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Further Analytical Studies on a Mercuri Thiol Adduct Isolated from a Human Prostate Cancer Cell Line (LNCaP)

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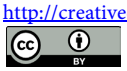


Abstract

Thiols play vital roles in cellular metabolism knowledge of which may be important in the design of future anticancer drugs. Previous work on the composition of the thiols present in human cancer cell lines has shown the presence of an unknown low molecular weight species, deemed to be a "Conthiol", which could be important in this respect. This was prepared and isolated from a human prostate cancer cell line (LNCaP) in the form of an adduct of 2-mercuri-4-nitrophenol; it accounts for 56.5% of the total cellular thiols present in this cell line. Initial LC-MS analysis of this adduct had indicated that the possible molecular weight of the thiol was in the region of 467 daltons. In further analytical studies to identify the thiol, attempts were made to release it from the adduct by passage through a Thiopropyl Sepharose6B column. LC-MS analysis of the column eluate revealed two components yielding negative ion fragments of 427 m/z and 449 m/z. Only the former component contained thiol, indicating that a breakdown and/or possible rearrangement of the Conthiol had occurred. Further investigations of the column thiol eluate using ICP-MS analysis showed that the sulfur content agreed with the spectrophotometric analysis result (Ellman assay) and that the molecule did not contain phosphate. Amino acid analyses of the eluate were negative. In an attempt to prevent the breakdown of the thiol released by the Thiopropyl Sepharose 6B column, the adduct was treated with 5% v/v bromine water prior to applying to the column. In this instance the thiol containing eluate obtained from the column was treated with an equimolar quantity of mercuric chloride forming a fresh adduct, RS-Hg-SR. LC-MS analysis of this mercurial adduct detected a negative ion fragment of 782 m/z which on further ionization gave a ladder like pattern showing loss of mass units of 58 in each rung. This would seem to suggest the presence of a repeat polymer like structure containing 5 monomers, which, plus the thiol atom, gives a possible formula

weight of 322; probably revealing only a part of the unknown Conthiol molecule whose properties and formula weight do not correlate with any known cellular thiol. Further analysis of the thiol released from the adduct on the Thiopropyl Sepharose 6B column by Infra-red (FTIR) provided little information except to confirm the presence of the thiol group and C=O stretch bands together with the possibility of a lactam ring at 1651 and 1634 cm·s^{-1}.

Keywords

Low Molecular Weight Thiol, Conthiol, LNCaP Prostate Tumour Cell Line, Thiol Adduct Analysis, LC-MS Analysis, ICP-MS Analysis

1. Introduction

Over the last 100 years, so many research papers and books have been published featuring one of the most reactive chemical groupings found in cells and living tissues, namely the thiol or sulfhydryl components found in proteins and other low molecular weight compounds. These studies have established that thiol compounds play major roles in cellular metabolism ranging from oxidative stress, cell signaling and redox control (e.g. see [1] [2] [3]) to reaction with xenophobic compounds, drugs and defense against ionizing radiation.

In recent times studies that have been focused on the low molecular weight components (LMWT) of cells, centred mainly around a common relatively stable thiol, the ubiquitous thiol glutathione, establishing its importance in cellular metabolism. It is now generally assumed that glutathione accounts for over 90% of the LMWT present in the cell. However, a series of studies by the author have shown that is not necessarily so. In an earlier study on the thiols of a two human prostate cell lines LNCaP (androgen-sensitive human prostate adenocarcinoma cells) and PNT2 cells (a normal human prostate epithelial cell line immortalized with SV40 virus) it was shown that an unknown LMWT termed a Conthiol is present in these cells and that it accounted for 56.5% and 61.4% of their cellular thiolomes [4].

In later work on the LNCaP cell line a thiol adduct of an unknown thiol, named a "Conthiol", was successfully prepared and isolated [5]. In this study 2-chloromercuri-4-nitrophenol (ClMNP—see supplementary information for details of preparation) was used as a thiol labelling agent to produce an adduct (RSMNP) as shown in **Figure 1** below.

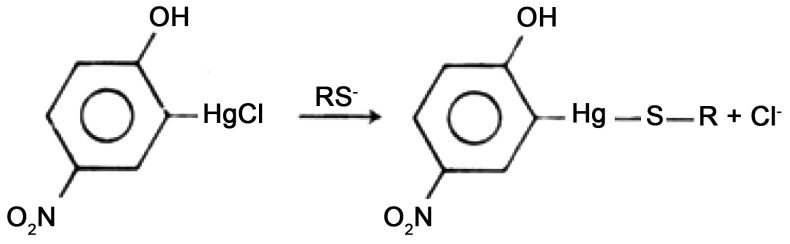


Figure 1. Preparation of thiol mercurial adduct.

Preliminary LC-MS studies of this adduct isolated from LNCaP cells indicated a thiol molecule with a molecular weight of approximately 467 daltons to be present.

Attempts to analyse and identify this thiol component of the nitrophenol mercurial adduct by mass spectroscopy proved too difficult to interpret and so it was deemed that, in order to facilitate further analysis, it was necessary to attempt to release the thiol from the adduct using an immobilized Thiopropyl Sepharose6B column (abbreviated as TPS) as illustrated in Figure 2 below.

