A doubly responsive probe for the detection of Cys4-tagged proteins

Naoko Kotera, Emmanuelle Dubost, Gaëlle Milanole, Eric Doris, Edmond Gravel, Nathalie Arhel, Thierry Brotin, Jean-Pierre Dutasta, James Cochrane, Emilie Mari, Céline Boutin, Estelle Léonce, Patrick Berthault and Bernard Rousseau

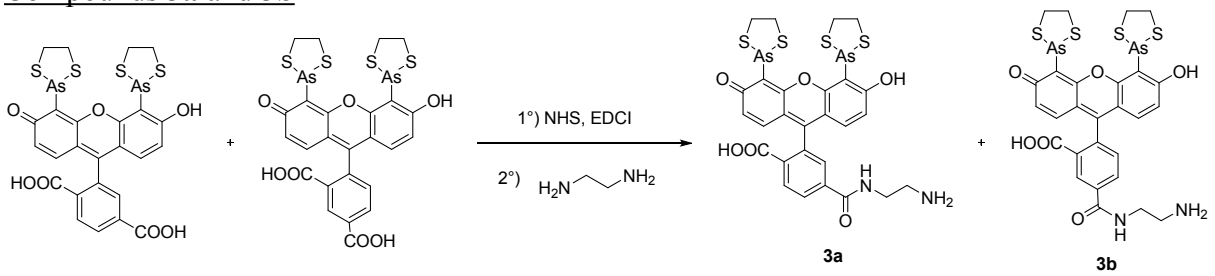
~~~~~~~~

Supporting Information

Content:
~~~~~~~~

