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# The Utilization of 3-Mercapto-2-Methylpropionate as Sulphur Source by a Phototrophic Bacterium

Mark R. Smith,[1a] Will J. J. van den Tweel[2] and Jan A. M. de Bont*[1]

[1]*Division of Industrial Microbiology, Department of Food Science, Agricultural University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands*

[2]*Division of Bio-Organic Chemistry, DSM Research, P.O. Box 18, 6160 MD Geleen, The Netherlands*

**Abstract**—A bacterium (strain photoB) photoassimilated 3-mercapto-2-methylpropionate as sole source of sulphur with methacrylate accumulating in the medium. This was thought to be the product of a sulphur-lyase type enzyme attacking the 3-mercapto-2-methylpropionate. Detailed examination of the biochemistry of the utilization of 3-mercapto-2-methylpropionate showed however that the thiol was first activated to 3-mercapto-2-methylpropionyl CoA. It appeared that this CoA-derivative then served as the substrate for a sulphur-lyase type enzyme, with methacrylyl-CoA as product. Further metabolism was via β-hydroxyisobutyryl-CoA and β-hydroxyisobutyrate.

## Introduction

The abiotic nucleophilic addition of bisulphide to unsaturated aliphatic compounds in marine sediments has been postulated and this is thought to account for the formation of low molecular weight thiols in organic-rich sediments in marine coastal environments.[1] 3-Mercaptopropionic acid was shown to be the most abundant species in sediment samples taken from intertidal zones of Biscayne Bay, Florida. This thiol was thought to be the result of bisulphide addition to acrylic acid; the former being generated by bacterial dissimilatory sulphate reduction, the latter is derived from the cleavage of β-dimethysulphoniopropionate, an osmolyte in many algae and sea grasses.[2,3] The addition of bisulphide to fumaric acid, acrylonitrile and methacrylic acid was also demonstrated.[1] The addition of bisulphide to the last of these α,β-unsaturated compounds results in the formation of 3-mercapto-2-methylpropionic acid.

This present communication reports on the biochemical mechanisms involved in the mineralization of such a thiol. 3-Mercapto-2-methylpropionate was selected for these studies as, in addition to its occurrence in natural sediments, it is a potential precursor for the synthesis of an antihypertensive agent, Captopril [*N*-(3-mercapto-2-D-methylpropanoyl)-L-proline]. Both compounds are shown in Figure 1. Previously we have proposed the use of esters of 3-chloro-2-methylpropionate as substrates of lipases,[4] esterases[5] and dehalogenases[6] for the preparation of precursors of this important drug. One of the aims of this current work was to screen for bacteria which could metabolize 3-mercapto-2-methylpropionate. In this work we used 3-mercapto-2-methylpropionate as the sole source of sulphur, in the hope of selecting bacteria which specifically removed the sulphur moiety without the need to further metabolize the compound. It was thought at the outset of this study that such (limited) metabolism of the thiol might be initiated by a sulphur lyase type enzyme. Such a reaction should be reversible and would involve the enzymatic nucleophilic addition of bisulphide to methacrylate (analogous to the abiotic reaction described above). At the same time it would have the potential to prepare 3-mercapto-2-methylpropionate from methacrylate and bisulphide. For the purpose of our studies we screened for phototrophic bacteria as these would be found in abundance in the type of environment in which the thiols are abiotically formed and because they have previously been shown to possess several novel sulphur metabolizing enzymes.[7] In this current communication the metabolic fate of the thiol is presented.

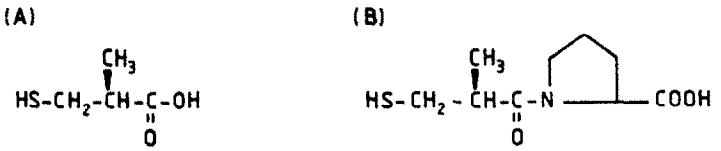


**Figure 1.** Structures of 3-mercapto-2-methylpropionic acid (A) and Captopril [*N*-(3-mercapto-2-D-methylpropionyl)-L-proline] (B).

[a]Present Address: Department of Microbiology, NIZO, Ede, The Netherlands.

*Author to whom correspondence should be addressed.

## Results and Discussion

*Isolation of phototrophic bacteria*

Within two days of initiating the selection procedure all of the test flasks, containing 3-mercapto-2-methylpropionate as sulphur source, showed good growth (thick, deep purple coloured culture). Growth was matched in colour and intensity by the sulphur 'free' control. After subculturing each of the test and control bottles to the respective fresh media and incubating under illumination for a further two days, growth was only observed in the case of tests. The original control bottles were also subcultured into fresh medium supplemented with $Na_2S \cdot xH_2O$. These positive controls grew, confirming that after the first subculture the cultures were S-limited. During repeated (4 times) subculturing to fresh medium only the bottles containing 3-mercapto-2-methylpropionate showed growth. This gave a positive impression that the phototrophic population was using the 3-mercapto-2-methylpropionate as sulphur source. Additional evidence for this was obtained by examining culture fluid filtrates for substrate and products (HPLC), revealing traces of methacrylate and a concomitant decrease in the level of the 3-mercapto-2-methylpropionate.