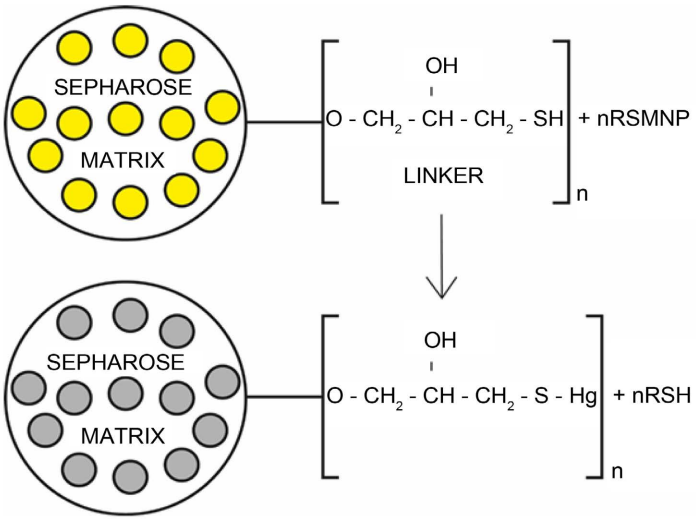


Figure 2. Use of an immobilized thiol column (TPS) to release a thiol from its mercurial adduct.

The thiol released from the column by elution with water was analyzed by a number of available analytical techniques.

2. Materials

All reagents and chemicals were of high analytical grade. Ellman reagent, (5,5'-dithio-bis-(2-nitrobenzoic acid), 2-mercaptoethanesulfonic acid, sodium salt (MESNA), mercaptoacetic acid, and other chemicals used were obtained from VWR Chemicals (BDH Prolab) and Sigma Aldrich (Merck) Thiopropyl Sepharose 6B (TPS) was supplied by GE Health Care cat. No. 17-0420-01).

LNCaP (androgen-sensitive human prostate adenocarcinoma cells, clone FGC-ECACC No. 89110211) [and PNT2 cells (a normal human prostate epithelial cell line immortalized with SV40 virus] were purchased from the Public Health England Laboratories (ECACC—HPA at Porton Down). Cells were grown to confluence in cell factories in a medium consisting of RPMI 1640 + 2 mM glutamine + 1 mM sodium pyruvate containing 10% Zone 2 FBS. They were then harvested by trypsinization (Tryple Express) and collected by centrifugation. The cell pellets were frozen until required.

LC-MS analyses were carried by Dr. Carolyn Hyde at the Bio-Analysis Centre,

London Bioscience Innovation Centre, 2 Royal College St., London NW1 0NH.

Full cysteine and other amino acid assays were carried on an ion exchange auto analyser by the Protein & Nucleic Acid chemistry facility of the Department of Biochemistry at the University of Cambridge.

Sulphur and Phosphorus analysis were carried out by Philip Holdship at the Department of Earth Sciences, via Oxford University Innovation Services by Inductively Coupled Plasma Mass-Spectrometry (ICP-MS) using Thermo Finnigan Element 2 Magnetic-Sector ICP-MS equipment.

Fourier Transform Infrared Spectroscopy, known as FTIR, was carried out by Intertek Pharmaceutical Services, Manchester, UK using a Bruker Tensor FTIR Spectrophotometer.

LNCaP cell conthiol adducts (RSMNP) were prepared in bulk from 5×10^8 cell batches as previously described [2] (for techniques used see supplementary information).

3. Methods

3.1. Preparation and Use of a Thiopropyl Sepharose 6B (TPS) Column

500 mg of the freeze dried powder supplied were rehydrated and re-suspended in an excess of water and mixed with enough Sepharose 6B to make up a 10×2 cm glass chromatography column and with the top sealed with a sintered glass disc. The column was eluted with 0.1 M solution the ammonium thioglycolate until all the covalently bound 2-thiol pyridyl ion had been removed (λ_{max} 343 nm) and the bound thiol had been fully rejuvenated. The column was then washed with 50 ml of 0.1 M mercaptoacetic acid, then exhaustively with water until no thiol could be detected in the eluate (using the Ellman reagent [6]). From the A_{343} released it was calculated that the column contained the equivalent of 40 µm oles of bound thiol.

3.2. Initial Studies

Samples of the thiol adduct of 2-mercuri-4-nitrophenol (RSMNP) (λ_{max} A_{404}) in 10 ml of water were run into the column and then the thiol was eluted with water; 5 ml fractions were collected. The thiol content of 100 µl of the fractions was determined with 900 µl the Ellman reagent (ESSE) [6] (0.1 mg per ml of 0.2 M phosphate buffer pH 7.8). Generally thiol appeared in the eluate after the first two fractions and was collected in a further two fractions. The extent of the exchange could be seen by the yellow 2-mercuri-4-nitrophenol (λ_{max} 410 nm) left bound on the column.

It was found that 5.17 ± 0.52 A_{404} optical units (1 cm cell) of the adduct released 1 micromole of thiol on passing through this column.

3.3. Further Studies after Treatment of the Adduct with Bromine Water

Following the initial work which indicated that the released thiol was unstable

breaking down to several components, the RSMNP was treated with a reagent capable of preventing polymerization of compounds containing alkene double bonds.

A sample of RSMNP calculated to contain approximately 6 μmoles of RSH/thiol dissolved in 5 ml of water was treated with 200 μl of 5% v/v of bromine water overnight at room temperature. After evaporation to dryness at 46˚C the resulting solid was re-dissolved in 5 ml of water. The yellow colour due to the 4-nitrophenol label had virtually disappeared indicating that bromination of the p-nitrophenol ring had occurred; less than 10% of the original A_{404} units/ml remained.