A) Synthesis and characterization of compounds *MM*-1a and *MM*-1b

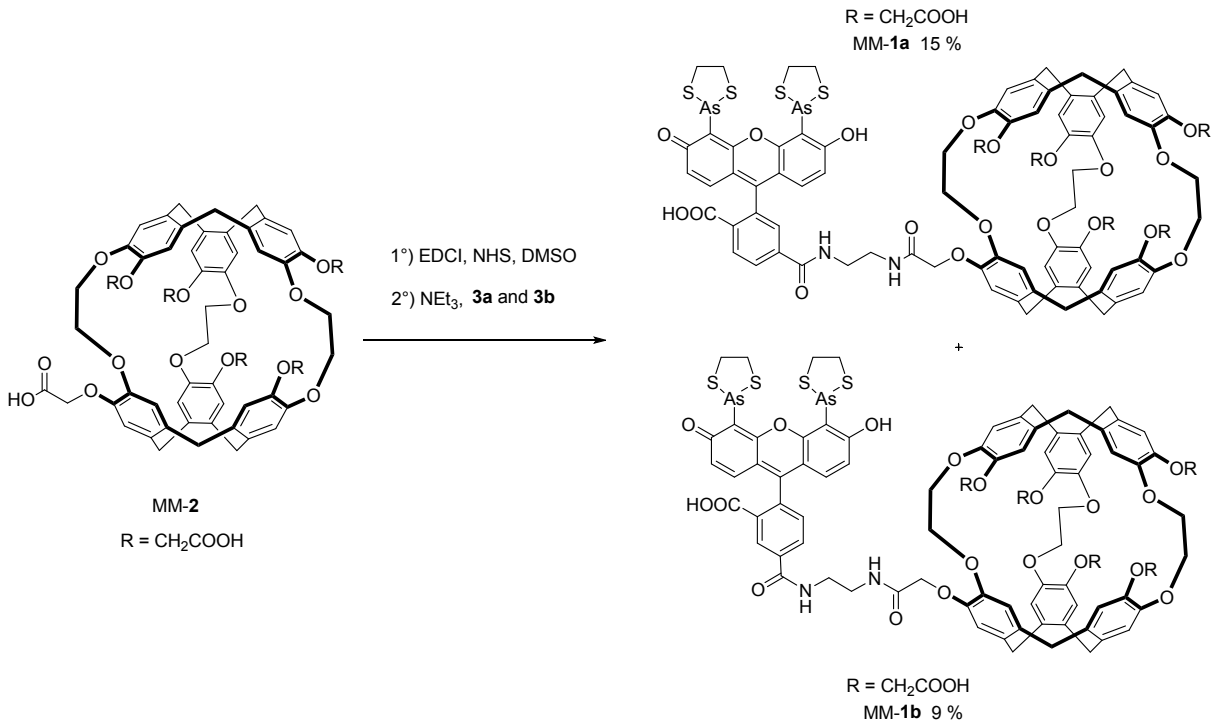
Compounds **3a** and **3b**



Diisopropylcarbodiimide (20 µL, 0.13 mmol) and N-hydroxysuccinimide (15 mg, 0.13 mmol) were added to a solution of 6- and 5-CrAsH isomers (70 mg, 0.10 mmol) in THF (15 mL). The mixture was stirred for 3 hours (the reaction was monitored by TLC or HPLC) then ethylene diamine (66 µL, 1.0 mmol) was added. The solution turned from orange to bright pink and a precipitate appeared. The solvent was evaporated, the residue was passed through a C18 silica patch, rinsed with water to remove the excess of ethylene diamine, and the rest of the mixture was eluted with acetonitrile. The fraction was evaporated and gave 45 mg of red solid used as is for the next reaction.
By HPLC, we estimated the amount of **3a** and **3b** obtained at around 30 mg (~ 40 % yield).

MS (ESI-TOF) *m/z*: 750.7 (100 %, $[M+H]^+$)

Compounds *MM*-**1a** and *MM*-**1b**



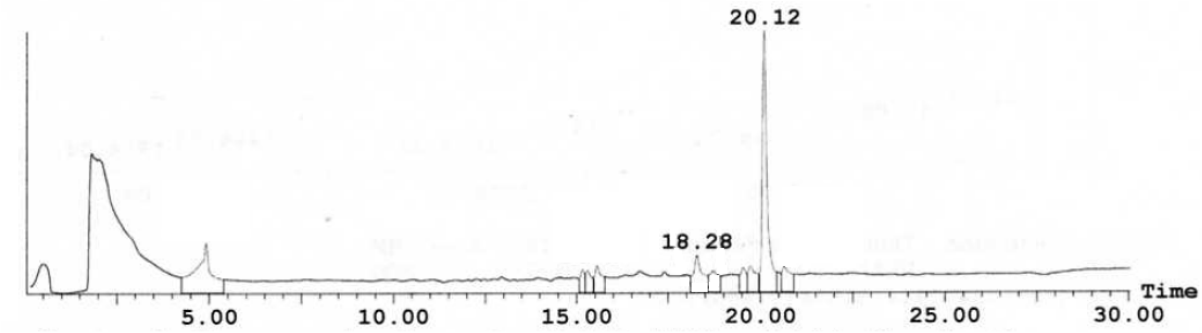
N-hydroxysuccinimide (1.0 mg, 8.6 μmol) and N'-ethyl-N-dimethylaminopropylcarbodiimide (1.8 mg, 9.4 μmol) were added under argon atmosphere to a stirred solution of *MM*-**2** (10 mg, 8.6 μmol) in DMSO (0.1 mL). The mixture was stirred for 5 hours at room temperature. Then a solution of crude **3a** and **3b** (4.5 mg) dissolved in DMSO (0.1 mL) and triethylamine were added. The solution was then stirred for an additional 16 hours at room temperature. The excess of triethylamine was removed under reduced pressure. The solution was then directly injected for purification on preparative HPLC chromatography (Luna PFP column. Size: 150x21, gradient: 95/5 to 30/70 H_2O/AcCN + 0.1 %HCOOH) to give *MM*-**1a** (2.4 mg, 15 %) and *MM*-**1b** (1.4 mg, 9 %) as orange solids.

Characterization of Compound *MM*-**1a**

^{1}H NMR (700 MHz, D_2O): δ 7.91 (d, 1H, J = 8.0 Hz), 7.78 (d, 1H, J = 8.0 Hz), 7.37 (s, 1H), 6.98 (d, 1H, J = 9.2 Hz), 6.94 (d, 1H, J = 9.2 Hz), 6.76-6.48 (m, 12H), 6.46 (d, 2H, J = 9.2 Hz), 4.60-4.18 (m, 24H), 4.16-3.89 (m, 4H), 3.84-3.46 (m, 8H), 3.37-3.22 (m, 6H), 3.21-3.03 (m, 6H)

HPLC chromatogram: Purity checked on Luna PFP. Gradient: H_2O/AcCN 95:5 to 30:70

Retention time: 20.12 min (Peak at 18.28 corresponds to the product without one arsenic).

