Phosphonated Trityl Probe for Concurrent *In vivo* Tissue Oxygen and pH Monitoring Using EPR-based Techniques

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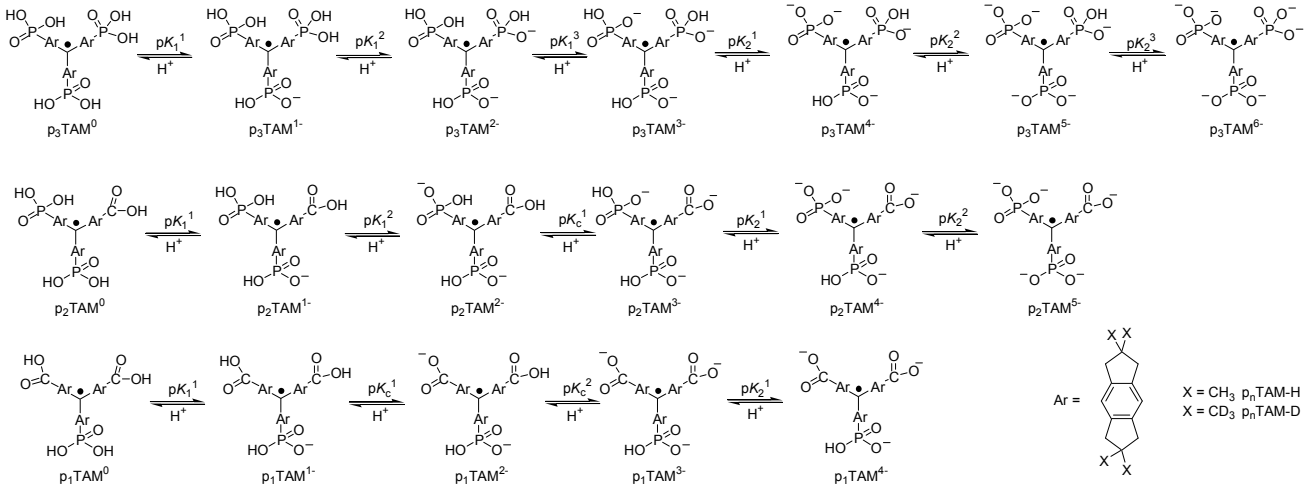
Scheme SI1. The scheme of acid-base equilibrium for p_nTAM trityl radicals. pK_1^i and pK_2^i denote the pK_a values of first and second ionization constants for each (i = 1, 2, 3) phosphonic acid residue; pK_c^i denote the pK_a values of ionization constants for each (i = 1, 2) carboxylic acid residue of p_nTAM probes.



X-band EPR spectra of p_1TAM-H, p_2TAM-H and p_2TAM-D at different pH values and titration curves for p_2TAM radicals

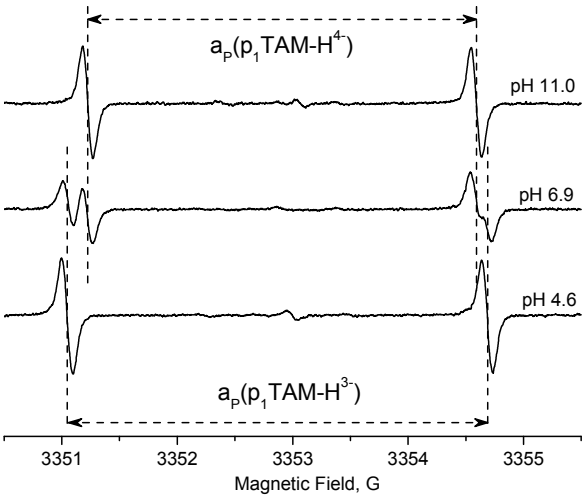


Figure SI1. EPR spectra of 100 μM p_1TAM-H solutions in 1 mM Na-phosphate buffer, 150 mM NaCl, measured at various pH values under nitrogen atmosphere and temperature 37 °C. X-Band EPR spectrometer settings: microwave power, 130 μW; time constant, 20.48 ms; conversion time, 5 ms; sweep time, 20.48 s; modulation frequency, 10 kHz; modulation amplitude, 0.05 G; sweep width 5.5 G; number of points, 4096.

