Biosynthesis and Isolation of Selenoneine from Genetically Modified Fission Yeast

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Selenoneine, a naturally occurring form of selenium, is the selenium analogue of ergothioneine, a sulfur species with health relevance not only as a purported antioxidant but likely also beyond. Selenoneine has been speculated to exhibit similar effects. To study selenoneine's health properties as well as its metabolic transformation, the pure compound is required. Chemical synthesis of selenoneine, however, is challenging and biosynthetic approaches have been sought. We herein report the biosynthesis and isolation of selenoneine from genetically modified fission yeast *Schizosaccharomyces pombe* grown in a medium containing sodium selenate. After cell lysis and extraction with methanol, selenoneine was purified by three consecutive preparative reversed-phase HPLC steps. The product obtained at the mg level was characterised by high resolution mass spectrometry, NMR as well as HPLC/ICPMS. Biosynthesis was found to be a promising alternative to chemical synthesis, and should be suitable for upscaling to produce higher amounts of this important selenium species in the future.

1 Introduction

Selenoneine is the selenium analogue of the sulphur metabolite ergothioneine (Fig. 1), a purported anti-oxidant occurring in microorganisms, fungi, plants and animals.¹ Ergothioneine has recently attracted considerable attention following the report that it is synthesized by strictly anaerobic bacteria strongly suggesting physiological roles beyond its discussed anti-oxidative potential.^{1,2} Selenoneine, which was first discovered in marine fish³ and later also detected in human blood,^{4,5} is expected to be of similar importance; it is thought to contribute significantly to selenium's beneficial health effects,⁶ and might also play a role in methyl mercury detoxification.^{7,8}

To gain further insight into selenoneine's toxicity and protective effects as well as its metabolic transformation in organisms, the pure compound needs to be produced. In contrast to ergothioneine, for which synthetic routes have been published,⁹⁻¹¹ the chemical synthesis of selenoneine has been described only in the form of a patent application exactly following a protocol published for its sulphur analogue ergothioneine.¹² However, our attempts to synthesize selenoneine following this route were unsuccessful and we sought other possibilities for its production.

Alternatively, selenoneine can be isolated from natural sources containing sufficient quantities of this selenium species. Recently, Pluskal *et al.* engineered fission yeast, *Schizosaccharomyces pombe*, to overexpress an enzyme of the biosynthetic pathway leading to ergothioneine and reported that selenoneine was also produced by the organism, when the growth medium was supplemented with sodium selenate.¹³ The possible biotechnological production of selenoneine has also been suggested by a recent patent application.¹⁴ In the light of the findings for the genetically modified strain of *S. pombe*¹³ and its availability from the Yeast Genetic Resource Center in Osaka,

Japan, we attempted to utilize this microorganism for the biosynthesis and isolation of selenoneine.

Figure 1: Structures of ergothioneine (bottom) and its selenium analogue selenoneine in its reduced and oxidized form (top).

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1 Experimental

2 Chemicals and Reagents

3 Analytical grade chemicals and purified water (18.2 MΩ, Milli-
4 Q, Academic water purification system, Millipore GmbH,
5 Vienna, Austria) were used for all isolation steps and analyses.
6 Ammonium formate (≥ 95%), formic acid (≥ 98%, p.a.), Tris-(2-
7 carboxyethyl)phosphine hydrochloride (TCEP; > 98%, for
8 biochemistry), nitric acid (65%, p.a.) and hydrogen peroxide
9 (30%, p.a., stabilized) were purchased from Carl Roth GmbH +
10 Co. KG (Karlsruhe, Germany). Further purification of nitric acid
11 was achieved by sub-boiling. Acetic acid (glacial, 100%,
12 anhydrous for analysis) and methanol (HPLC gradient grade)
13 were obtained from Merck (Darmstadt, Germany) and VWR
14 International (Fontenay-sous-Bois, France), respectively.
15 Ergothioneine (≥ 98%) and D,L-selenomethionine (> 99%) were
16 purchased from Tetrahedron (Paris, France) and Fluka (Buchs,
17 Switzerland), respectively, and were utilized for the preparation
18 of stock standard solutions in water stored at 4 °C. Single-
19 element standard solutions of selenium (1000 mg Se L⁻¹ ± 0.2%
20 in 2% HNO₃) and germanium (1000 ± 3 mg Ge L⁻¹ in 2% HNO₃)
21 for the determination of total selenium were obtained from Carl
22 Roth GmbH + Co. KG and CPI International (Santa Rosa, USA),
23 respectively. Standard solutions for total selenium and
24 selenium species determination were prepared fresh daily by
25 dilution with water from stock standard solutions. Sodium
26 selenate (98%) for treatment of *S. pombe* was obtained from
27 Sigma-Aldrich (Vienna, Austria). Deuterated water (D₂O, 99.95%
28 D) for NMR spectroscopy was purchased from Deutero
29 (Kastellaun, Germany).
30 Chemicals for cultures of *S. pombe* were: Ammonium chloride
31 (≥99.5%, puriss. p.a.), D-(+)-glucose (≥99.5%), Na₂HPO₄
32 (≥98.0%), potassium hydrogen phthalate (≥99.5%), MgCl₂ ·
33 6H₂O (≥99.0%, BioUltra), CaCl₂ · 2H₂O (98+%, A.C.S. reagent
34 grade), KCl (≥99.5%), Na₂SO₄ (≥99.0%, BioUltra), MnSO₄ · H₂O
35 (≥99.0%, ReagentPlus®), KI (≥99.5%, BioUltra), CuSO₄ · 5H₂O
36 (≥98.0%, Bioreagent), ZnSO₄ · 7H₂O (≥99.0%, BioReagent), FeCl₃
37 · 6H₂O (97%, ACS reagent), boric acid (≥99.5%, Bioreagent),
38 citric acid (≥99.5%, ACS reagent), molybdic acid (≥85.0% MoO₃
39 basis, ACS reagent), Biotin (≥99%, BioReagent), Inositol (≥99.0%,
40 Plant Cell Culture Reagent), pantothenic acid (as hemicalcium
41 salt, ≥99.0%), nicotinic acid (≥98%, Bioreagent), L-Lysine (≥98%),
42 L-Leucine (≥99.5%, BioUltra) and adenine (≥99%, BioReagent)
43 from Sigma-Aldrich; uracil (≥99%, puriss.) and histidine (≥99%,
44 puriss.) from Fluka.
45 YES and EMM2-medium as well as the necessary stock solutions
46 were prepared according to literature.¹⁵ YPD-medium (pH 6.5)
47 was prepared by dissolving 10 g yeast extract (Oxoid L21,
48 Basingstoke, UK), 20 g peptone (Oxoid L37, Basingstoke, UK),
49 and 20 g glucose in 1 L water. Agar (20 g L⁻¹, Oxoid LP11,
50 Basingstoke, UK) was added to obtain plates.