The brominated solution was applied to TPS column as before and the thiol eluted with water. In this study, to prevent oxidation of the released thiol, the eluate was reacted with calculated amount of mercuric chloride dissolved in water. This reacts to form an adduct as follows:

$$2RSH + HgCl_2 = RS - Hg - SR + 2HCl$$

No precipitation occurred in this eluate or after addition of the mercuric chloride.

3.4. LC-MS Studies

Liquid Chromatography was carried out on a Kinetex EVOC18 column (5 μ, 100 A, 50 × 2.1 mm) with a mobile phase A as 0.1% aqueous formic acid and mobile phase B as acetonitrile containing 0.1% formic acid. The gradient was 5% to 95% B over 10 mins. The photodiode array was set to scan from 190 - 800 nm. The Mass Spectrometer was set to scan in both positive and negative modes. 50 μl of each sample was injected.

4. Results

4.1. LC-MS Analysis of TPS Column Eluates

The thiol containing eluate obtained from passage of the RSMNP adduct through the TPS column was slightly acid, pH around 5, and slowly developed a precipitate overnight which was collected by centrifugation. The thiol content was estimated using the Ellman reagent. The precipitate did not contain thiol but the supernatant thiol content had not changed.

The two fractions obtained, supernatant and redissolved precipitate from the TPS column, were analysed by LC-MS. Both fractions gave a peak at 2.12 mins with a λ_{max} of 317 nm. However, the thiol containing supernatant had an additional 254 nm peak emerging after only 0.732 - 0.893 mins as shown in **Figure 3** below. This is almost certainly the thiol moiety; possibly an artefactual form produced on the TPS column.

The initial A_{254} peak emerging at 0.75 mins (0.732 - 0.893) mins had a MS fragment spectral pattern as shown in **Figure 4** below.

Prominent Positive ions were seen at 205, 236, 239, 256, 278 and 403 m/z.

Negative ions at 213, 363, 395 and 427 m/z.

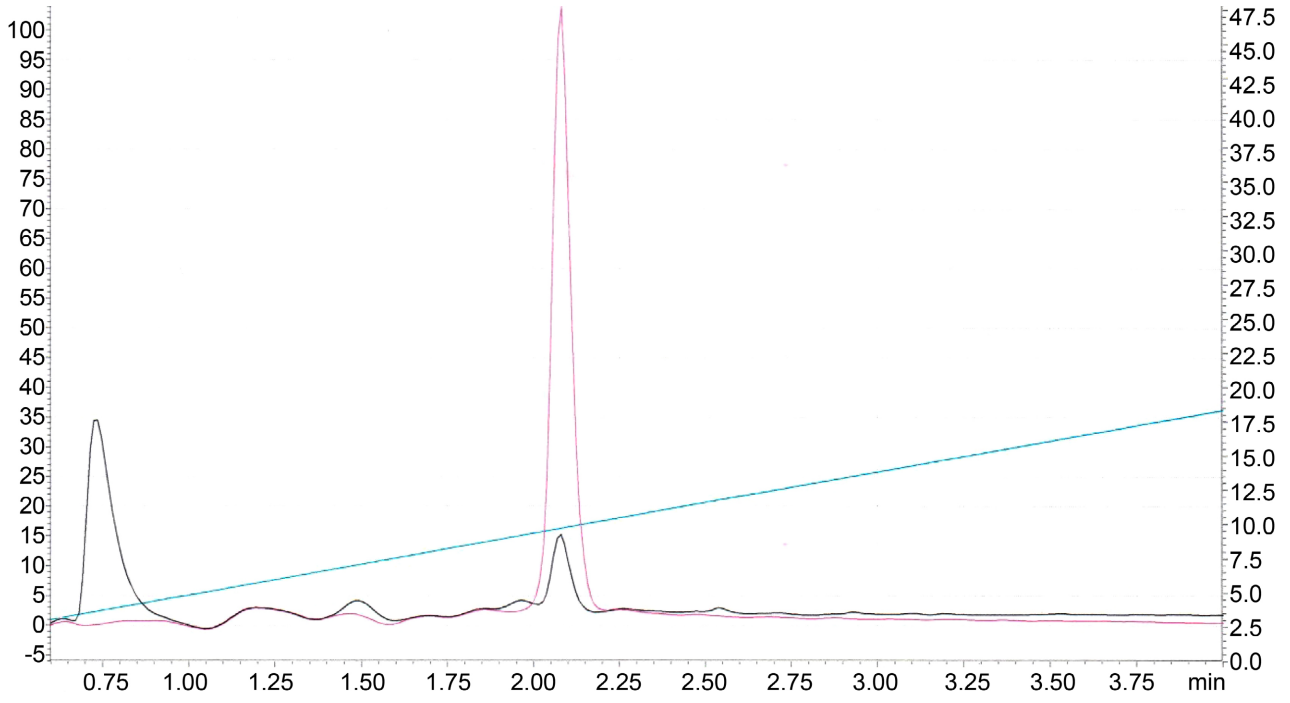


Figure 3. LC-MS Spectral pattern of the thiol eluate: black A_{254}, red A_{317}, and gradient blue; abscissa: column elute emergence time; ordinate: UV response (AU).

Figure 4. LC-MS scans of peak at 0.75 mins. Upper—positive ions; Lower—negative ions.

A second peak seen in both supernatant and redissolved precipitate emerged at 2.12 mins which UV spectral analysis showed had a pronounced A_{317} peak as illustrated in **Figure 5**.