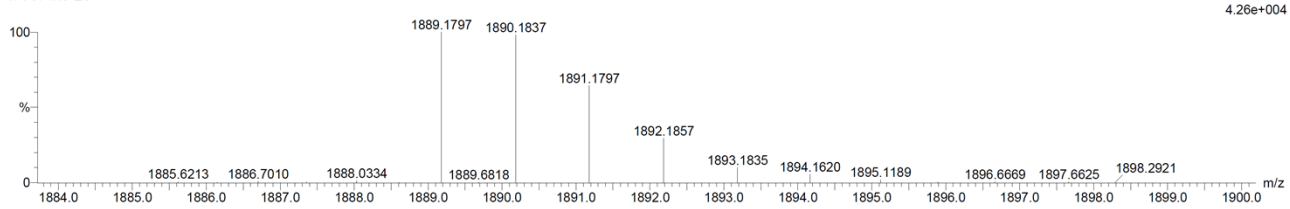


HRMS-ES$^-$ (m/z) calcd for $C_{87}H_{75}N_2O_{29}S_4As_2$, 1889.1770 found 1889.1797 ([M-H]$^-$).

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 80.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

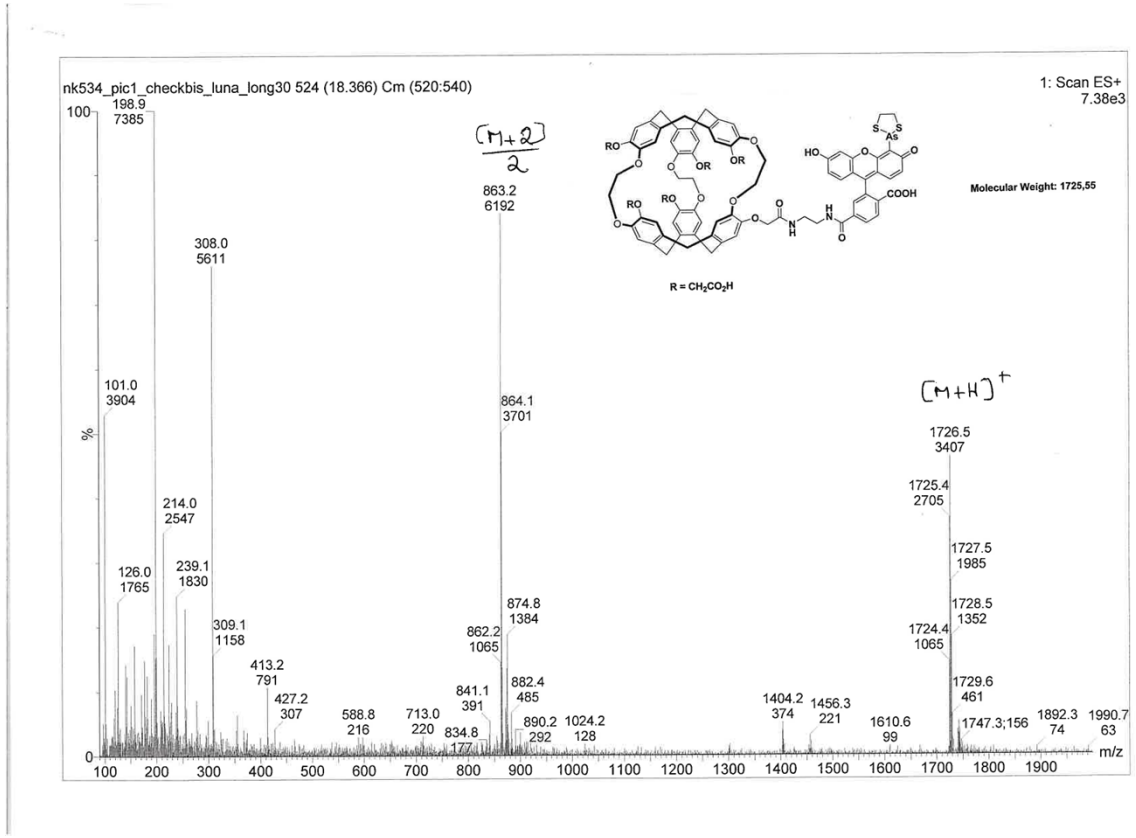
Monoisotopic Mass, Even Electron Ions
356 formula(e) evaluated with 3 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 79-100 H: 0-100 N: 0-2 O: 29-30 S: 0-4 As: 0-2