51

52 Instrumentation

53 Sterilised spatula, scalpel and pincers were used for the
54 preparation of cell cultures. An Eppendorf BioPhotometer plus
55 (Eppendorf, Hamburg, Germany) was employed for optical
56 density measurements of yeast cultures at 595 nm (OD₅₉₅). Cell
57 lysis was achieved using a Branson Digital Sonifier (Branson,

58 Danbury, USA). Isolation of selenoneine from *S. pombe* was
59 carried out on an Agilent 1100 high performance liquid
60 chromatography (HPLC) system (Agilent Technologies,
61 Waldbronn, Germany) consisting of a binary pump (G1312A), a
62 thermostated autosampler (G1329A) equipped with an
63 extended loop capillary (G1313-87308), a diode array detector
64 (G1315B), and a 1200 series preparative fraction collector
65 (G1364B). Monitoring of selenium species in yeast cultures was
66 achieved by splitting ca. 4% of the chromatographic flow via a
67 T-piece installed between the diode array detector and the
68 fraction collector to an Agilent 7500ce inductively coupled
69 plasma mass spectrometer (ICPMS, Agilent Technologies)
70 equipped with a Burgener Ari Mist HP nebulizer (Burgener
71 Research International, Berkshire, UK), a cyclone spray
72 chamber, and an octopole reaction cell. The ICPMS was
73 operated in He-mode (He flow rate: 4.0 mL min⁻¹) for detection
74 of sulphur and selenium species at *m/z* 34, 77 and 78. The
75 combined fractions were evaporated to dryness after each
76 clean-up step with a Rotary Evaporator (RV 10 digital, IKA Works
77 Inc., Wilmington, North Carolina, USA) equipped with an LVS
78 105 T – 10 ef diaphragm vacuum pump (Welch by Gardner
79 Denver, Ilmenau, Germany).

80 An UltraCLAVE IV High Performance Microwave Reactor (MLS
81 GmbH, Leutkirch, Germany) was used for microwave-assisted
82 acid digestion before determination of total selenium in the
83 culture compartments as well as in the isolated product. Total
84 selenium determination and final characterisation of selenium
85 species was performed on an Agilent 7900 ICPMS or 8800
86 ICP-QQQ-MS operated in H₂-mode (H₂ flow rate: 3.5 mL min⁻¹)
87 equipped with a Micro Mist nebulizer and a Scott-type spray
88 chamber, either using an ASX-500 series ICP-MS autosampler or
89 an Agilent 1100 HPLC system including a degasser (G1379A), a
90 binary pump (G1312A), a thermostated autosampler (G1330A)
91 and a thermostated column compartment (G1316A) for sample
92 introduction during total selenium or selenium species
93 determination, respectively. Monitored masses were *m/z* 77,
94 78, 80, and 82; quantification was performed using *m/z* 78. 1%
95 CO₂ in Ar as optional gas at a flow rate of 12% of the carrier gas
96 flow was used for signal enhancement during total selenium
97 determination.

98 A Dionex Ultimate 3000 HPLC system (Thermo Scientific,
99 Waltham, USA) consisting of a Rapid Separation (RS) pump, an
100 RS autosampler and an RS column compartment connected to
101 a Q Exactive Orbitrap Mass Spectrometer (Thermo Scientific)
102 equipped with a heated electrospray ionization (HESI) source
103 (Thermo Scientific) was used for identification of selenoneine in
104 yeast cultures, fractions monitored during the isolation, and the
105 final isolated product. The electrospray ionization source was
106 operated in positive mode with the following source settings:
107 gas temperature 440 °C, gas flow rates 53 (sheath) and 14 (aux)
108 instrument units, spray voltage 3500 V and capillary
109 temperature 270 °C. A resolution of 70,000 (full width half
110 maximum, FWHM) was used for a full scan range of *m/z*
111 100 – 1000. The automatic gain control (AGC) target and the
112 maximum injection time (IT) were 3 × 10⁶ and 100 ms,
113 respectively. MS/MS (ddMS/MS) was performed with collision
114 energies of 10, 20, 30 (stepped) instrument units for the
115 precursor ion at *m/z* 553. Settings for MS/MS were as follows:

1 resolution 17,500 FWHM, maximum IT 50 ms, AGC target 1 x
2 10^5 and isolation window 8 Thomson (mass/charge).
3 NMR spectra were measured on a Bruker Avance III 500 MHz
4 NMR spectrometer, equipped with a 5 mm TXI probe with z-axis
5 gradients. Chemical shifts are reported in ppm (δ), with the
6 solvent reference relative to TMS ($\delta = 0.00$ ppm) as an internal
7 standard (D_2O , $\delta = 4.79$ ppm); coupling constants J are given in
8 Hz.