The MS of this peak shown in **Figure 6** below was different to the 0.75 mins

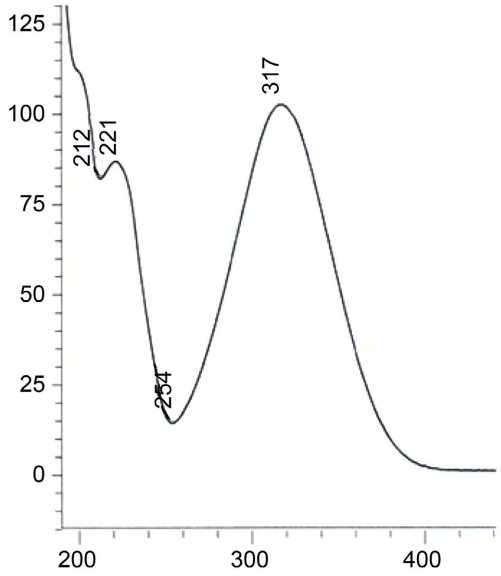


Figure 5. UV spectrum of 2.12 min peak in 0.1% formic acid; abscissa: UV adsorption; ordinate: optical units.

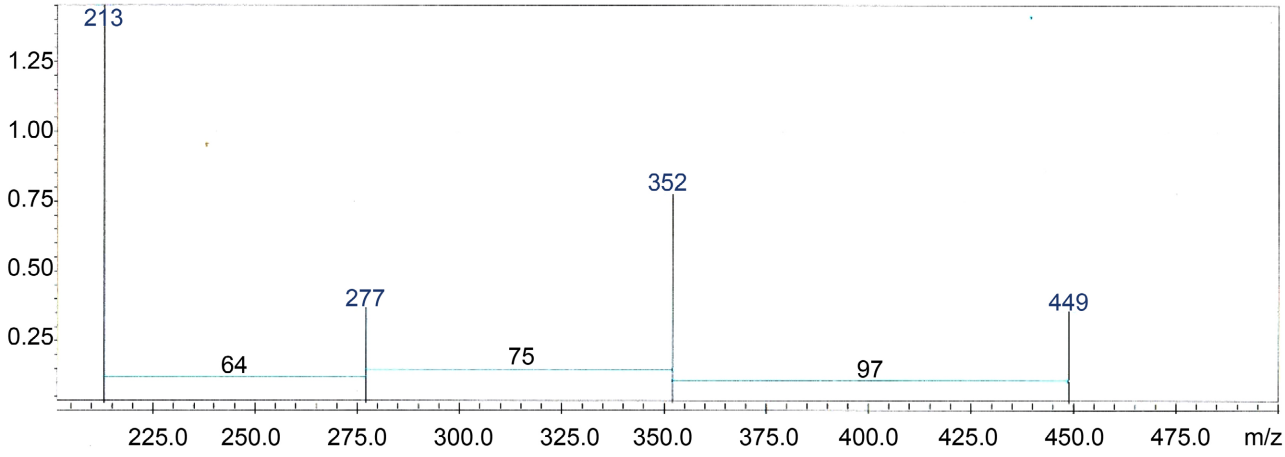


Figure 6. Negative ion MS of 2.12 min peak.

peak. The supernatant almost certainly has some of this component still in solution after the bulk had precipitated out.

Prominent negative ions are seen at 217, 352 and 449 m/z.

None of the TPS column aqueous eluates had any of the typical seven mercurial (Hg) clusters seen in the original LC-MS analysis of the RSMNP samples indicating that the 2-mercuri-4-nitrophenol component had been left firmly bound to the TPS column. However, when the column was eluted with ammonium thioglycolate (0.1 M) it was found that only 70% of the RSMNP A_{404} applied could be recovered. It is therefore possible that on release the thiol it somehow incorporated p-nitrophenol onto the part of its structure which was then cleaved to give the non-thiol A_{317}/2.12 min peak.

With regard to the 0.75 min thiol containing peak, the highest fragment size

in the negative ion pattern was at 427 m/z and, in the 2.12 min peak at 449 m/z, indicating that RSH could have a formula weight in the region of 450 daltons. This is close to a previous estimate-based LC-MS work on RSMNP when it was estimated that RSH could have a FW of around 467 daltons.

4.2. Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) Analysis of Column Eluate

This technique can be used for analysis of certain elements at nanomolar levels, Thiol samples released from TPS column runs were quantified by the Ellman assay and analysed by ICP-MS. The sulfur contents correlated well as shown in Table 1 below. The phosphorus content was also measured but only contaminated traces were present showing that phosphate was not present in the thiol molecule and could not be responsible for the high water solubility of the RSMNP adduct. No selenium could be detected.

[Unfortunately C, H, O, N and the halogens cannot be measured accurately by ICP methods and there was not enough eluate sample available for a classical Carbon (C, H and N) analysis to be carried out].

Table 1. Inductively coupled plasma-mass spectrophotometry analysis of thiol TPS column eluate.