LCT Premier XE KE483 29-Mar-2012 CEA_NK447P1_inf 131 (0.662) Cm (101:131)
1: TOF MS ES- 4.26e+004



Minimum:				-1.5			
Maximum:		5.0	5.0	80.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
1889.1797	1889.1770	2.7	1.4	51.5	33.4	0.7	C87 H75 N2 O29 S4 As2
	1889.1737	6.0	3.2	56.5	33.7	1.1	C90 H71 N2 O29 S3 As2
	1889.1703	9.4	5.0	61.5	34.4	1.7	C93 H67 N2 O29 S2 As2

MS-ESI (m/z). The spectrum shows two peaks



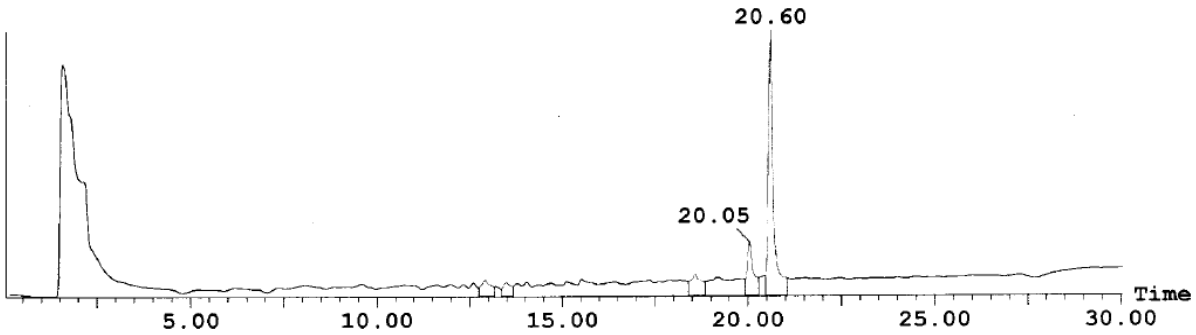
unambiguously assigned to the monoarsenate species: 1726 $[M+H]^+$ and 863 [M+2]/2.

Compound *MM*-**1b**

After HPLC purification, this compound was obtained as a mixture of *MM*-**1a** and *MM*-**1b** (1:8).

^{1}H NMR (700 MHz, D_2O): δ 8.21 (s, 1H), 7.52 (d, 1H, J = 7.6 Hz), 7.12-7.05 (m, 3H), 6.89-6.50 (m, 12 H), 6.46 (d, 1H, J = 9.2 Hz), 6.42 (d, 1H, J = 9.2 Hz), 4.73-4.20 (m, 22H), 4.18-3.98 (m, 6H), 3.96-3.52 (m, 8H), 3.50-3.22 (m, 8H), 3.21-3.01 (m, 4H)

HPLC chromatogram: Purity checked on Luna PFP. Gradient: H_2O/AcCN 95:5 to 30:70
Retention time: 20.60 min (Peak at 20.05 corresponds to the isomer *MM*-**1a**)



MS (ESI-TOF) *m/z*: 1892.5 (100%, $[M+H]^+$).

B) ^{129}Xe NMR experiments with compounds *MM*-1a and racemic 1a.

Preparation of the NMR samples

The following solutions of reducing agents in H_2O are prepared:
- β-mercaptoethanol (10 mM)
- TCEP (10 mM)
- EDT (10 mM)

The fluorophore is dissolved in PBS to give a solution of:
- Biosensor *MM*-**1a** (500 μM)

The peptide (1 mg) is dissolved in 1.03 mL H_2O to give a solution of 500 μM.

Each sample is composed of 70 μL of each reducing agent and 35 μL of fluorophore solution. Then, peptide solution and PBS are added as follows:
Sample without peptide 1: 405 μL PBS + 50 μL D_2O
Sample with 10 equivalents of peptide: 350 μL peptide solution + 55 μL PBS + 50 μL D_2O
pH was adjusted to 7 by adding K_2HPO_4

Final concentrations in solution are:
- β-mercaptoethanol (1 mM)
- TCEP (1 mM)
- EDT (1 mM)
- Fluorophore solution (25 μM)

Preparation of laser-polarized xenon.