10 Culturing of *S. pombe* TP1803

11 *S. pombe* TP1803 cells on sterile filter paper were purchased
12 from the Yeast Genetic Resource Center (YGRC), Osaka, Japan.
13 Part of the yeast was cut from the "pellet" and a streak on a YES-
14 agar-plate was made. The streak was incubated at 30 °C for 2
15 days. Overnight cultures (ONCs, 10 mL each) were prepared in
16 EMM2-medium in 50 mL polypropylene tubes and grown in the
17 shaker at 28 °C and 140 rpm. The ONCs were used to inoculate
18 the main cultures. These were prepared in EMM2-medium (300
19 mL in baffled 1 L flasks, containing 10 μ M sodium selenate),
20 which were grown in the shaker at 26 °C and 120 rpm. Another
21 aliquot of sodium selenate (300 μ L of a 10 mM solution in water)
22 was supplemented after a reaction time of 48 hours. The
23 experimental design is shown in Fig. 2. To monitor cell growth,
24 OD_{595} of the pre- and main cultures was measured against
25 EMM2-medium.

27 Harvesting of *S. pombe* TP1803

28 Harvesting of the cells was performed using a procedure
29 adopted from Pluskal *et al.*¹³ (Fig. 2). On day 5 after the
30 inoculation of the main culture, the cells were separated from
31 the medium by centrifugation (6000 g, 20 min, 4 °C). The media
32 were removed; the cell pellets were re-suspended in 20 mL
33 methanol each and sonicated (50% amplitude, 5 min, 2 s pulse
34 on, 4 s pulse off). The suspensions were centrifuged (4000 g, 20
35 min, 4 °C) and the supernatants were evaporated. The residues
36 from evaporation were taken up in 5 mL water each and filtered
37 into HPLC vials through nylon syringe filters (CHROMAFIL® PA,
38 0.20 μ M, Macherey-Nagel, Germany) pre-washed twice with
39 methanol and twice with water.

41 Total selenium and selenium speciation in *S. pombe* TP1803

42 The distribution of selenium between culture medium, cell
43 lysate and cell pellet after harvesting was estimated in selected
44 samples by subjecting 1 mL of medium, 20 μ L of cell lysate, or 1
45 mL of a suspension of the cell pellet in 20 mL water to
46 microwave-assisted acid digestion with 5 mL of nitric acid.
47 Digests were made up to 50 mL with water and total selenium
48 was determined by ICPMS using external calibration and Ge as
49 internal standard.

50 Selenium speciation in the re-dissolved residues was monitored
51 by reversed phase HPLC/ICPMS (for experimental conditions
52 see Supplementary Material Table S-1, HPLC conditions A.1).
53 The presence of selenate was investigated by anion-exchange
54 HPLC/ICPMS using a Dionex IonPac™ AS14A-

79 Figure 2: Growth and harvest of *S. pombe* TP1803

81 5 μ m RFIC™ column (3 x 150 mm, Thermo Scientific) with 5 mM
82 malonate at pH 9.5 as the mobile phase, a flow rate of 0.7 mL min⁻¹,
83 a column temperature of 40 °C and an injection volume of 1 μ L. The
84 analytical columns were protected by their respective guard
85 columns.

87 Isolation of selenoneine from cell lysates of *S. pombe* TP1803

88 Selenoneine from the lysates of *S. pombe* was isolated by
89 preparative reversed-phase HPLC in three consecutive steps
90 (NEUTRAL 1, ACIDIC and NEUTRAL 2; Fig. 3; for experimental
91 conditions see Supplementary Material Table S-1, B). Collection
92 of fractions was monitored by UV absorbance. During the first
93 clean-up step (NEUTRAL 1, Fig. 3), part of the effluent from the
94 column was directed via a T-piece, installed between the diode
95 array detector and the preparative fraction collector, to an
96 ICPMS to monitor profiles of selenium and sulphur.
97 Furthermore, 1.5% aqueous H₂O₂ (1000 μ L) was injected
98 between the cell lysates to support oxidative conditions in the
99 LC to promote the presence of the oxidised form of
100 selenoneine.

1 Characterisation of selenoneine isolated from *S. pombe* TP1803

2 Selenoneine isolated by preparative reversed-phase HPLC was
 3 characterised by HPLC/ICPMS, high resolution Orbitrap MS and
 4 NMR spectroscopy. Total selenium in the isolated product was
 5 determined by ICPMS after dissolution in water and subsequent
 6 microwave-assisted acid digestion with nitric acid as detailed
 7 above. For characterisation by HPLC/ICPMS two
 8 chromatographic conditions differing in the pH of the mobile
 9 phase were used (for experimental conditions see
 10 Supplementary Material Table S-1, conditions A.2).
 11 Quantification of isolated selenoneine was performed using
 12 selenomethionine standards, assuming similar instrument
 13 response for both selenium species. The same chromatographic
 14 conditions were also used for the characterisation of isolated
 15 selenoneine by HPLC coupled to high resolution Orbitrap MS.
 16 Additionally, flow injection into 3% aqueous methanol (flow
 17 rate 0.4 mL min⁻¹) was performed to detect potential impurities.
 18 To check for contamination by selenate the isolated
 19 selenoneine was dissolved in water and subjected to anion-
 20 exchange HPLC/ICPMS under the conditions described before.
 21 For characterisation by NMR spectroscopy the isolated, dry
 22 selenoneine was re-dissolved in D₂O.