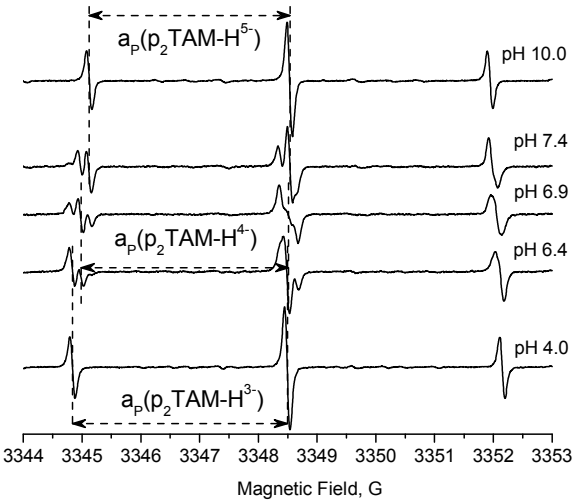


Figure SI2. X-band EPR spectra of 250 μM p_2TAM-H solutions in 1 mM Na-phosphate buffer, 150 mM NaCl, measured at various pH values under nitrogen atmosphere and temperature 37 °C. Spectrometer settings: microwave power, 130 μW; time constant, 20.48 ms; conversion time, 5 ms; sweep time, 20.48 s; modulation frequency, 10 kHz; modulation amplitude, 0.05 G; sweep width 10 G; number of points, 4096.

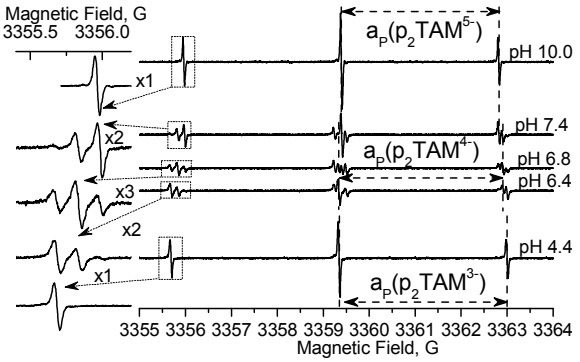


Figure SI3. X-band EPR spectra of 0.2 mM p_2TAM-D solutions in 1 mM Na-phosphate buffer, 150 mM NaCl, measured at various pHs under nitrogen atmosphere, at 37 °C. Spectrometer settings: microwave power, 40 μW; modulation frequency, 10 kHz; modulation amplitude, 0.02 G; number of points, 8192.

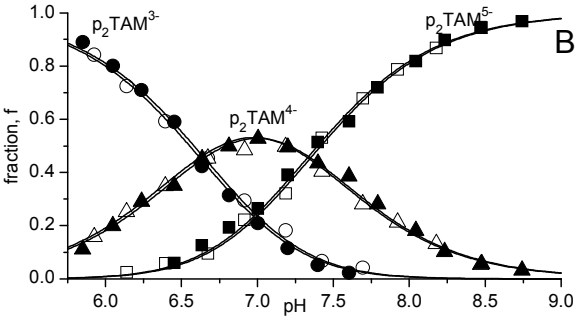


Figure SI4. The pH dependencies of fraction of the ionization states of diphosphonated p_2TAMs calculated from the corresponding EPR spectra (Figs. SI2 and SI1-3). The closed and open symbols denote the data obtained for p_2TAM-D and p_2TAM-H, correspondingly. The squares denote p_2TAM^{5-}, triangles denote p_2TAM^{4-} and circles denote p_2TAM^{3-} ionization states (See Scheme SI1). Solid lines represent the best fits using standard titration equations yielding pK_a values presented in the Table SI1.