83%-enriched ^{129}Xe from EurisoTop was polarized by spin-exchange optical pumping with rubidium[i] using a recently installed home-built setup based on laser diodes. The photons exiting from a duo-FAP system (2x30W) and circular polarizer from Coherent illuminated a Pyrex cell placed at the center of a 100G magnetic field. The bandwidth of the laser diodes being about 2 nm, pressure-broadening was necessary. Therefore, the pressure in the cell rose to 3 atm (measured at room temperature) with a mixture of 2% Xe – 10% N_2 – 88% He. The pumping cell was heated for 2 min to 400K via a flow of hot N_2 in an external envelope, in a fashion similar to what was developed for our previous experimental setup.[ii] Then xenon was

condensed in a cold finger inside a 3kG solenoid immersed in liquid nitrogen, and thus separated from helium and nitrogen. The average polarization value with this experimental setup was 15%, measured for the gaseous phase in the NMR spectrometer. Hyperpolarized xenon was introduced on top of the solution in the upper part of the screwed NMR tube by using a vacuum line in the fringe field of the NMR magnet. Then a vigorous shaking of the tube followed by a 10 s delay ensured complete dissolution and equilibration of both gaseous and dissolved phases.
Each NMR tube received about 1 atm of hyperpolarized xenon on top of the solution.

NMR experiments.

The ^{129}Xe NMR experiments were performed on a narrow-bore 11.7T Avance II Bruker spectrometer, equipped with a 5 mm BBI (broadband inverse) probehead. All the NMR spectra were recorded at 293K. The signals are referenced with respect to xenon in water (196 ppm).