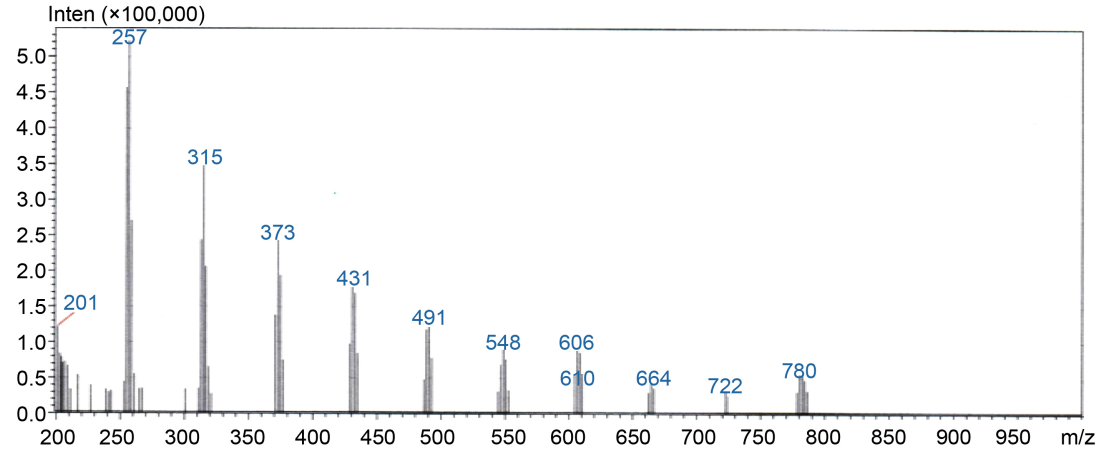
TPS eluate Sample	Thiol content in nanomoles per ml eluate by Ellman reagent	Thiol content in nanomoles per ml as measured by ICP-MS analysis	Phosphorus content in nanomoles per ml as measured by ICP-MS analysis
A	350	327	0.56
B	430	400	4.2
C	770	636	4.3
D	938	900	2.9

4.3. LC-MS Analysis of RSMNP Treated with Bromine Water

TPS eluates obtained from aqueous solutions of bromine treated samples, to which the calculated amount of mercuric chloride solution to form a thiol adduct had been added, were analysed by LC-MS on a Shimadzu LC-MS system fitted with a photodiode array detector. Mobile phase: A-water and B-methanol as before.

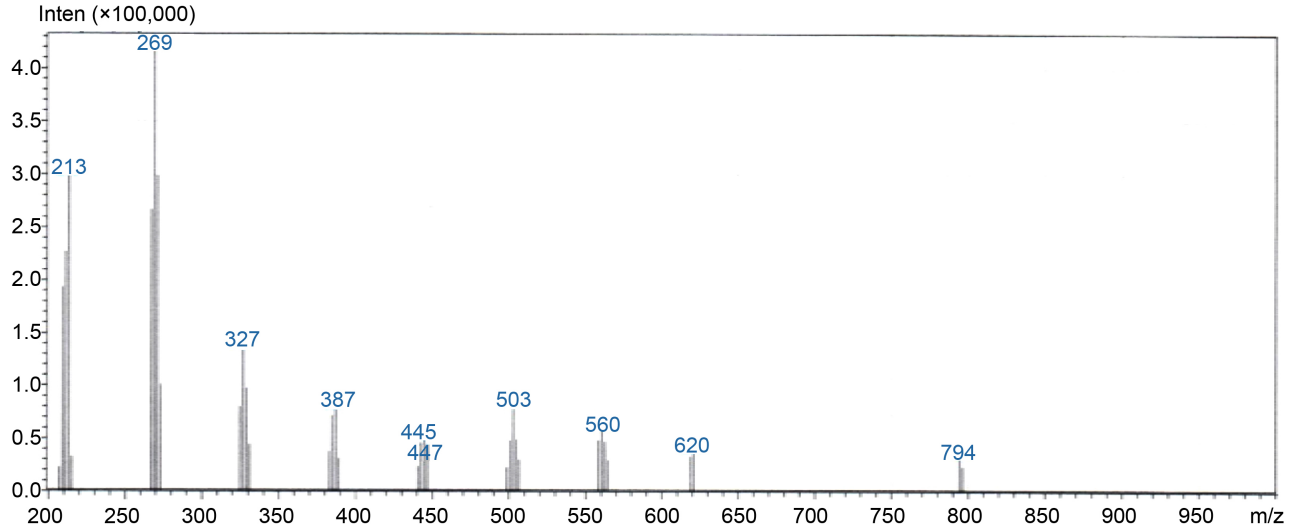
There are 7 stable isotopes of Hg which can be clearly seen in clusters appearing in the LC-MS patterns as shown in Figure 7 and Figure 8 below, showing the mass differences the "ladder" fragments between the peaks.

These positive and negative scan patterns are completely different to those obtained in the original RSMNP LC-MS patterns. The "ladder" fragments between the peaks clearly show that no bromine atoms (FW 80) have been incorporated into the thiol molecule ruling out the presence of alkene groups in the thiol. However, the loss of UV spectrum noted after bromine treatment does



201		257		315		373		431		491		548		606		664		722		780	+ve ion
	56		58		58		58		60		57		58		58		58		58		mass diff.

Figure 7. MS positive ions for peak eluting at 0.4 mins showing mercuri clusters and mass differences in stepwise fragments released.



213		269		327		387		445		503		560		620		794	-ve ion
	56		58		60		58		58		57		60		174		mass diff.

Figure 8. MS negative ions for peak at 0.4 mins showing mercuri clusters and mass differences in stepwise fragments released.

show that the nitrophenol ring of the RSMNP had been brominated.

The repeat units of 56 - 60 m/z shown in both figures that appear in the brominated adduct had not been detected or seen in the MS scan of the original RSMNP adduct.

Assuming a monomer ladder average mass of 58 daltons this 10 units per dual thiol Hg adduct giving a possible monomer molecular mass of 5 × 58 = 290 daltons. This plus the sulfur atom gives a possible formula weight of the thiol as 322.

The 58 dalton subunit found could be a combination of C, H, O and N found in many biological structures (e.g. N-CO_2). Further LC-MSn studies or a more

sophisticated form of analysis for nitrogen containing compounds will be necessary to resolve this issue.