Table SI1. The pK_a Values and X-Band EPR Spectral Parameters for Different Ionization States of p_2TAM-D.

Ionization State	pK_a	a_{P1} (PO_3^{2-}), mG	a_{P2} (HPO_3^-), mG	a_{P3} (H_2PO_3), mG	ΔHpp, mG	Δg, mG
p_2TAM^{5-}	7.35±0.05	3420±5 (2)[a]	Na	na	34±2	0
p_2TAM^{4-}	6.65±0.05	3420±5 (1)[a]	3660±5 (1)[a]	na	37±2	37±5
p_2TAM^{3-}	≈2.6±0.3[b]	na	3660±5 (2)[a]	na	42±2	72±8
p_2TAM^{2-}	≈1.3±0.3	na	≈3650 (2)[a]	na	≈42	≈72
p_2TAM^{0}		na	Na	≈3750 (2)[a]	≈42	≈100

[a] the values in parenthesis denote the number of phosphono groups.

[b] the pK_a value for carboxyl group.

Concentration self broadening of p_1TAM-D

Concentration-induced line self-broadening of TAM probes is one order of magnitude less than that of nitroxide probes[1] and is not an issue for low concentrations of the pTAM probes. However at high probe concentration line-broadening (see Fig.SI5) is observed.

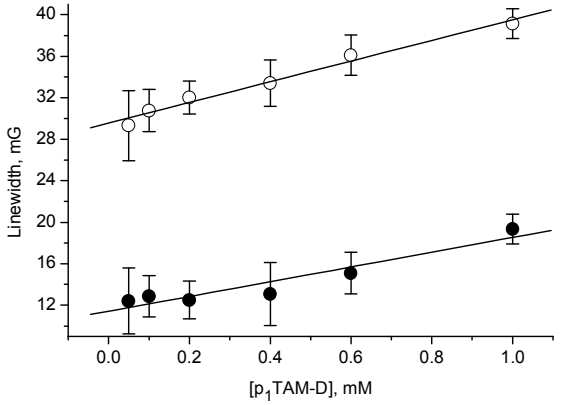


Figure SI5. The concentration dependence of Lorenz linewidths of p_1TAM^{3-}(○) and p_1TAM^{4-}(●) components of p_1TAM-D probe. Line represents the best linear fit with the slopes of (10±1) mG/mM and (7±1) mG/mM, correspondingly. L-band EPR spectra were recorded in the presence of 150 mM NaCl and 1mM Na-phosphate buffer under anoxic conditions and temperature of 37 °C.

Partition coefficients for p_1TAM-D probe

To support high aqueous solubility of p_1TAM probe, we measured their partition coefficients in octanol/water solutions. The solutions of p_1TAM-D (200 μM) in the presence of 0.1 M Na-phosphate buffer at various pHs in the range from 6.0 to 8.0 were vigorously mixed with equal volume of octanol for 5 min and allowed to stand for another 5 min until separation of the phases was completed. The values of the partition coefficient, Kp, were calculated as a ratio of the integral intensities of the EPR spectra of p_1TAM-D probe in octanol and aqueous phases, correspondingly. The spectrum of p_1TAM-D probe was found to be below detection limit at these pH values providing an upper limit of Kp value being < 0.001. For comparison, Kp values for deuterated Finland TAM-D probe was found to be equal to 0.36±0.13 (pH=6), 0.02±0.01 (pH=6.5) and ≤0.003 (pH=7). The data support superior aqueous solubility of the phosphonated compound.

Solubility of p_nTAMs at acidic pH

The aggregation of Finland TAM in acidic solutions was previously reported[2]. An introduction of phosphono groups significantly extends the pH range of solubility of the p_nTAM probes (Fig. SI6) apparently due to low pK_a for the phosphonic acid dissociation (pK_{a1}≈1.3), and therefore maintaining a negatively charged structure at any reasonable pH above pH 3.