From this mixed population a pure culture, designated photoB, was obtained which only grew when 3-mercapto-2-methylpropionate (or an alternative S-source such as $Na_2S$) was included in the medium. No growth was detected when the cultures were incubated in the dark. In addition, growth of an illuminated culture ceased when it was transferred to the dark.

Strain photoB was sensitive to the concentration of 3-mercapto-2-methylpropionate in the medium, with concentrations in excess of 150 μM proving toxic to growth. Strain photoB could not grow with 3-mercapto-2-methylpropionate as sole carbon and sulphur source, nor on methacrylate as sole carbon source.

*Growth characteristics of strain photoB*

Figure 2 shows the anaerobic, light-dependent growth of strain photoB on malate (2 g/L) as the carbon source and 3-mercapto-2-methylpropionate (50 μM) as the sole sulphur source. Similar data were obtained with acetate as carbon source. Methacrylate accumulated in the medium (Figure 2); the concentration formed was always less than the concentration of 3-mercapto-2-methylpropionate used.

*Studies with washed whole cells of photoB*

The observation that methacrylate accumulated during growth of strain photoB in the presence of 3-mercapto-2-methylpropionate suggested that the bacterium might remove the sulphur group using a lyase type reaction. Washed whole cells of strain photoB, grown on malate with 3-mercapto-2-methylpropionate as sulphur source, only metabolized 3-mercapto-2-methylpropionate (Figure 3) when incubated in the presence of either light or acetate. Permeabilized cells of strain photoB gave exactly the same results. The data suggest that metabolism of 3-mercapto-2-methylpropionate is energy requiring, but not at the level of uptake of the sulphur compound.

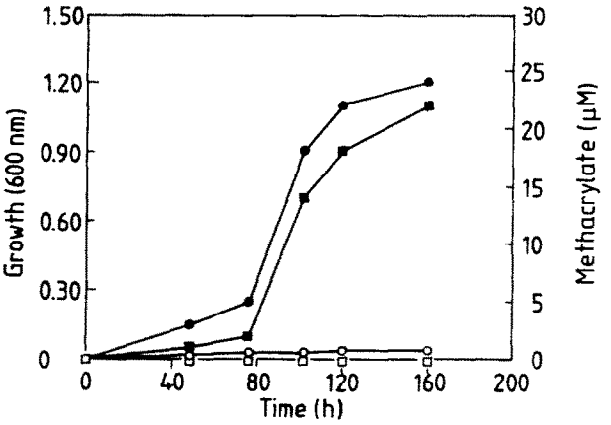


**Figure 2.** Anaerobic growth of strain photoB cells on malate with 3-mercapto-2-methylpropionate as sulphur source under illumination. (●) Growth with addition of 3-mercapto-2-methylpropionate; (○) growth in absence of added thiol compound; (■) accumulation of methacrylate during growth; (□) methacrylate concentrations in the absence of thiol compound.

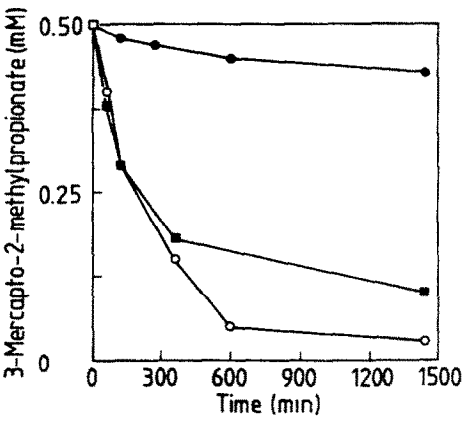


**Figure 3.** Anaerobic metabolism of 3-mercapto-2-methylpropionate by washed whole cells of strain photoB. All incubations were carried out at 30 °C in sealed 30 mL serum bottles containing 5 mL of washed whole cell suspensions in phosphate buffer (50 mM, pH 7.0) (○) illuminated; (●) dark; (■) in the dark with acetate as co-substrate.

Metabolism of methacrylate by washed whole cell and permeabilized cell preparations was likewise energy dependent (Figure 4).

*Studies with cell free extracts of strain photoB*

When dialysed cell free extracts prepared from cells of strain photoB were incubated with 3-mercapto-2-methylpropionate in a buffered environment, no utilization of substrate could be detected (Figure 5). If, however, CoA and ATP were included in the incubation mixture all the substrate was utilized (Figure 5). Similarly, the utilization of methacrylate by dialysed cell free extracts was CoA/ATP dependent (Figure 6).