It was also noted that treatment of the adduct with an oxidizing agent, hydrogen peroxide, did not produce this ladder pattern in the TPS eluate.

4.4. Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

FTIR analyses were carried out using a Bruker Tensor FTIR Spectrophotometer.

The spectrum of an RSH eluate obtained from the passage of the adduct RSMNP through a Thiopropyl Sepharose (TPS) column is shown in Figure 9 below.

The FTIR analyst's conclusions on this pattern are given in Table 2 below.

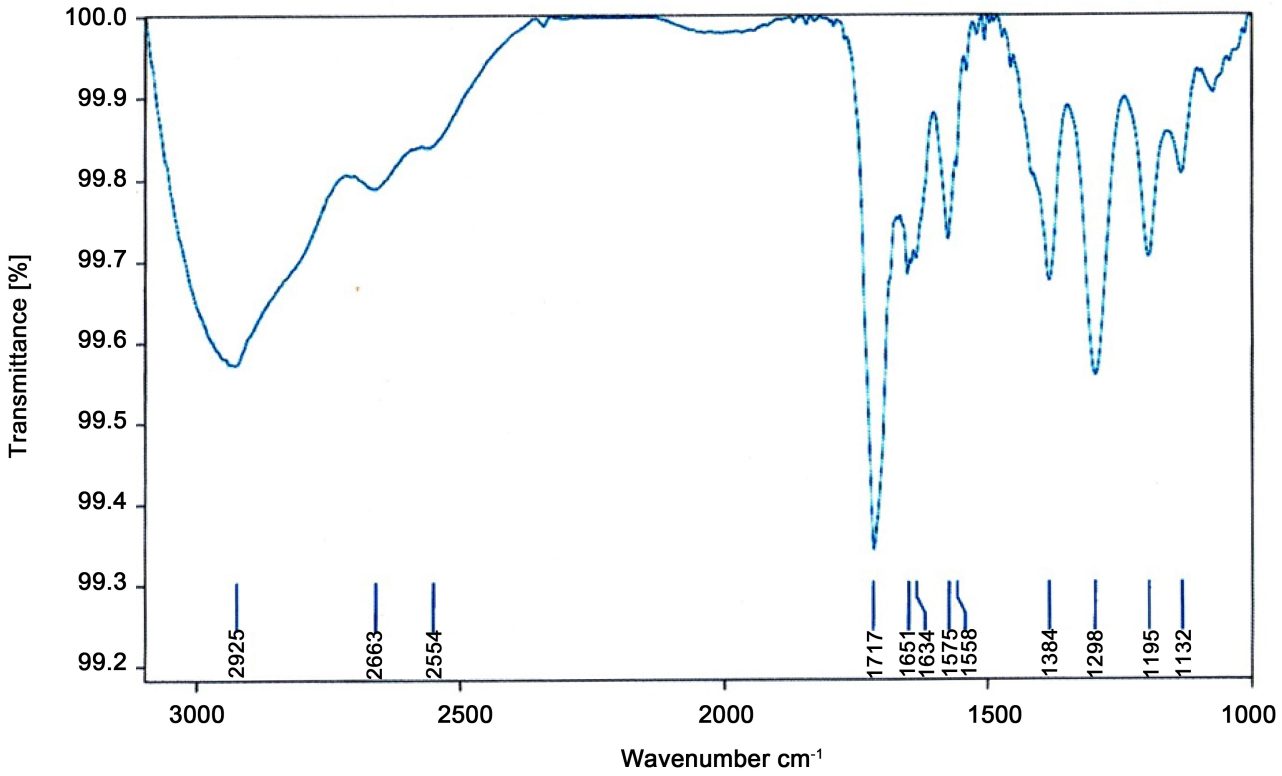


Figure 9. FTIR spectrum of eluate from passage of mercurial adduct (RSMNP) through a TPS column.

Table 2. Analysis of FTIR spectrum bands.

Wavenumber (cm^{-1})	Assignments
2925	C-H or C=C stretch, presence of aromatics
2663, 2554	SH stretch
1717	C=O stretch
1651,1634	C=O stretch, presence of aromatics (lactam ring)
1575,1558	C=C, C=N stretching, N-H (Nitro) deformation
1384	C-H and/or 0-H deformation and/or C-0 stretch
1298, 1195,1132	C-H or 0-H bending, C=O stretch

Very little information was obtained from this analysis except to confirm the presence of the thiol group and C=O stretch bands together with the possibility of a lactam ring at 1651 and 1634 $cm \cdot s^{-1}$. The C=O bands could be in a grouping present in the 58 dalton fragment found in the brominated sample. [It is notable that tertiary amides (a nitrogen links itself to 3 carbon atoms) give a band at 1651 ± 15 $cm \cdot s^{-1}$. This grouping could account for the cationic nature observed with this thiol adduct.]

5. Conclusions and Discussion

Elucidation of the nature of the Conthiols remains as elusive as ever! Apart from being "hidden" in the cellular protein matrices and being difficult to extract from cell preparations, little has been revealed on its structure despite the use of many different simple or complex analytical techniques that have been used in the author's studies. Conclusions that can be reached from the available data are as follows:

Both the RSMNP adduct and the thiol released (RSH) on the TPS column column are very soluble in water at neutral pH (in contrast to the labelling compound itself (ClMNP) which is poorly ionized). The RSMNP is easily adsorbed on either cation or anion exchangers indicating the presence of a zwitterion group or groupings—making it a very effective chelating agent in combination with the thiol group?