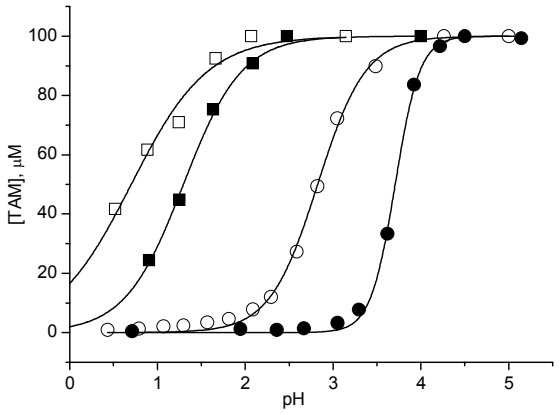


Figure SI6. The dependence of the EPR peak signal intensities of deuterated Finland TAM probe, TAM-D (●, 100 μM), p_1TAM-D (○, 100 μM), p_2TAM-D (■, 200 μM), and p_3TAM-D (□, 200 μM) on the pH of the sample. Lines represent the best fits using equation $[TAM]_{pH} = [TAM]_0 - [TAM]_0/(1 + 10^{(pH-pKagg)/q})$ yielding pKagg = 3.7±0.05, 2.82±0.05, 1.30±0.05 and 0.70±0.05; and q = 0.32, 0.63, 0.74 and 1 for TAM-D, p_1TAM-D, p_2TAM-D, and p_3TAM-D, correspondingly. The samples were measured in the presence of 1 mM Na-phosphate buffer, 150 mM NaCl and temperature 37 °C.

An exemplified analysis of in vivo L-band EPR spectra of p_1TAM-D probe

Lineshape analysis of the EPR spectrum was performed as previously described[3] fitting experimental spectrum by rational approximation of Voigt function given by Hui *et al*.[4] The independent character of the signal intensities ratio and line broadenings provides dual pH & oxygen functionality, as illustrated below.

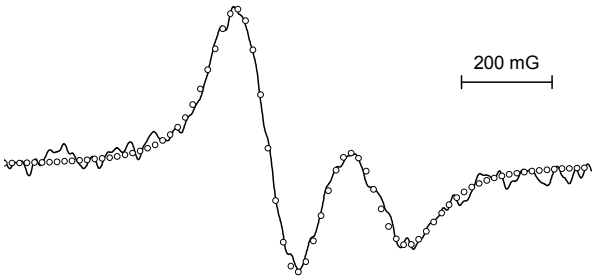


Fig. SI7. L-band EPR spectrum of high-field component of p_1TAM-D probe injected into the mammary gland (0.5 mM, 10 μl). Circles indicate simulated spectrum yielding the values of pO_2 and pH equal to 54 mmHg and 7.14 pH units, correspondingly.

For the spectrum shown in Fig. SI7, the lineshape analysis yields: $[p_1TAM^{4-}]/[p_1TAM^{3-}]$ =1.73 (which corresponds to pH 7.14); line distance between p_1TAM^{4-} and p_1TAM^{3-} lines, 115 mG (which corresponds to 1.4 mM buffer concentration, see Fig. 6B); and Lorenzian linewidths, $L_{tot}(p_1TAM^{4-})$ = 46 mG and $L_{tot}(p_1TAM^{3-})$ =53 mG. Subtracting from the latter values the Lorenzian linewidths in deoxygenated 1.4 mM phosphate buffer, $L_{buf}(p_1TAM^{4-})$ = 21 mG and $L_{buf}(p_1TAM^{3-})$ = 28 mG (see calibration in Fig. 6A), we obtain oxygen-induced line broadenings, $\Delta L_{ox}(p_1TAM^{4-})$ =25 mG and $\Delta L_{ox}(p_1TAM^{3-})$ =25 mG. The latter corresponds to the oxygen partial pressure, pO_2=52 mmHg (see calibration in Fig. 4). Note that concentration-induced line self-broadening is insignificant at low probe concentrations used. From our previous imaging studies of the membrane-impermeable extracellular probe[5] we should expect fast p_1TAM-D distribution throughout the tissue of the mammary gland or tumor, therefore final concentration of the probe is about an order lower (50-100 μM) than the threshold for self-broadening (≈400 μM, Fig. SI5).

