DMSO-d_6) δ 7.79 (s, 3H), 7.66 (d, J = 8.1, 6H), 7.25 (d, J = 8.1, 6H), 4.60 (s, 6H)
^{13}C NMR (75 MHz, DMSO-d_6) δ 192.93, 167.34, 166.45, 147.73, 132.90, 128.64, 124.86, 120.17, 45.16, 30.73.
MS (ESI$^-$) m/z 847.3 [M-H]-
HRMS (ESI$^-$) calcd for $C_{36}H_{22}N_4O_9S_6$ 845.9711, found 845.9730

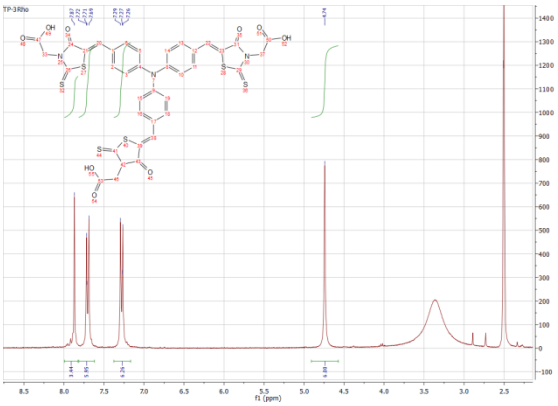
2,2'-((5Z,5'Z)-5,5'-(((phenylazanediyl)bis(4,1-phenylene))bis(methanylylidene))bis(4-oxo-2-thioxothiazolidin-3-yl-5-ylidene))diacetic acid (TP-2Rho)

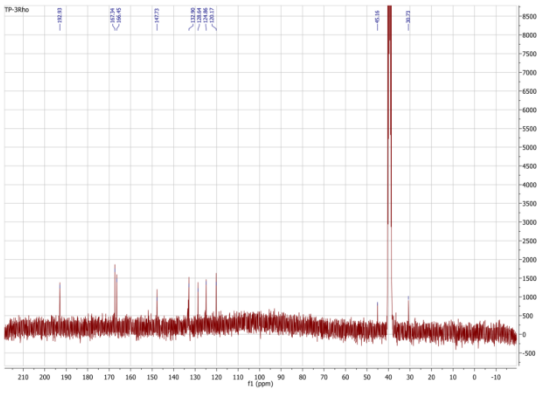
^{1}H NMR (300 MHz, DMSO-d_6) δ 7.82 (s, 2H), 7.64 (d, J = 8.6 Hz, 4H), 7.47 (t, J = 7.5 Hz, 2H), 7.31 (t, J = 7.5 Hz, 2H), 7.22 (d, J = 7.5 Hz, 2H), 7.16 (d, J = 8.6 Hz, 3H), 4.74 (s, 4H).
^{13}C NMR (75 MHz, DMSO-d_6) δ 192.92, 167.36, 166.46, 148.56, 144.93, 133.47, 132.79, 130.37, 127.17, 127.00, 126.34, 122.99, 119.15, 45.18, 30.73.
MS (ESI$^-$) m/z 646.0 [M-H]-
HRMS (ESI$^-$) calcd for $C_{30}H_{20}N_3O_6S_4$ 646.0235, found 646.0267