Both RSH and RSMNP samples were repeatedly analysed by automated Moore and Stein analysis for amino acids but none were detected. As a positively charged group is present in the Conthiol, and this is not due to the presence of a primary amine, it is possible that a tertiary or quaternary amine is present; e.g. as is found a molecule as such as ergothioneine.

ICP-MS analysis of the TPS eluate coupled with thiol measurement with the Ellman reagent shows that all the sulphur present is in the thiol form and that it does not contain phosphate. It is therefore highly likely that its negative charge is due to the presence of a carboxylic acid group or groupings.

Tests for ribose/pentoses using classic analytical techniques, for ribose using Bial's Orcinol method and for deoxyribose (2'-deoxypentose sugars) using the Dische Diphenylamine reagent, were negative [5].

The low UV adsorption at 260 nm of the TPS eluates showed that nucleic acid bases are not present even at high concentrations of Conthiol, ruling out the presence of the well-known cellular thiol Coenzyme A.

Analysis of the thiol released from bromine water treated RSMNP adduct showed that no aliphatic alkene groups were present. For an unknown reason a repeat 58 m/z fragment has been generated in the brominated RSMNP sample. This was not detected in the MS analysis of RSMNP itself or the thiol released by its passage through the TPS column.

It seems that Conthiols do not contain any fragments of the thiols normally found in eukaryote cells (see Supplementary Information 2 for examples). How-

ever, it has been found to be present in high quantities in the cells studied. LNCaP cells contain 31.8 ± 2.2 femtomoles of Conthiol per cell constituting 56.5% of the total thiol present as opposed to their glutathione content of only 8.3 ± 0.7 femtomoles per cell (14.7%) [5].

With regard to the known cellular low molecular weight thiol components, many studies have indicated that, apart from the presence in small quantities of some rarer thiols, the major thiol present in eukaryote cells is the ubiquitous tripeptide glutathione (*γ*-glutamylcysteinylglycine). This thiol is often quoted as constituting over 90% of the low molecular weight thiols present in these cells; however, during the author's studies evidence has been mounting that this is not necessarily always the case, particularly in tumour cells (e.g. [7]).

All of this information points to this "Conthiol" as having an extremely important role in cellular metabolism, possibly in the synthesis of nucleic acids as it has been found in high quantities in the non-histone proteins isolated from isolated nuclei from various tissues and cells [8].

Further research in this thiol may reveal a potentially new platform for the development of new therapeutic agents to treat metastatic cancers.

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Conflicts of Interest

The author has no conflicts of interest (political, personal, religious, ideological, academic, intellectual, commercial or any other) to declare in relation to this manuscript.

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Supplementary Information

1) Method for the isolation of protein bound thiols (Conthiols) from LNCaP cells using p-Chloromeruri-nitrophenol (ClMNP)

Procedure for 2 × 10^9 cells—(approx. 6 - 7 ml cell vol. containing 86 - 88 μmoles of Protein bound–SH in residue after ASF extracted).

Cells suspended in 2 × 30 ml water at 0 - 4˚C = 2 × 50 ml centrifuge tubes.

Briefly sonicated each for 4 × 30 sec bursts checking temp does not rise above 12 deg., then 2 × 3 ml (=6 ml total) of 100% TCA added (to give a final concentration of approx. 10% TCA)

Then sonicated for a further 2 × 30 secs and left for 15 min in ice.

The mixture is centrifuged at 3200 ×g for 4 mins. The supernatant (ASF) is aspirated off and the cell residue extracted with a further 2 × 30 ml of 10% TCA (this removes the cellular glutathione plus smaller amounts of cysteine and cysteinylglycine).

[see Childs, S., Haroune, N., Williams, L. and Gronow, M. (2017) Investigation of the Low Molecular Weight Thiol Composition in a Metastatic Prostate Cancer Cell Line (LNCaP) by LC-UV-MS and NMR after labelling with the Ellman Reagent. American Journal of Analytical Chemistry, 8, 1-18.]

Each cell residue (ca 3 - 4 ml) is then re-suspended in 30 ml of water combined to give 60 ml in total and the thiol content determined*. It is a very fine suspension, almost an emulsion, giving reliable repeat aliquots.

*Three samples are made up:

50 μl of cell suspension is added to 2950 μl of 8 M urea 0.5 M Na phosphate buffer pH 7.6 containing 0.2 mg/ml of Ellman's reagent-ESSE (will need to make up at least 50 ml containing 10 mg ESSE).

The A_{412} is read using the urea/ESSE solution as a blank (although it is not yellow it has a significant adsorption value at A_{412}).

This is a 1 to 60 dilution, therefore A_{412} (usually ca 0.4) × 60 units/ml of cell suspension × 80 gives the total A_{412}. From this protein thiol content can be calculated by dividing by 13.7 (€ millimolar ES in 8 M urea).

Should yield approx. 88 μmoles of –SH

For labelling with pClMNP dissolve 45.2 mg·s in 24 ml·s of acetone (or 18.8 mg in 10 ml) to give 5 μmoles/ml (Can't get any higher concentration). This will require 10 ml. all glass pipette).

The aqueous cell suspension is centrifuged as before and the pellet obtained is resuspended in 20 ml·s of water. This is added dropwise, with rapid mixing (magnetic stirrer), into 160 ml (1 to 7 dilution) of 8 M urea 50 mM phosphate pH 7.5 containing the half (50%) the calculated quantity of Hg reagent (at a 1:1molar ratio). When half of the residue suspension has been added the rest of the acetone 2-chloromercuri-4-nitrophenol—ClMNP (λ_{max} 410 nm) is added to the mixture. (You can check that all the –SH has reacted but you will need 500 μl plus 2.5 ml of the Ellman reagent, however since the reagent itself is yellow you will need another 500 μl in 8 M urea phosphate for a blank).