23 Results and discussion

24 Biosynthesis of selenoneine in *S. pombe* TP1803

25 While being a cheap selenium donor, sodium selenate was
 26 reported to be toxic to the cells in concentrations higher than
 27 10 µM.¹³ Being limited to this maximum concentration, we
 28 conducted a time study to monitor the consumption of selenate
 29 from the culture medium. ICPMS measurements revealed that
 30 the selenium added to the medium was consumed almost
 31 completely within two days (less than 10% of the added Se
 32 remaining in the medium). As the cells were growing for up to 5
 33 days, we added another aliquot of sodium selenate after two
 34 days thereby avoiding toxic concentrations of selenate while
 35 increasing the amount of selenoneine formed by the
 36 microorganism.

37 After five days of growth, the medium was removed and the
 38 cells were lysed and extracted with methanol. Determination of
 39 total selenium in the cell culture medium, cell pellet, and cell
 40 lysate revealed that only about 5% of the added selenium had
 41 remained in the medium, whereas ca 25% could be found in the
 42 pellet and about 50% was detected in the lysate (overall
 43 recovery ca 80% of the added selenium).

44 After evaporation of the methanol from the cell lysate, we
 45 obtained a yellow oily residue containing the desired product
 46 selenoneine along with its sulphur analogue ergothioneine and
 47 other matrix constituents. Based on selenium speciation
 48 analysis by reversed-phase HPLC/ICPMS after re-dissolution in
 49 water we estimated that 300 mL of culture yielded about 180 µg
 50 Se in the form of selenoneine (sum of reduced and oxidised
 51 form) along with other minor selenium species. A typical
 52 chromatogram is shown in Supplementary Material Fig.

71 **Figure 3:** Isolation of selenoneine from *S. pombe* TP1803. During each clean-up step,
 72 three fractions (F1, F2 and F3) per injection were collected from the preparative HPLC
 73 column using a fraction collector working in the time-based collection mode. Fraction 2
 74 containing selenoneine was combined from all injections in one clean-up step and
 75 evaporated to dryness with a rotary evaporator. The residue was then re-dissolved in
 76 water and subjected to the next clean-up step or to characterisation of the isolated
 77 product.

78
 79 S-1. Selenate was not detectable in the lysates by anion-
 80 exchange HPLC/ICPMS (Supplementary Material Fig. S-2).

82 Isolation and clean-up of selenoneine from *S. pombe* TP1803

83 A crucial step in the clean-up of selenoneine was its separation
 84 from its sulphur analogue ergothioneine. Selenoneine and
 85 ergothioneine only differ by one heteroatom and are, hence,
 86 expected to elute at similar chromatographic retention times.
 87 As selenol groups are more readily oxidised than are thiol
 88 groups¹⁶ we aimed to selectively oxidise selenoneine to achieve
 89 a chromatographic behaviour different from that of
 90 ergothioneine.

91 However, analytical reversed-phase HPLC/ICPMS coupled to
 92 elemental or high resolution MS using a chromatographic
 93 system routinely applied in our lab for the separation of
 94 organoselenium species including selenoneine^{17,18} revealed
 95 that the oxidised form of selenoneine eluted only marginally
 96 later than the reduced form as shown below (see
 97 "Characterisation of selenoneine isolated from *S. pombe*
 98 TP1803"). When the mobile phase (20 mM ammonium formate
 99 3% methanol pH 3.0) was changed to 3% aqueous methanol, the
 100 oxidised form of selenoneine was shifted towards longer
 101 retention times, whereas the reduced form still eluted close to
 102 ergothioneine (Supplementary Material Fig. S-3). Although
 103 column recoveries were only around 70%, we decided to
 104 proceed with this mobile phase as it can be easily removed by
 105 evaporation when clean-up by preparative chromatography is
 106 employed.

For the oxidation of selenoneine in the lysates of *S. pombe* we aimed to avoid complex and time-consuming procedures like treatment with organic solvents followed by evaporation to dryness as previously suggested.³ Preliminary investigations using oxidation by addition of aqueous H₂O₂ at different concentrations resulted in the degradation of selenoneine as well as the formation of mixed selenium-sulphur species and oxidised ergothioneine (Supplementary Material Fig. S-4). We noticed, however, that regular rinsing of the LC system with 0.3% H₂O₂ (Supplementary Material Fig. S-5) as well as freezing and thawing of the lysates led to a higher ratio of oxidised to reduced selenoneine without the undesired by-products that had formed when H₂O₂ was added directly to the samples. Hence, we decided to apply those mild and straight-forward methods, although quantitative oxidation was not always achieved.

Transfer of the analytical reversed-phase HPLC separation employing 3% aqueous methanol as the mobile phase to the preparative scale resulted in a broad signal for oxidised selenoneine (Supplementary Material Fig. S-5b). Applying a methanol gradient improved the peak shape and concurrently ensured the removal of late eluting sample constituents from the column before injection of the next sample (Supplementary Material Fig. S-6). In this way, fractions of *S. pombe* lysates containing the oxidised form of selenoneine were collected and evaporated to dryness (preparative HPLC, NEUTRAL 1, Fig. 3). Although HPLC/ICPMS monitoring of the residue re-dissolved in water indicated selenium purity as well as the absence of sulphur species like ergothioneine (Supplementary Material Fig. S-7), recording of the corresponding UV signal showed the presence of non-selenium containing matrix constituents in the collected fraction (Supplementary Material Fig. S-8a). High resolution MS of the re-dissolved fraction also indicated impurities (Supplementary Material Fig. S-9a).