Display Report

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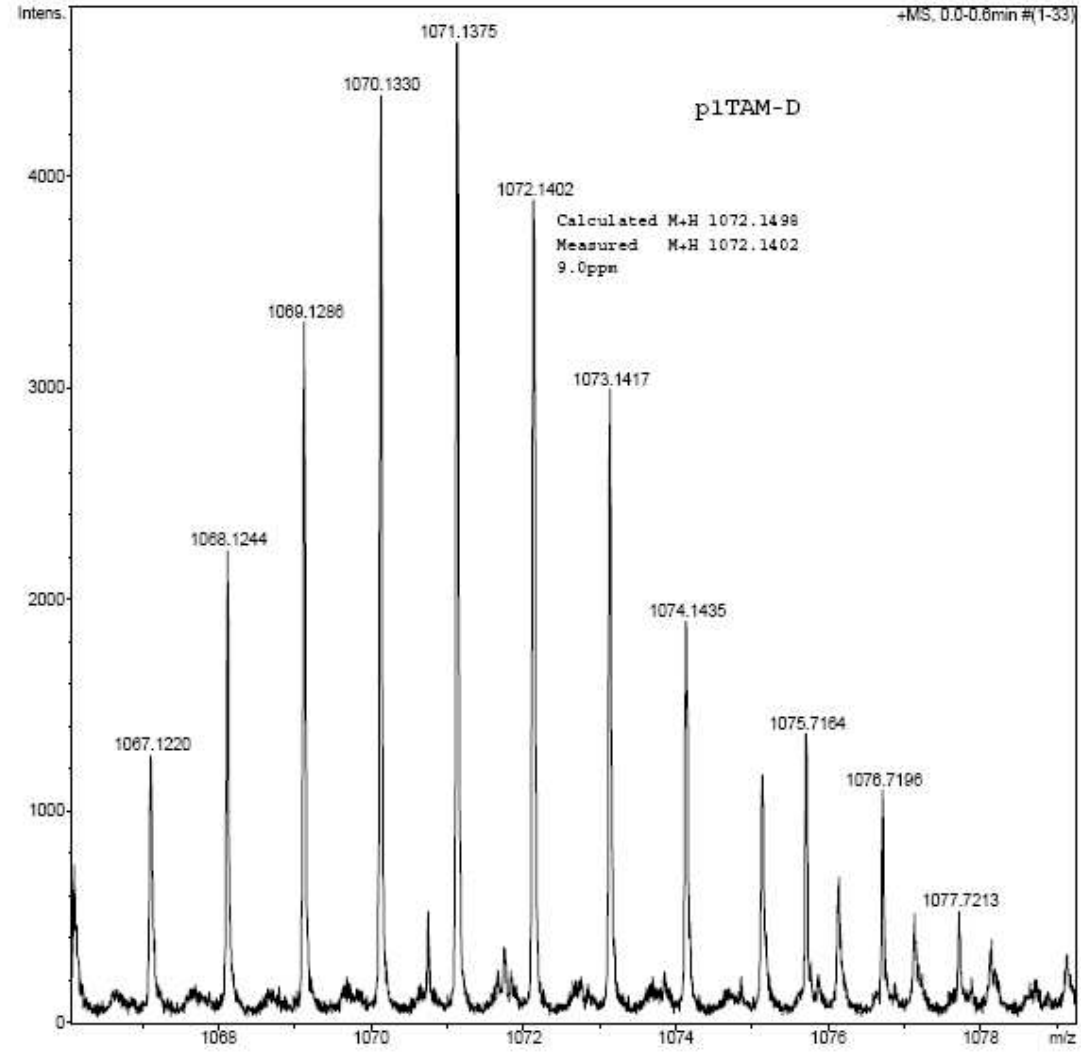