After stirring at RT for about 15 mins the resultant mixture is then centrifuged at 3300 rpm for 10 mins to sediment the chromatin (DNA-histone) complex.

The supernatant is then checked for A_{410} content (approx. 0.5 ml of 8 M extract + 2.5/ml 8 M/ESSE gives A_{410} of about 0.5).

For gel filtration 50 ml of 8 M urea extract is made up to 1 M NaCl (ie 50 ml extract + 3 g NaCl), the mixture heated at 50˚C for 1 - 2 hrs, shaking occasionally and then added to 10 × 6 cms column of Sephadex G-15 (bead size 40 - 120 μm; fractionation range excluded above 1500 MW) in 8 M urea 50 mM phosphate pH 7.5 Followed by 20 ml of 8 M urea 50 mM phosphate 1 M NaCl pH 7.5.

Using the yellow A_{410} as a guide, 10 ml fractions are collected. The protein MNP, which is excluded from the gel, emerges after about 100 ml of eluant. When all the protein has been collected, in about 60 - 70 mls, elution is continued with 8 M urea 10 mM ammonium bicarbonate pH ~7.8 After a further 180 ml of faint yellow, slightly turbid intermediate fraction has been collected the low molecular weight adduct emerges and elution continued until no yellow colour can be seen in the fractions.

The intense yellow LMW comes off in approximately 150 ml, the conductivity of which should be around 820 μS).

This can be adsorbed onto a 10 × 3 cm·s column of Whatman DE52 cellulose anion exchanger (Cl^- form). After washing with 150 ml of water to remove urea the RSMNP adducts can be eluted in a gradient of 0 to 1 M NaCl containing 50 mM ammonium bicarbonate. (This column will take up to 200 ml, (containing approximately 120 A_{410}) of G15 LMW eluate.)

A final Separation of the yellow low molecular weight RSMNP can be achieved on a 20 × 4 cm·s column of Phenyl Sepharose in water.

It is possible to fractionate up to 200 ml of the low molecular weight RSMNP eluted from the DE52 on column. After adsorbing this onto the column a wash of 20 ml of 0.5 M NaCl (or 1 M ammonium bicarbonate) is applied followed by water. After continuing elution with H_2O the yellow band concentrates and, about ~250 ml of H_2O, the RSMNP elutes in 8 - 10 ml at a conductivity of less than 1 mS.

(If the sample needs to be re-run on this column care must be taken as, if the pH drops on the column during passage of the sample, it sticks and loses colour.)

Solutions required for preparation:

1000 ml 8 M urea 50 mM phosphate. (480 g urea + 50 ml of 1 M phosphate buffer).

250 ml 8 M urea 0.5 M phosphate (120 g urea + 125 ml 1 M phosphate buffer pH 7.8 made up to 250 ml).

100% TCA ca 10 g made up to 10 ml in water.

50 ml of 8 M urea 0.5 M Na phosphate buffer pH 7.6 containing 10 mg ESSE

(5,5'-dithiobis-(2-nitrobenzoic acid) (0.2 mg/ml).

pClMNP reagent—dissolve 42.5 mg·s in 24 ml·s of acetone to give 5 μmoles/ml (Can't get any higher concentration). (5 × 24 = 120 umoles).

Column chromatography media.

Sephadex G-15.

Whatman DE52 cellulose anion exchanger (or equivalent).

Phenyl Sepharose 6 Fast Flow (high substitution).

Equipment required:

Ultrasonic cell disruptor.

Bench centrifuge.

Conductivity monitor (optional).

UV/visible spectrophotometer.

5 place balance.

25 × 6 cm·s glass column (for G15 Sephadex)

30 × 2 cm·s glass column (for DE52 anion exchanger)

40 × 4 cm·s glass column (for Phenylsepharose)

(NOTE—using a very similar preparation Conthiol adducts can also be prepared with other aromatic mercurials such as p-mercuribenzoic acid or p-mercuribenzenesulfonic acid)

2) Formula weights of LMW thiols commonly found in eukaryote cells (in $g \cdot mol^{-1}$)

Hydrogen Sulphide 34.08 (Present in trace amounts, involved with signaling processes).

Cysteine 121.16 (present mainly in protein structures).

Homocysteine 135.18 (mainly extra-cellar, found in some pathological conditions).

α-Lipoic acid (reduced) 206.32 (Naturally occurring lipoic acid is always covalently bound to macromolecules).

Pantetheine 278.379 (intermediate in the production of Coenzyme A).

Glutathione 307.32 (assumed to be major non protein thiol in cells).

Coenzyme A 767.535 (present in trace amounts, can scarcely be detected in cell extracts).

Some other possible low molecular weight thiols not normally found or synthesised in eukaryote cells.

Ovothiol A 201 (+ MNP 338 = 539 expected RSMNP mass) Ovothiol 228 + 338 = 566?

Ergothionine 229 (+MNP = 567).

Abbreviations

ASF: acid soluble fraction; ClMNP: 2-chloromercuri-4-nitrophenol; FTIR: Fourier-transform infrared spectroscopy; LC-MS: liquid chromatography-mass spectrographic analysis: LMWT: low molecular weight thiols; LNCaP: Lymph node androgen-sensitive human prostate adenocarcinoma cells (clone); RSMNP: thiol adduct of 2-mercuri-4-nitrophenol; TPS: Thiopropyl Sepharose 6B column.