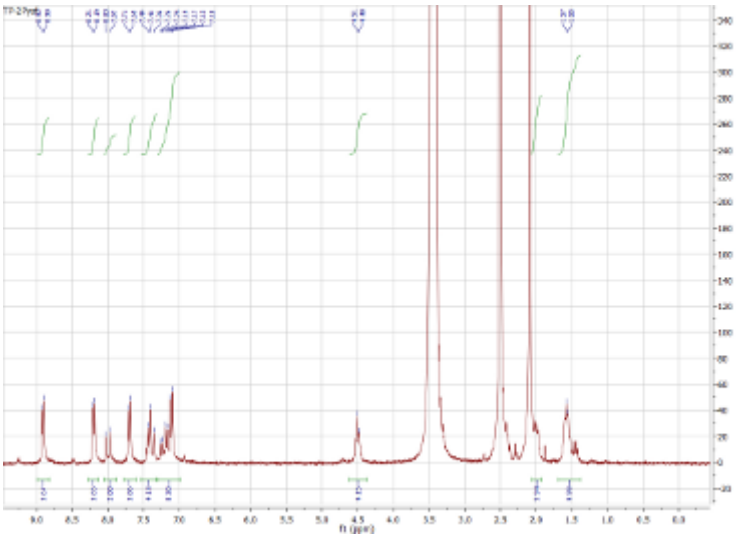
Analyses
- NMR spectra

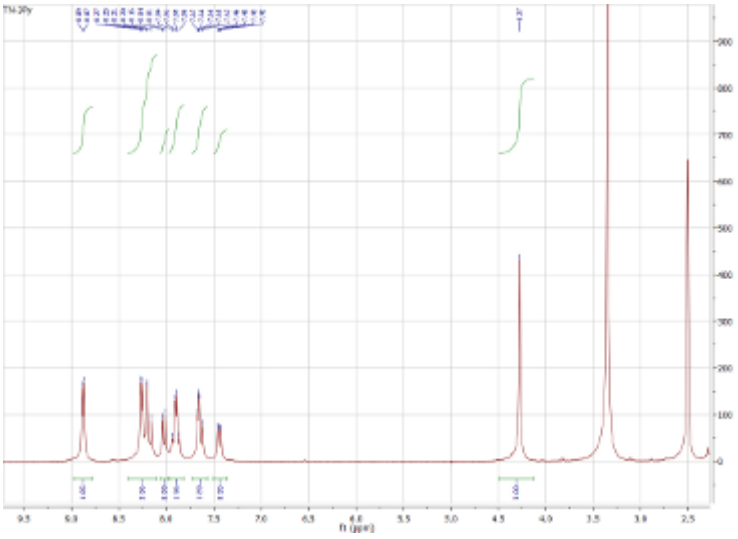




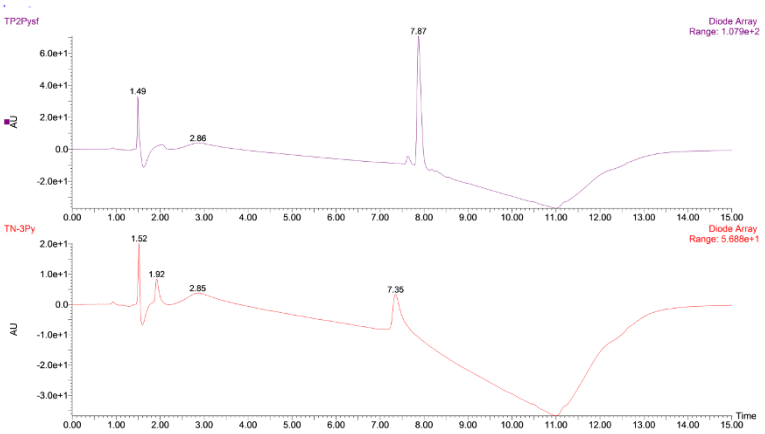




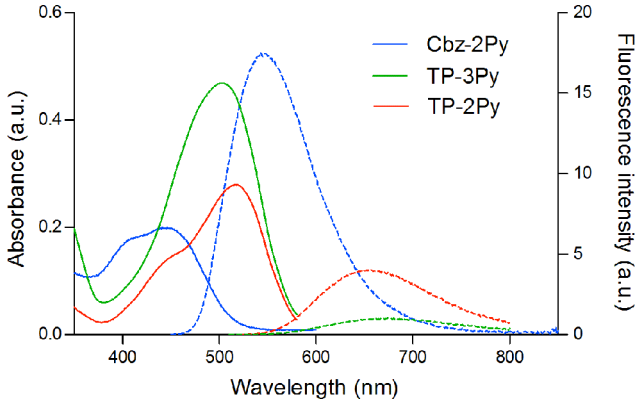




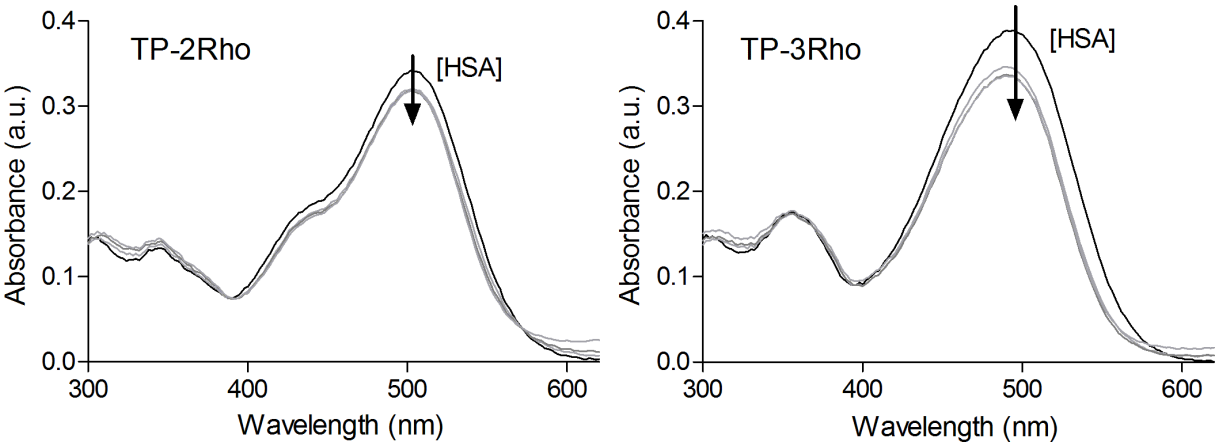
- HPLC chromatograms



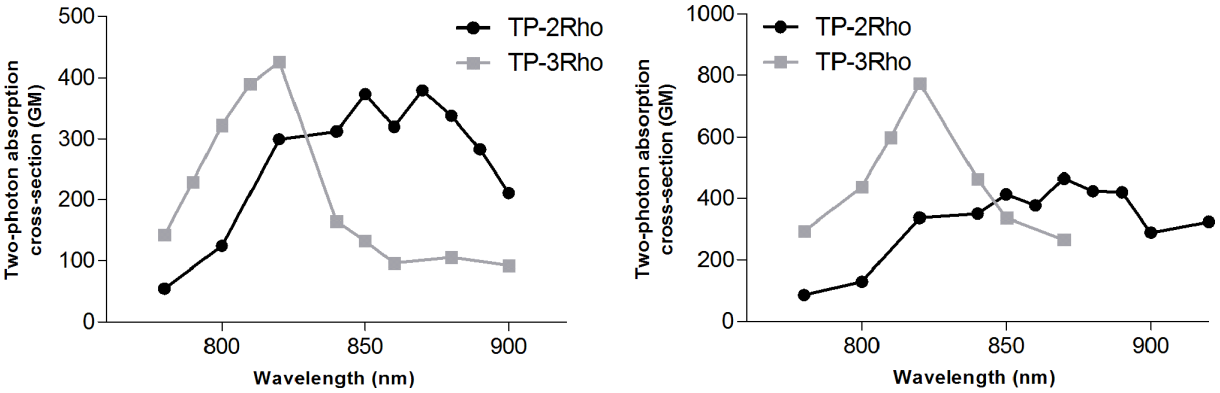
Additional figures



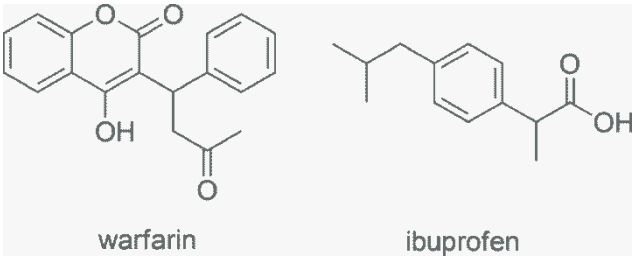
SI 1. UV (plain) and fluorescence (dotted lines) spectra of the pyridinium compounds in presence of DNA in 10 mM sodium cacodylate buffer pH7.2 + 100 mM NaCl. UV: [dye] = 10 µM with 1 molar equivalents of d(CGCGAAATTTCGCG)$_2$. Fluorescence: [dye] = 1 µM with 10 equivalents in base pairs of herring testes DNA.



SI 2. UV titrations of TP-2Rho and TP-3Rho by HSA in 10 mM sodium cacodylate buffer pH 7.2. [dye] = 5 µM, [HSA] = 0-5 µM.



SI 3. Two-photon absorption spectra of the TP-Rho in dichloromethane (left) and in presence of 5 molar equivalents of HSA in 10 mM sodium cacodylate buffer pH 7.2.(right).



SI 4. Structure of warfarin and ibuprofen

Cytotoxicities of TP dyes

Percentage inhibition of cellular growth

	KB 1 µM / 10 µM	HT29 10µM	MRC5 10µM
TP-2Py	15 / 71	nd	43
TP-3Py	n.d. / 0	nd	0
TP-2Rho	n.d.	0	0
TP-3Rho	n.d.	1	0

1 T. Peters, *All about albumin: biochemistry, genetics, and medical applications*, Elsevier Academic Press, 1996.

2 M. A. Albota, C. Xu, and W. W. Webb, *Appl. Opt.*, 1998, **37**, 7352-6.

3 C.-H. Yang, H.-L. Chen, Y.-Y. Chuang, C.-G. Wu, C.-P. Chen, S.-H. Liao, and T.-L. Wang, *J.Power Sources*, 2009, **188**, 627-634.