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First preparative biocatalytic hydrolysis and *S*-methylation of cyclic trithiocarbonates

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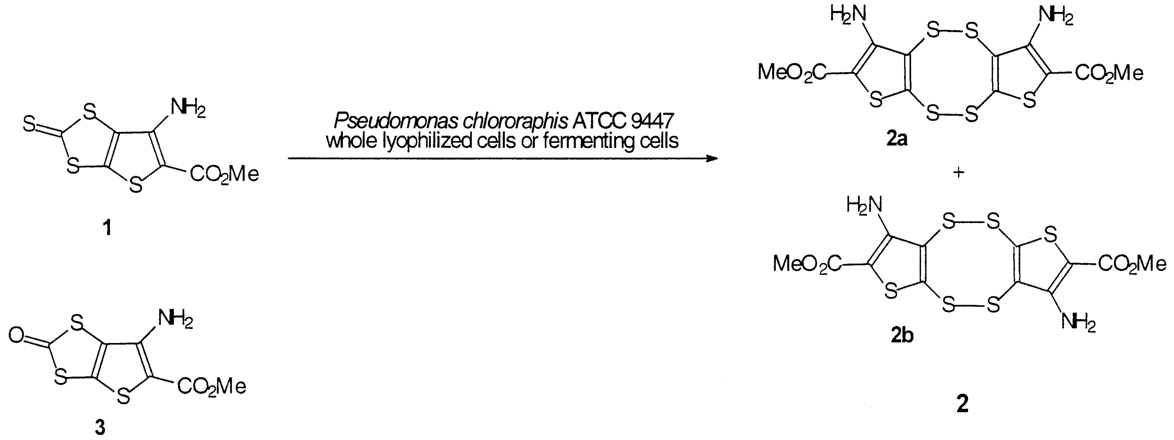
Abstract—The biocatalytic degradation of a cyclic trithiocarbonate, 6-amino-5-methoxycarbonyl-thieno[2,3-*d*]-1,3-dithiole-2-thione **1**, is reported. The product of the hydrolysis of the five-membered ring by *Pseudomonas chlororaphis* ATCC 9447 oxidatively dimerized to form the tetrathiocin derivative **2**. Furthermore, we performed the first preparative biocatalytic methylation of an unnatural compound employing *Emericella unguis* ATCC 10032 by cleaving the dithiole ring **1** followed by methylation of both thiol groups to form the methylated product **4** in 64% isolated yield. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Different types of enzymes involved in the metabolism of sulfur in natural compounds, like sulfurtransferases,[1] monooxygenases and dioxygenases, like cysteine oxygenase,[2] oxidases, like the methyl mercaptan oxidase,[3] sulfur methylating enzymes[4] and others have been described before.

Using non-natural compounds as substrates, monooxygenases of fermenting cells[5–7] as well as isolated peroxidases[8,9] have been used for the synthesis of chiral sulfoxides on preparative scale. Three different monooxygenases and one desulfinase were used for the desulfurization of petroleum fractions containing dibenzothiophene.[10] Employing baker's yeast, a carbon–sulfur double bond C=S was reduced to give optically active thiols.[11]

However, to the best of our knowledge no preparative methylation of a thiol moiety of non-natural substrates has been described. We wish to report the first enzymatic cleavage of trithiocarbonates and the first biocatalytic sulfur methylation on a preparative scale.



Scheme 1. Enzymatic hydrolysis of **1** followed by an oxidation reaction.

Keywords: biotransformation; sulfur methylation; *Emericella unguis*; *Pseudomonas chlororaphis*.

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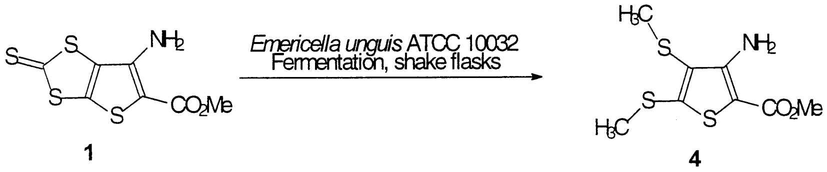

Scheme 2. Formation of a dimethyl thioether from cyclic trithiocarbonate **1**.

2. Results and discussion

In a screening for identifying microorganisms capable of degrading 6-amino-5-methoxycarbonyl-thieno[2,3-*d*]-1,3-dithiol-2-thione **1** two interesting strains were found. Fermenting cells of *Pseudomonas chlororaphis* ATCC 9447 in Luria broth as well as lyophilized whole cells in phosphate buffer (100 mM, pH 7.0) transformed the poorly soluble substrate **1** to a yellow precipitate (67% isolated yield) which is proposed to be the tetrathiocin **2** (Scheme 1) based on mass spectrometry data.[12–16] Due to the low solubility of **2** it was not possible to analyze it further by NMR spectroscopy. However, in analogy to the findings of Zhang et al., it seems likely that **2** consists of a mixture of **2a** and **2b**.[17]

Using 6-amino-5-methoxycarbonyl-thieno[2,3-*d*]-1,3-dithiol-2-one **3** as substrate and employing lyophilized cells of *P. chlororaphis* ATCC 9447 as catalyst the same insoluble product **2** was obtained.