Figure S1: ^{129}Xe NMR spectra (red
color) of the racemic biosensor
7

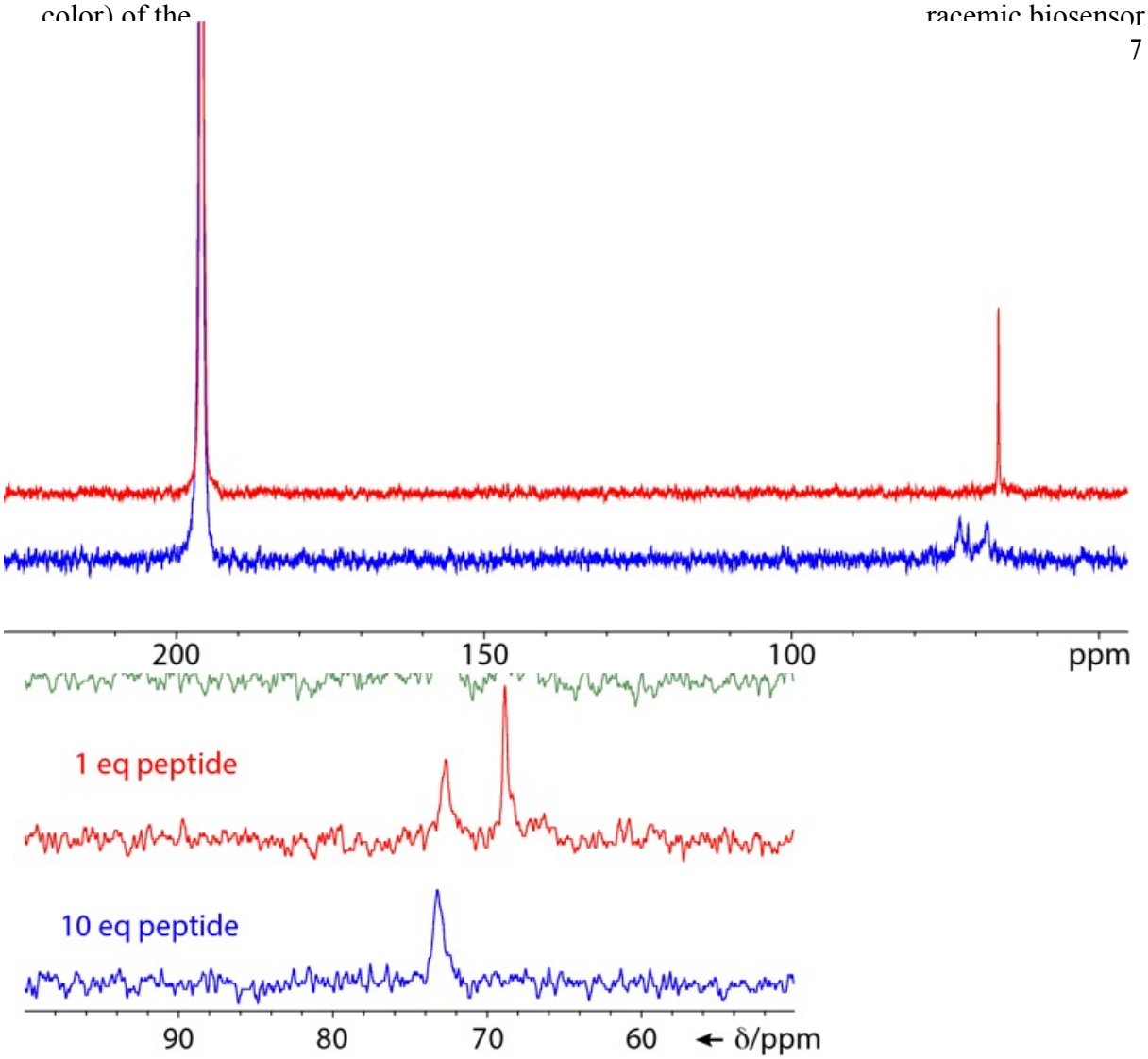


Figure S2: High field part of the ^{129}Xe NMR spectrum of a 25 µM solution of *MM*-**1a** in the presence of various quantities of peptide.

C) Fluorescence experiments with compound *MM*-1a

With Peptide : tests with 25 µM biosensor

The following solutions of reducing agents in H_2O are prepared:
- β-mercaptoethanol (10 mM)
- TCEP (10 mM)
- EDT (10 mM)

The fluorophore is dissolved in PBS to give a solution of:
- Biosensor *MM*-**1a** (500 µM)

The peptide (1 mg) is dissolved in 1.03 mL H_2O to give a solution of 500 µM.

Each sample is composed of 10 µL of each reducing agent 5 µL of fluorophore solution. Then, peptide solution and PBS are added as follows:
Sample 1: 0 µL peptide solution + 65 µL PBS (0 equivalent of peptide)
Sample 2: 50 µL peptide solution + 15 µL PBS (10 equivalents of peptide)

Final concentrations in solution are:
- β-mercaptoethanol (1 mM)
- TCEP (1 mM)
- EDT (1 mM)
- Fluorophore solution (25 µM)

Labeling reaction is monitored by spectrofluorescence using a microplate fluorometer with the following settings: fluorophore excitation 485 nm, emission 535 nm.

D) LC/MS monitoring of reaction between *MM*-1a and the peptide:

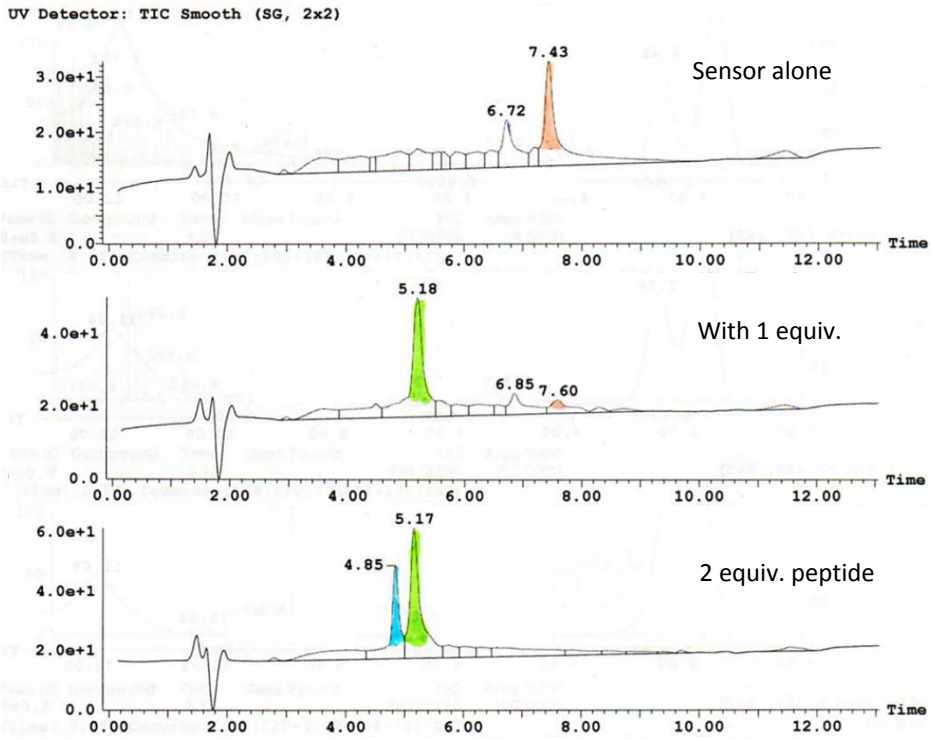


Figure S3: LC/MS analysis

The formation of the biosensor/peptide complex was observed after 24 h incubation by LC/MS as the biosensor alone, the peptide and the complex were identified and characterized at different retention times.

a) *MM*-**1a** is detected, as the biosensor alone, at a retention time of 7'43. The monoarsenate biosensor was also observed at 6'72.
b) With one equivalent of peptide, the peptide-sensor complex was detected at 5'18 min (detected ion $[M+2H]^{2+}$ with m/z = 1822,8).
c) With two equivalents of peptide, the tetracysteine-biosensor complex (5'17) and the excess peptide (4'85).

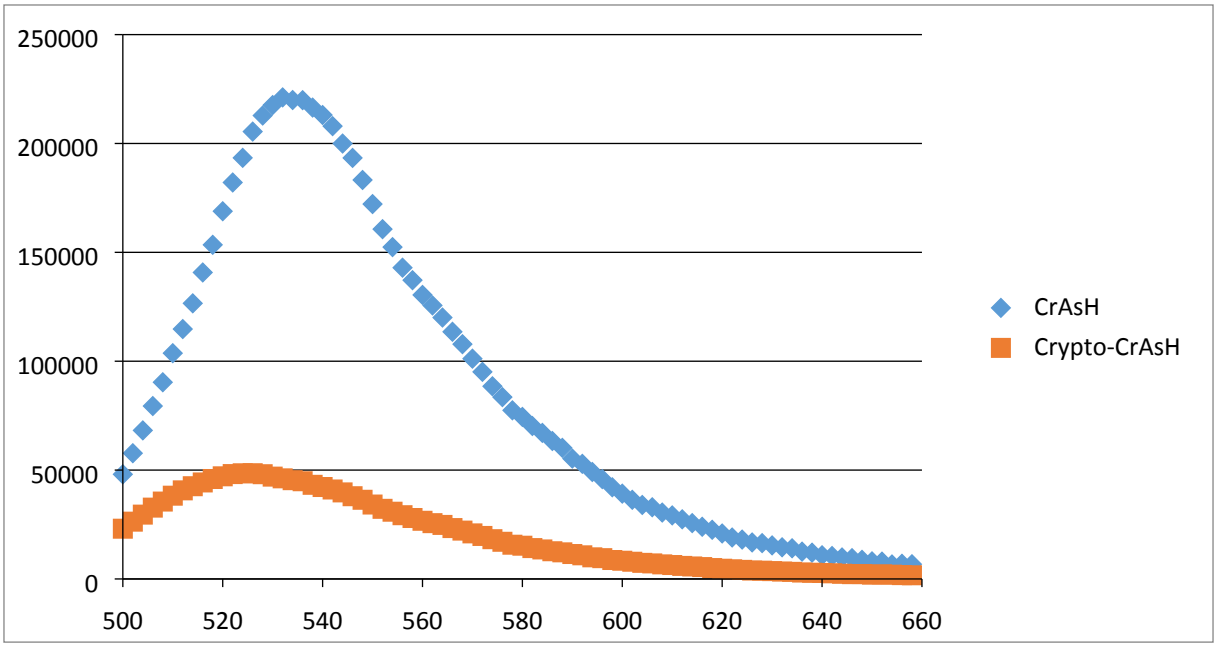


Figure S4: Comparison of the fluorescence spectra (excitation wavelength 480 nm) of the CrAsH moiety (blue) and *MM*-**1a** (orange).

[i] Walker, T. G.; Happer, W. *Rev. Mod. Phys.* **1997**, *69*, 629-641.
[ii] Desvaux, H.; Gautier, T.; Le Goff, G.; Pétro, M.; Berthault, P. *Eur. Phys. J. D* **2000**, *12*, 289-296.