Hence, a second clean-up step was applied, whereby the pH of the mobile phase was decreased again by the addition of acetic acid (preparative HPLC, ACIDIC, Fig. 3). Monitoring of the UV trace (Supplementary Material Fig. S-8b) as well as high resolution MS (Supplementary Material Fig. S-9b) indicated efficient clean up. However, NMR (500 MHz) analysis of the selenoneine-containing fraction revealed that the oxidised selenoneine was in the form of the acetate (data not shown). Therefore, another preparative clean-up step at neutral pH was employed (preparative HPLC, NEUTRAL 2, Fig. 3; Supplementary Material Fig. S-8c).

Characterisation of selenoneine isolated from *S. pombe* TP1803

The oxidised selenoneine collected from the third preparative chromatographic step (preparative HPLC, NEUTRAL 2, Fig. 3) was characterised by NMR, high resolution MS, and HPLC/ICPMS. The proton NMR spectrum (500 MHz) yielded signals corresponding to those obtained after isolation from tuna blood³ but without the impurities observed when selenoneine was purified from the fish matrix (Supplementary Material Fig. S-10a). Furthermore, the structure was confirmed by Heteronuclear Single Quantum Coherence Spectroscopy (HSQC) (Supplementary Material Fig. S-10b) and Heteronuclear

Multiple Bond Correlation (HMBC) between ¹³C and ¹H (Supplementary Material Fig. S-10c).

High resolution Orbitrap MS showed a clean spectrum for selenoneine with masses corresponding to the protonated molecule, its doubly-charged species, as well as their sodium and potassium adducts (Fig. 4). The structure was confirmed by fragmentation (Fig. 4).

HPLC/ICPMS of an aqueous solution of the isolated compound was employed to exclude the presence of selenium-containing contaminations. Reversed-phase chromatography with 3% aqueous methanol as mobile phase showed an intense signal at the retention time of oxidised selenoneine and a second albeit smaller signal at the retention time of its reduced form, whereby the baseline of the chromatogram did not return to background levels between the two peaks (Supplementary Material Fig. S-11a). This pattern of chromatographic peaks persisted when the pH of the mobile phase was decreased to 3.0, although the signal of the oxidised form was shifted to lower retention times (Supplementary Material Fig. S-11b).

HPLC/Orbitrap MS experiments under the same chromatographic conditions (Supplementary Material Fig. S-12) indicated that besides in-source reduction/oxidation of selenoneine, an equilibrium between the reduced and the oxidised selenoneine existed on the column confirming the unusual peak shape found in HPLC/ICPMS. Addition of TCEP to the mobile phase resulted in reduction of selenoneine leading to a single peak with improved peak shape (Supplementary Material Fig. S-13). Under these conditions the recovery of selenoneine when quantified against selenomethionine standard solutions in comparison to total selenium was found to be 108 ± 1%. The selenium purity was ≥ 98%.

As determined from total selenium measurements of the final product, we succeeded in isolating about 700 µg of oxidised selenoneine from 1 L of original cell culture (mean OD₅₉₅ 9.5 ± 1.6 corresponding to ca. 1.5 × 10¹¹ cells¹⁹). When comparing the sum of reduced and oxidised selenoneine determined in the lysates by HPLC/ICPMS with the total selenium concentration after isolation, the isolation yield was about 35%.

Conclusions

The successful isolation of the oxidised form of selenoneine from genetically modified *S. pombe* opens up a broad field of future applications such as its use as a standard compound for its analysis as well as its toxicological and metabolic characterisation. Such investigations will help to increase our understanding of selenoneine's role and health effects, and indicate its potential usefulness as a food supplement or therapeutic agent.

Conflicts of interest

There are no conflicts to declare.

Figure 4: Flow injection ESI-Orbitrap-MS of the cleaned-up oxidised selenoneine isolated from a lysate of *S. pombe* grown in selenate containing medium (a) spectrum of oxidised selenoneine, (b) zoom of the measured isotope pattern for oxidised selenoneine (c) calculated isotope pattern for oxidised selenoneine, (d) fragmentation of m/z 553.0603 (the signal at m/z 422 remained unassigned but was suspected to be the oxide of m/z 406); mobile phase: 3% aqueous methanol; flow rate: 0.4 mL min⁻¹, injection volume: 1 μ L.

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Electronic Supplementary Information

Biosynthesis and Isolation of Selenoneine from Genetically Modified Fission Yeast

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Table S-1: Chromatographic conditions for selenium species determination and clean-up of selenoneine from *S. pombe* TP1803

Figure S-1: Typical HPLC/ICPMS chromatogram (^{78}Se) of a lysate of *S. pombe* grown in selenate-containing medium; Chromatographic conditions: Column: Atlantis dC18, 4.6×150 mm, column temperature: 30°C , mobile phase: 3% aqueous methanol, flow rate: 1 mL min^{-1} , injection volume: $1\text{ }\mu\text{L}$.