Lyophilized cells of *P. chlororaphis* ATCC 9447 were disintegrated and the water-soluble enzyme fraction was partly purified on an anion-exchange column (Mono Q). Testing the fractions for activity using **1** and **3** as substrates resulted in three fractions which degraded substrate **3**, but only one fraction was capable of degrading **1**. This fraction was identical with one of the active fractions for substrate **3**.

Fermenting cells of *Emericella unguis* ATCC 10032 in Luria broth converted the 1,3-dithiole-2-thione **1** within 5 days to the dimethyl thioether **4** on preparative scale (Scheme 2). After 5 days, all the starting material was converted to product, and formation of side products could not be detected by HPLC (Fig. 1). The product **4** was isolated in 64% yield (15 mg).

In contrast to fermenting cells, lyophilized cells of *E. unguis* ATCC 10032 did not degrade the starting material **1**. Work will be continued concerning the elucidation of the mechanism involved in the formation of **4** (e.g. methyl donor, cofactor, enzymes).

One of the major problems for these biotransformations was the low solubility of **1** in water. Only a high amount of detergent (Tween 40) and the addition of a co-solvent (*N*-methyl-2-pyrrolidone) could solubilize **1**. How far the co-solvent, the detergent or their degradation products (e.g. palmitic acid) influenced the reaction (enzyme induction or inhibition), remains to be clarified.

After the enzymatic hydrolysis of **1** and **3**, an oxidative dimerization leads to **2**. The latter reaction might also be catalyzed by an oxydoreductase of *P. chlororaphis* ATCC 9447, in analogy to a similar reaction reported recently with a plant enzyme.[17]

3. Experimental

NMR-spectra were measured on a Varian Unity 500 MHz. Electron impact (EI) mass spectrometric measurements were carried out on a Finnigan MAT 90 (Finnigan MAT, Bremen D). EI spectra were recorded at 70 eV from 33 to 1000 Da. The source temperature was 200°C. Sample introduction was via the direct inlet probe. High resolution measurements were made by peak matching at a resolution power of 10,000 using perfluorokerosene as reference compound.

Electrospray (ESI) ionization mass spectrometric measurements were done on a micromass Quattro II (Micromass, Manchester UK) with a Waters 2690 and a Waters PDA 966

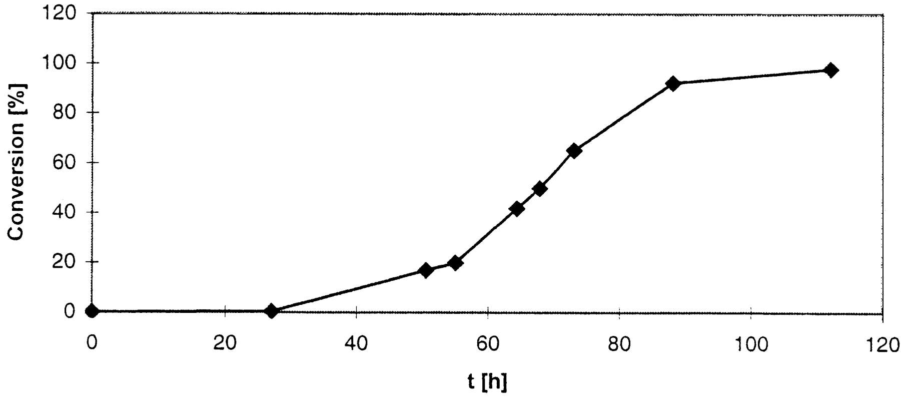

Figure 1. Formation of the dimethyl thioether **4** from **1** by fermenting cells of *E. unguis*.

(Waters, Milford MA). HPLC separation was accomplished with a YMC ODS-AQ 125×2 mm^2 120A S-5 μm (Stagroma, Reinach CH) operated at 40°C at a flow rate of 0.5 ml/min and a linear gradient from 100% A (water, 0.01% TFA) to 100% B (acetonitrile, 0.01% TFA) within 15 min and staying at 100% B for five minutes. The spray voltage was set to 4 kV and the cone voltage was at 30 V. Source temperature was 80°C. The quadrupole scanned over a mass range from 80 to 1500 Da. 6-Amino-thieno[2,3-*d*]-1,3-dithiol-2-thione **1** and its derivative **3** were synthesized according to a procedure described in the literature.[18]

P. chlororaphis ATCC 9447 and *E. unguis* ATCC 10032 were cultivated in Luria medium (16 g/l Difco Luria broth; 1.4 g/l Merck KH_2PO_4; 4.4 g/l Merck K_2HPO_4) at 28°C. Reactions were followed by HPLC: Merck Hitachi (L-4200 UV-VIS, L-6200 pump, HS-2000A autosampler); Column: Kromasil 100-5 C18 5μ, (125×4 mm^2, Macherey-Nagel); Linear gradient: 50% A (acetonitrile, 0.0075% TFA) and 50% B (water, 0.01% TFA) to 70% A within 8 min. Retention times of **1**: 6.6 min; **3**: 3.3 min; **4**: 4.5 min.