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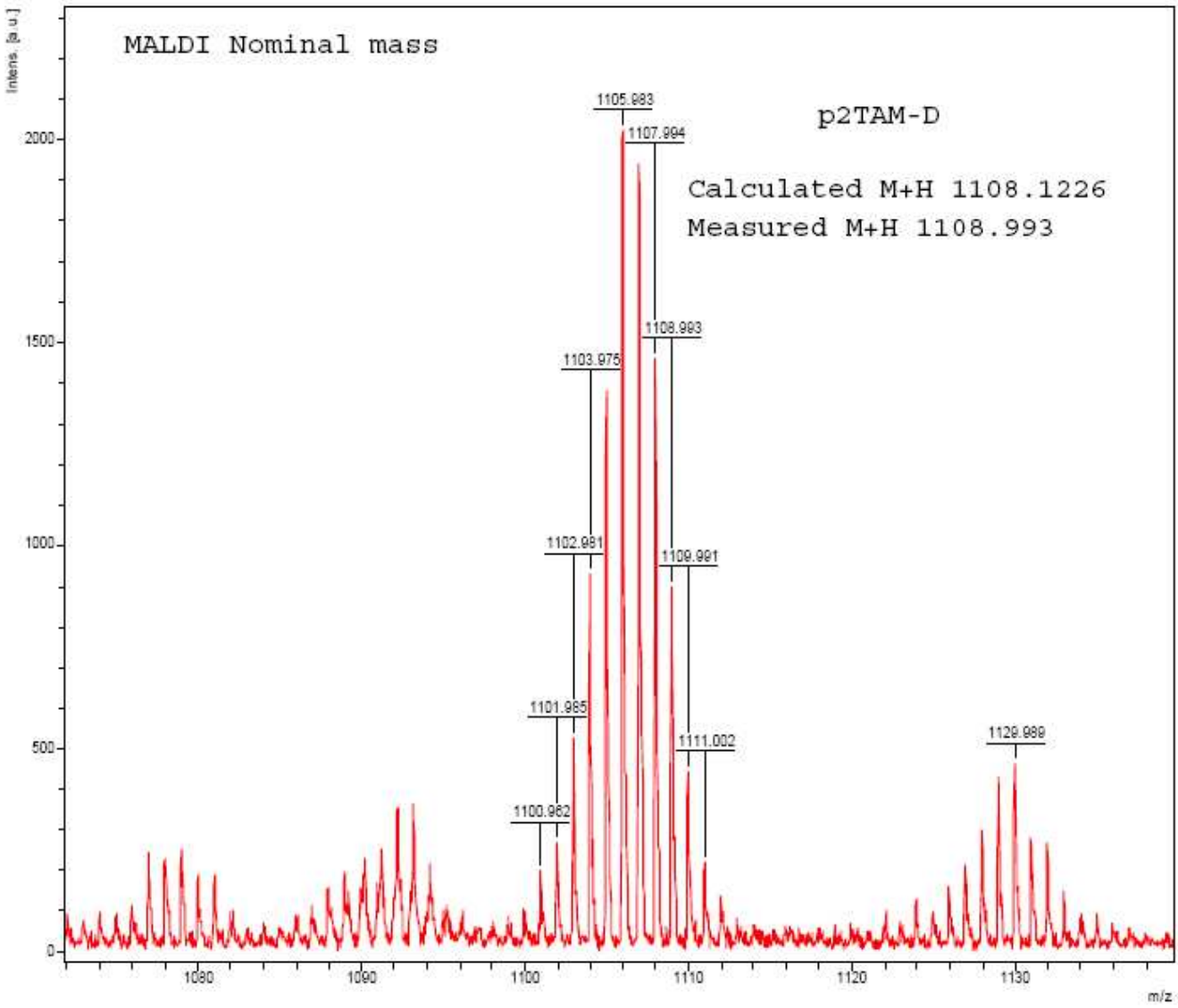