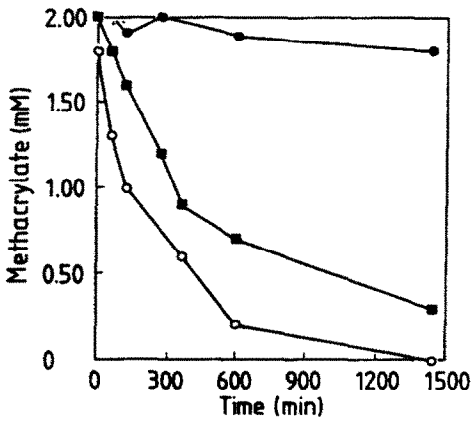


**Figure 4.** Anaerobic metabolism of methacrylate by washed whole cells of strain photoB. All incubations were carried out at 30 °C in sealed 30 mL serum bottles containing 5 mL of washed whole cell suspensions in phosphate buffer (50 mM, pH 7.0) (O) illuminated; (●) dark; (■) in the dark with acetate as co-substrate.

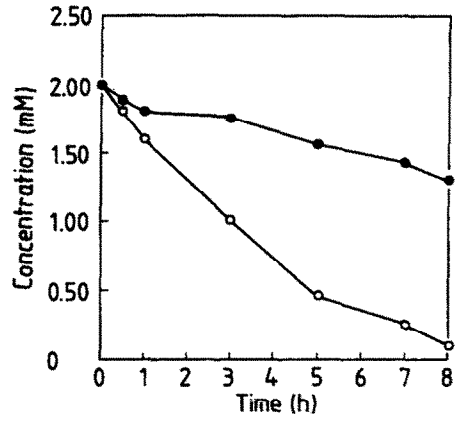


**Figure 5.** 3-Mercapto-2-methylpropionate utilization by dialysed cell free extract of strain photoB. Incubations were carried out anaerobically at 30 °C in sealed serum bottles containing cell free extract (21 mg protein) in 5 mL phosphate buffer (50 mM containing 10 mM $Mg^{2+}$, pH 7.0) in the presence (O) and absence (●) of ATP (5mM) and CoA (5 mM).

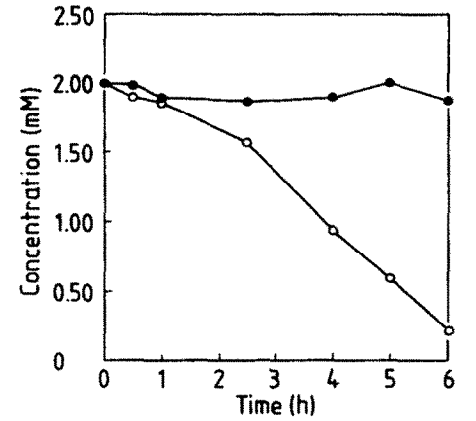


**Figure 6.** Methacrylate utilization by dialysed cell free extract of strain photoB. Incubations were carried out anaerobically at 30 °C in sealed serum bottles containing cell free extract (44 mg protein) in 5 mL phosphate buffer (50 mM containing 10 mM $Mg^{2+}$, pH 7.0) in the presence (O) and absence (●) of ATP (5 mM) and CoA (5 mM).

If 3-mercapto-2-methylpropionate is initially activated to 3-mercapto-2-methylpropionyl-CoA and this is subsequently metabolized by a lyase, the product would be methacrylyl-CoA which is a common intermediate in microbial valine metabolism.[8] Methacrylyl-CoA did not appear to accumulate in the incubations with 3-mercapto-2-methylpropionate, CoA and ATP. However, two other intermediates in the metabolism of valine[8] were detected in these incubations. Firstly, a peak with a retention time of 28 min was detected by HPLC analysis. This unknown was thought to possibly be methacrylyl-CoA. A sample was therefore made alkaline (pH 11) and re-analysed for methacrylate, without success (free CoA was detected confirming that the pretreated compound was a CoA derivative). Subsequent analysis of this sample by GC revealed a peak with the same retention time as β-hydroxyisobutyrate. The accumulation of the HPLC peak at 28 min, now identified as β-hydroxyisobutyryl-CoA, was transient and therefore the untreated samples were re-analysed (by GC) for β-hydroxyisobutyrate. The accumulation of this product is shown in Figure 7. It was also noteworthy that the appearance of β-hydroxyisobutyrate mirrored the disappearance of the HPLC peak with a retention time of 28 min (data not shown).