Figure S-2: Typical anion-exchange HPLC/ICPMS chromatogram (^{78}Se) of a lysate of *S. pombe* grown in selenate-containing medium; native lysate (solid line) and lysate spiked with $150\text{ }\mu\text{g Se L}^{-1}$ selenite and selenate (dashed line); insert shows the full chromatogram; Chromatographic conditions: Column: Dionex IonPacTM AS14A- $5\text{ }\mu\text{m}$ RFITM 3×150 mm, column temperature: 40°C , mobile phase: 5 mM malonate pH 9.5, flow rate: 0.7 mL min^{-1} , injection volume: $1\text{ }\mu\text{L}$.

Figure S-3: HPLC/ICP-MS chromatogram (oxygen mode, ca $0.3\text{ mL O}_2\text{ min}^{-1}$; mass shift $^{78}\text{Se} \rightarrow ^{78}\text{Se}^{16}\text{O}$ and $^{32}\text{S} \rightarrow ^{32}\text{S}^{16}\text{O}$) of a lysate of *S. pombe* grown in selenate-containing medium; Chromatographic conditions: Column: Atlantis dC18, 4.6×150 mm, column temperature: 30°C , mobile phase: 3% aqueous methanol, flow rate: 1 mL min^{-1} , injection volume: $1\text{ }\mu\text{L}$.

Figure S-4: Flow injection ESI-Orbitrap-MS of fraction 2 obtained after cleanup-step 1 (NEUTRAL 1, Supplementary Material Table S1-B) of a lysate of *S. pombe* grown in selenate-containing medium directly treated with H_2O_2 before clean-up resulting in the formation of the mixed oxidised S-Se-species and oxidised ergothioneine; (a) spectrum of the mixed oxidised S-Se-species, (b) calculated isotope pattern for the mixed oxidised S-Se-species, (c) spectrum of oxidised ergothioneine, (d) calculated isotope pattern for oxidised ergothioneine; mobile phase: 3% aqueous methanol; flow rate: 0.4 mL min^{-1} , injection volume: $1\text{ }\mu\text{L}$.

Figure S-5: HPLC/ICPMS chromatogram (^{78}Se) of a lysate of *S. pombe* grown in selenate-containing medium before (a) and after (b) rinsing of the HPLC system with 0.15% H_2O_2 ; Chromatographic conditions: Column: Atlantis dC18 OBT, 19×100 mm, column temperature: ambient, mobile phase: 3% aqueous methanol, flow rate: 5 mL min^{-1} , injection volume: $100\text{ }\mu\text{L}$ (a), $200\text{ }\mu\text{L}$ (b).

Figure S-6: HPLC/ICPMS chromatogram (^{78}Se) of a lysate of *S. pombe* grown in selenate-containing medium applying a methanol gradient; Chromatographic conditions: Column: Atlantis dC18 OBT, 19×100 mm, column temperature: ambient, solvent gradient from 3% aqueous methanol to 30% aqueous methanol (for gradient details see Table S-1B, Clean-up step NEUTRAL 1), flow rate: 5 mL min^{-1} , injection volume: $100\text{ }\mu\text{L}$; vertical bars indicate the collected fraction containing oxidised selenoneine.

Figure S-7: HPLC/ICPMS chromatogram (^{78}Se (a) and ^{34}S (b)) of a lysate of *S. pombe* grown in selenate-containing medium after application of the first clean-up step (Table S-1B, Clean-up step NEUTRAL 1); Chromatographic conditions: Column: Atlantis dC18, 4.6×150 mm, column temperature: 30°C , mobile phase: 3% aqueous methanol, flow rate: 1 mL min^{-1} , injection volume: $1\text{ }\mu\text{L}$.

Figure S-8: HPLC/UV chromatograms (wavelength 260 nm) for monitoring of the clean-up of oxidized selenoneine from a lysate of *S. pombe* grown in selenate-containing medium using three consecutive preparative chromatographic steps (Table S1-B NEUTRAL 1 (a), ACIDIC (b), and NEUTRAL 2 (c); vertical bars indicate the collected fractions containing the oxidised selenoneine); Chromatographic conditions: Column: Atlantis dC18 OBT, 19×100 mm, column temperature: ambient, flow rate: 5 mL min^{-1} , injection volume: $1200\text{ }\mu\text{L}$. For mobile phases, solvent gradients, and collected fractions see Table S-1B.

Figure S-9: Flow injection ESI-Orbitrap-MS of an aqueous solution of oxidised selenoneine for monitoring of the clean-up from a lysate of *S. pombe* grown in selenate-containing medium using two consecutive preparative chromatographic steps (NEUTRAL 1 (a), ACIDIC (b), Fig. 2, Supplementary Table S1-B); mobile phase: 3% aqueous methanol; flow rate: 0.4 mL min^{-1} , injection volume: $1\text{ }\mu\text{L}$.

Figure S-10: ^1H -NMR spectrum (a), Heteronuclear Single Quantum Coherence Spectroscopy (b), and Heteronuclear Multiple Bond Correlation (c) of the isolated oxidised selenoneine; frequency: 500-MHz , solvent: D_2O .

Figure S-11: HPLC/ICPMS chromatograms of an aqueous solution ($\text{ca } 130\text{ }\mu\text{g Se L}^{-1}$) of the isolated oxidised selenoneine; Chromatographic conditions: Column: Atlantis dC18, 4.6×150 mm, column temperature: 30°C , mobile phase: 3% aqueous methanol (a) or 20 mM ammonium formate 3% methanol $\text{pH } 3.0$ (b), flow rate: 1 mL min^{-1} , injection volume: $20\text{ }\mu\text{L}$.