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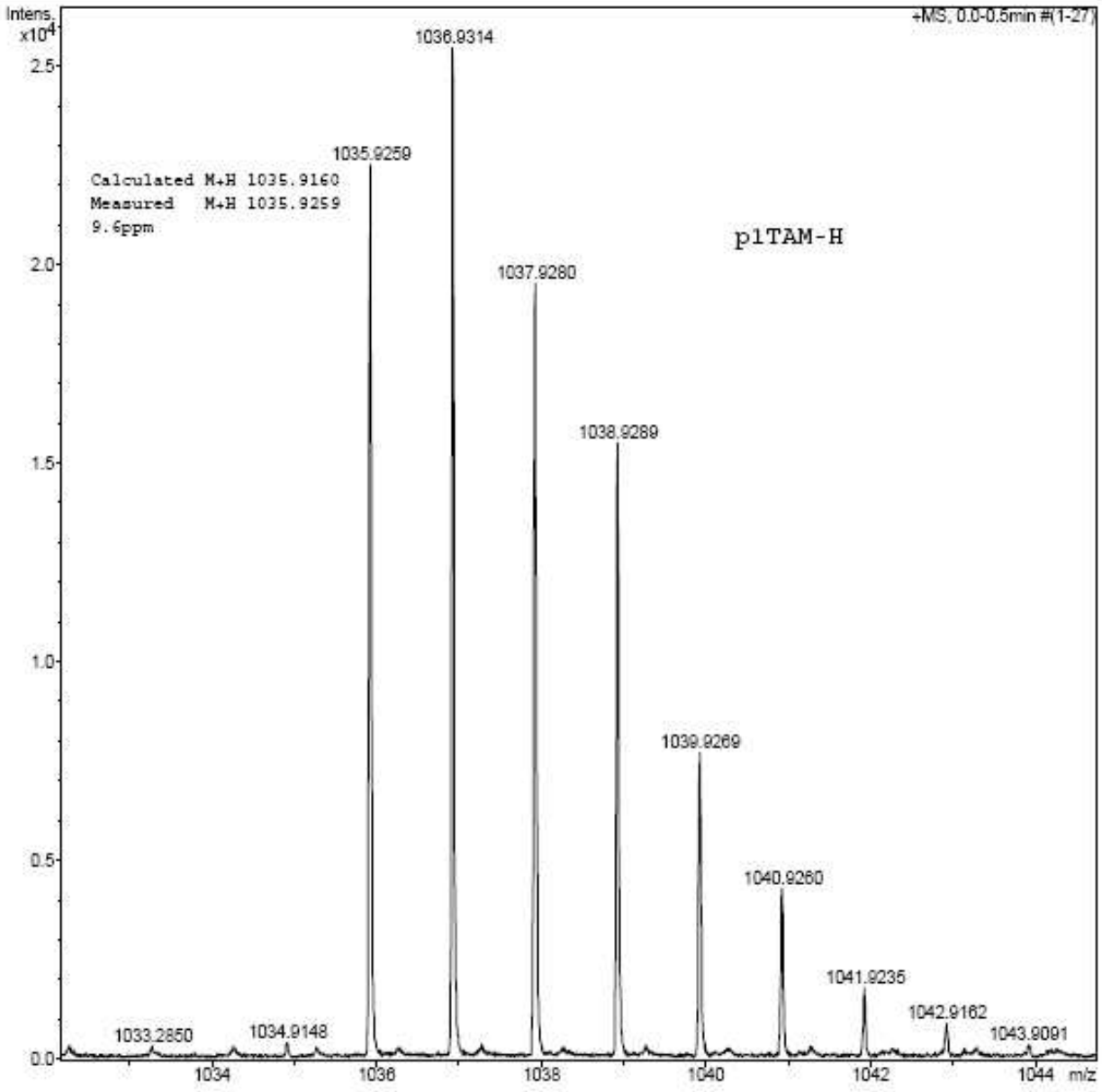