In a typical reaction employing lyophilized cells, the catalyst (170 mg cells) was suspended in phosphate buffer (12 ml, pH 7.0, 100 mM) and rehydrated for 30 min. The substrate **1** (50 mg, 0.19 mmol) was dissolved in *N*-methyl-2-pyrrolidone (400 μl) before Tween 40 (200 μl) was added. After the addition of the substrate solution to the catalyst suspension the mixture was shaken at 200 rpm at 28°C in a round-bottomed flask. Every 24 h 100 mg of lyophilized rehydrated cells were added. After 10 days the cells were removed by centrifugation (5000 rpm, 15 min). The product **2** which was suspended on the surface of the supernatant was filtered and washed first with diluted $NaHCO_3$ solution (2×30 ml), to remove palmitic acid as a degradation product of Tween 40, and then consecutively with water (2×30 ml) and ethyl acetate (2×30 ml) and finally lyophilized to give 25 mg (60%) of product **2**. A NMR spectrum could not be recorded, because the solubility of **2** was too low in all organic solvents tested. However, the structure could be proposed using the MS data. EI-MS: M^+ 438 (77%), 406 (15%), 374 (67%), 342 (14%), 315 (34%), 251 (14%), 219 (60%), 187 (29%). HR-MS: $C_{12}H_{10}N_2O_4S_6$ observed: 437.8966±0.0001 Da, calcd: 437.8965.

Partial protein purification: Lyophilized cells of *P. chlororaphis* ATCC 9447 (200 mg) were suspended in Tris buffer (4 ml, pH 7.5, 100 mM). The suspension was disintegrated in portions (0.75 ml) in Eppendorf tubes (2 ml) with glass beads (1.6 g/vial, 200–300 μm) in a MM200 disintegrator (Retsch-Germany, 30 s^{-1}, 9 min). The samples were centrifuged in vials (2 ml) in an Eppendorf centrifuge (2 min, 14,000 rpm), the beads were washed with buffer (1 ml), centrifuged again, and one milliliter of the combined supernatant was applied to an anion exchange column (Pharmacia, Mono Q HR 5/5, flow: 0.5 ml/min, fraction size: 1 ml). A linear gradient was run starting from 100% buffer A (Tris, 100 mM, pH 7.5) to 100% buffer B (Tris, 100 mM, pH 7.5, 1 M NaCl) within 30 min. The fractions were assayed using substrate **1** and **3**, thus for each substrate a certain amount (350 μl) of each fraction was transferred to an Eppendorf tube (1.5 ml), and substrate (0.3 mg dissolved in 3.5 μl *N*-methyl-2-pyrrolidone and 3.5 μl Tween 40) was added. The tubes were shaken at 200 rpm at 28°C for 2 days. The samples were analyzed by HPLC after the addition of acetonitrile (1 ml) and centrifugation.

In a typical transformation under fermentation conditions, Luria medium (250 ml) was inoculated with *E. unguis* ATCC 10032 in shake flasks (not baffled). After 2 h of shaking (200 rpm, 28°C) the substrate (25 mg, 0.095 mmol; dissolved in 1 ml *N*-methyl-2-pyrrolidone; and 1 ml Tween 40) was added by sterile filtration (Acrodisc, No. 4433, 0.2 μm). The shaking (200 rpm, 28°C) was continued for 6 days. After the addition of acetone (20 ml) and centrifugation (8000 rpm, 15 min) the medium was extracted with ethyl acetate (3×150 ml). The combined organic phases were dried (Na_2SO_4) and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (silica gel, hexane/ethyl acetate=4:1) to give 15 mg (64%) of **4**. ^1H NMR: ($CDCl_3$): δ 5.5 (2H, br, NH_2); 3.83 (3H, s, CH_3–O); 2.59, 2.22 (2×3H, s, CH_3–S). ^{13}C NMR: ($CDCl_3$): δ 51.7 (CH_3O–); 16.4, 17.1 (2×CH_3S–). EI-MS: M^+ 249 (5%), 219 (2%), $C_8H_{11}N_1S_3O_2$ calculated 248.9952 Da, observed 248.9944±0.0001 Da. ESI-MS: $[M+H]^+=250$ Da.

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