Figure S-12: HPLC/Orbitrap MS chromatograms (m/z 553.06 and 278.04) of an aqueous solution ($\text{ca } 130\text{ }\mu\text{g Se L}^{-1}$) of the isolated oxidised selenoneine; Chromatographic conditions: Column: Atlantis dC18, 4.6×150 mm, column temperature: 30°C , mobile phase: 3% aqueous methanol (a) or 20 mM ammonium formate 3% methanol $\text{pH } 3.0$ (b), flow rate: 1 mL min^{-1} , injection volume: $20\text{ }\mu\text{L}$.

Figure S-13: HPLC/ICPMS chromatogram of the isolated selenoneine; Chromatographic conditions: Column: Atlantis dC18, 4.6×150 mm, column temperature: 30°C , mobile phase: 3% aqueous methanol (a) or 20 mM ammonium formate $0.1\text{ mM TCEP } 3\%$ methanol $\text{pH } 3.0$ (b), flow rate: 1 mL min^{-1} , injection volume: $20\text{ }\mu\text{L}$.

Table S-1: Chromatographic conditions for selenium species determination and clean-up of selenoneine from *S. pombe* TP1803

A. Analytical reversed-phase HPLC			
Column	Atlantis dC18, 4.6 × 150 mm (Waters Corporation, Milford, USA) with guard column		
Column temperature	30 °C	Flow rate	1.0 mL min ⁻¹
1. Speciation analysis of <i>S. pombe</i> cell lysates			
Mobile phase	A: water B: methanol	Injection volume	1 µL
		DAD	260 nm
Gradient	Time [min] 0 – 20 20 – 21 21 – 27 27 – 28 28 – 35	Eluent B [%] 3 3 – 80 80 80 – 3 3	
Position of 6-port-valve between column and ICPMS	0 – 20 min 20 – 33 min 33 – 35 min	position 1; effluent from column to ICPMS position 2; effluent from column to waste position 1; effluent from column to ICPMS	
2. Determination of isolated product from <i>S. pombe</i>			
Mobile phase	3% MeOH or 20mM ammonium formate, 3% methanol, pH 3 (adjusted with formic acid) or 20mM ammonium formate, 3% methanol, 0.1 M TCEP, pH 3 (adjusted with formic acid)	Injection volume	1µL or 20 µL

B. Preparative reversed-phase HPLC			
Column	Atlantis dC18 OBT, 19 × 100 mm (Waters Corporation, Milford, USA)		
Column temperature	ambient	Injection volume	1200 µL
Flow rate	5.0 mL min ⁻¹	DAD	260 nm
1. Clean-up step 1 (NEUTRAL 1)			
Mobile phase	A: water B: methanol		
Gradient	Time [min] 0 – 20 20 – 21 21 – 30 30 – 31 31 – 45	Eluent B [%] 3 3 – 30 30 30 – 3 3	
Fraction collection	24 – 25 min 25 – 28 min 28 – 30 min	Fraction 1 (F1); before target species Fraction 2 (F2); containing target species Fraction 3 (F3); after target species	
2. Clean-up step 2 (ACIDIC)			
Mobile phase	A: 0.05% CH ₃ COOH in water B: methanol		
Gradient	Time [min] 0 – 11 11 – 12 12 – 17 17 – 18 18 – 25	Eluent B [%] 2 2 – 50 50 50 – 2 2	
Fraction collection	7 – 8 min 8 – 10 min 10 – 11 min	Fraction 1 (F1); before target species Fraction 2 (F2); containing target species Fraction 3 (F3); after target species	
3. Clean-up step 3 (NEUTRAL 2)			
Same settings as for clean-up step NEUTRAL 1 (see above), except for fraction collection			
Fraction collection	24 – 25 min 25 – 29 min 29 – 31 min	Fraction 1 (F1); before target species Fraction 2 (F2); containing target species Fraction 3 (F3); after target species

Figure S-1: Typical HPLC/ICPMS chromatogram (^{78}Se) of a lysate of *S. pombe* grown in selenate-containing medium; Chromatographic conditions: Column: Atlantis dC18, 4.6 $\times$ 150 mm, column temperature: 30 $^{\circ}\text{C}$, mobile phase: 3% aqueous methanol, flow rate: 1 mL min $^{-1}$, injection volume: 1 μL .

Figure S-2: Typical anion-exchange HPLC/ICPMS chromatogram (^{78}Se) of a lysate of *S. pombe* grown in selenate-containing medium; native lysate (solid line) and lysate spiked with $150\ \mu\text{g Se L}^{-1}$ selenite and selenate (dashed line); insert shows the full chromatogram; Chromatographic conditions: Column: Dionex IonPacTM AS14A-5 μm RFICTM 3 $\times$ 150 mm, column temperature: 40 $^{\circ}\text{C}$, mobile phase: 5 mM malonate pH 9.5, flow rate: 0.7 mL min⁻¹, injection volume: 1 μL .

Figure S-3: HPLC/ICP-QQQ-MS chromatogram (oxygen mode, ca 0.3 mL O₂ min⁻¹; mass shift ⁷⁸Se→⁷⁸Se¹⁶O and ³²S→³²S¹⁶O) of a lysate of *S. pombe* grown in selenate-containing medium; Chromatographic conditions: Column: Atlantis dC18, 4.6 × 150 mm, column temperature: 30 °C, mobile phase: 3% aqueous methanol, flow rate: 1 mL min⁻¹, injection volume: 1 µL.