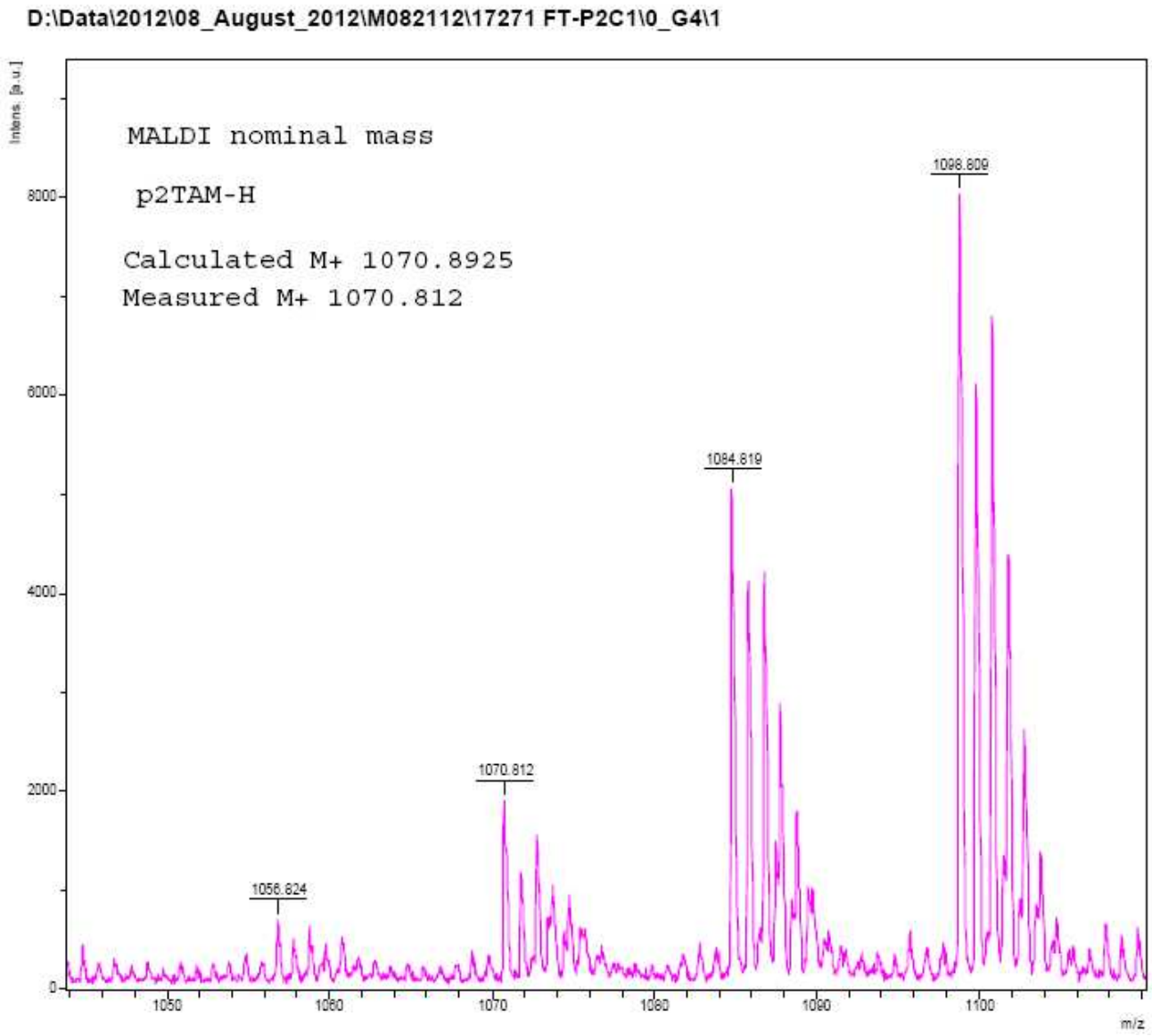

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