Figure S-4: Flow injection ESI-Orbitrap-MS of fraction 2 after cleanup-step 1 (NEUTRAL 1, Supplementary Material Table S1-B) of a lysate of *S. pombe* grown in selenate-containing medium and treated by direct addition of H_2O_2 to the lysate; (a) spectrum of the mixed oxidised S-Se-species, (b) calculated isotope pattern for the mixed oxidised S-Se-species; mobile phase: 3% aqueous methanol; flow rate: 0.4 mL min^{-1} , injection volume: $1\text{ }\mu\text{L}$.

Figure S-4: Flow injection ESI-Orbitrap-MS of fraction 2 after cleanup-step 1 (NEUTRAL 1, Supplementary Material Table S1-B) of a lysate of *S. pombe* grown in selenate-containing medium and treated by direct addition of H_2O_2 to the lysate; (c) spectrum of oxidised ergothioneine, (d) calculated isotope pattern for oxidised ergothioneine; mobile phase: 3% aqueous methanol; flow rate: 0.4 mL min^{-1} , injection volume: $1 \mu\text{L}$.

Figure S-5: HPLC/ICPMS chromatogram (^{78}Se) of a lysate of *S. pombe* grown in selenate-containing medium before (a) and after (b) rinsing of the HPLC system with 0.3% H_2O_2 ; Chromatographic conditions: Column: Atlantis dC18 OBT, 19×100 mm, column temperature: ambient, mobile phase: 3% aqueous methanol, flow rate: 5 mL min^{-1} , injection volume: $100 \mu\text{L}$ (a), $200 \mu\text{L}$ (b).

Figure S-6: HPLC/ICPMS chromatogram (^{78}Se) of a lysate of *S. pombe* grown in selenate-containing medium applying a methanol gradient; Chromatographic conditions: Column: Atlantis dC18 OBT, 19×100 mm, column temperature: ambient, solvent gradient from 3% aqueous methanol to 30% aqueous methanol (for gradient details see Table S-1B, Clean-up step NEUTRAL 1), flow rate: 5 mL min^{-1} , injection volume: $100 \mu\text{L}$; vertical bars indicate the collected fraction containing oxidised selenoneine.

Figure S-7: HPLC/ICPMS chromatogram (^{78}Se (a) and ^{34}S (b)) of a lysate of *S. pombe* grown in selenate-containing medium after application of the first clean-up step (Table S-1B, Clean-up step NEUTRAL 1); Chromatographic conditions: Column: Atlantis dC18, 4.6 $\times$ 150 mm, column temperature: 30 $^{\circ}\text{C}$, mobile phase: 3% aqueous methanol, flow rate: 1 mL min $^{-1}$, injection volume: 1 μL .

Figure S-8: HPLC/UV chromatograms (wavelength 260 nm) for monitoring the clean-up of oxidised selenoneine from a lysate of *S. pombe* grown in selenate-containing medium using three consecutive preparative chromatographic steps (Table S1-B NEUTRAL 1 (a), ACIDIC (b), and NEUTRAL 2 (c); vertical bars indicate the collected fractions containing the oxidised selenoneine); Chromatographic conditions: Column: Atlantis dC18 OBT, 19 × 100 mm, column temperature: ambient, flow rate: 5 mL min⁻¹, injection volume: 1200 µL. For mobile phases, solvent gradients, and collected fractions see Table S-1B.

Figure S-9: Flow injection ESI-Orbitrap-MS of an aqueous solution of oxidised selenoneine for monitoring of the clean-up from a lysate of *S. pombe* grown in selenate-containing medium using two consecutive preparative chromatographic steps (NEUTRAL 1 (a), ACIDIC (b), Fig. 2, Supplementary Table S1-B); mobile phase: 3% aqueous methanol; flow rate: 0.4 mL min⁻¹, injection volume: 1 μ L.

Figure S-10(a): ^1H -NMR spectrum of the isolated oxidised selenoneine; frequency: 500-MHz, solvent: D_2O .

Figure S-10(b): Heteronuclear Single Quantum Coherence Spectroscopy of the isolated oxidised selenoneine; frequency: 500-MHz, solvent: D₂O.

Figure S-10(c): Heteronuclear Multiple Bond Correlation of the isolated oxidised selenoneine; frequency: 500-MHz, solvent: D_2O .

Figure S-11: HPLC/ICPMS chromatograms of an aqueous solution (ca 130 $\mu\text{g Se L}^{-1}$) of the isolated oxidised selenoneine; Chromatographic conditions: Column: Atlantis dC18, 4.6 $\times$ 150 mm, column temperature: 30 $^{\circ}\text{C}$, mobile phase: 3% aqueous methanol (a) or 20 mM ammonium formate 3% methanol pH 3.0 (b), flow rate: 1 mL min^{-1} , injection volume: 20 μL .

Figure S-12: HPLC/Orbitrap MS chromatograms (m/z 553.06 and 278.04) of an aqueous solution (ca $130 \mu\text{g Se L}^{-1}$) of the isolated oxidised selenoneine; Chromatographic conditions: Column: Atlantis dC18, 4.6×150 mm, column temperature: 30°C , mobile phase: 3% aqueous methanol (a) or 20 mM ammonium formate 3% methanol pH 3.0 (b), flow rate: 1 mL min^{-1} , injection volume: $20 \mu\text{L}$.

Figure S-13: HPLC/ICPMS chromatogram of the isolated selenoneine; Chromatographic conditions: Column: Atlantis dC18, 4.6×150 mm, column temperature: 30 °C, mobile phase: 3% aqueous methanol (A) or 20 mM ammonium formate 0.1 mM TCEP 3% methanol pH 3.0 (B), flow rate: 1 mL min⁻¹, injection volume: 20 μ L.