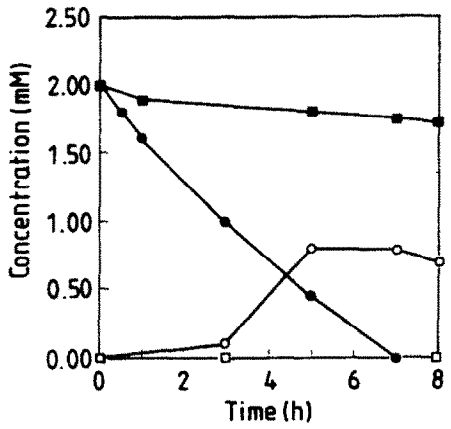


**Figure 7.** The accumulation of β-hydroxyisobutyrate during the Co-enzyme A dependent utilization of 3-mercapto-2-methylpropionate. Utilization of the thiol in the presence (●) and absence of CoA and ATP (■); accumulation of β-hydroxyisobutyrate in the presence (O) and absence (□) of CoA and ATP. Experiments were the same as outlined in Figure 5 except that 27 mg of protein was used.

From the foregoing a pathway, depicted in Figure 8, for the dissimilation of 3-mercapto-2-methylpropionate in strain photoB is proposed.

β-Hydroxyisobutyrate was not further metabolized. Initially the accumulation of β-hydroxyisobutyrate by the dialysed cell free extracts of strain photoB was thought to be due to the lack of $NAD^+$, required for the following reaction (formation of methylmalonate semialdehyde by β-hydroxyisobutyrate dehydrogenase).[8] This enzyme can be determined using a spectrophotometric assay[6] and this was attempted using dialysed cell free extracts of strain photoB. However, no activity was detected. Further investigation showed that strain photoB was unable to grow on β-

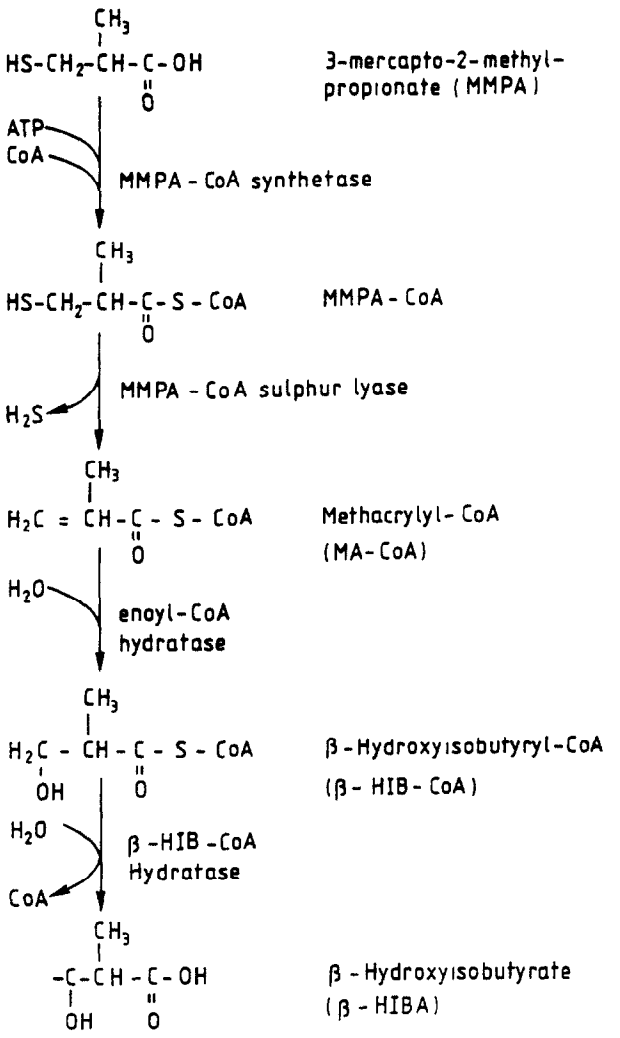


**Figure 8.** Proposed pathway for the anaerobic, light dependent utilization of 3-mercapto-2-methylpropionate as sole sulphur source by photoB cells.

hydroxyisobutyrate when supplied with either $Na_2S$ or 3-mercapto-2-methylpropionate as sulphur sources. Thus explaining why the utilization of 3-mercapto-2-methylpropionate is energy requiring. From the foregoing observations it would be expected that β-hydroxyisobutyrate would be the end product of the metabolism of 3-mercapto-2-methylpropionate during the growth of strain photoB. However, methacrylate was shown to accumulate (Figure 2) and not β-hydroxyisobutyrate. This anomaly presumably reflects the complexity of the enzyme control mechanism and clearly warrants further investigation.

The proposed metabolic pathway for the utilization of 3-mercapto-2-methylpropionate as the sole sulphur source by a photoheterotroph, depicted in Figure 8, shows similarity not only with the general bacterial metabolism of valine[8] but also with the specific aerobic degradation of a substrate analogue, 3-chloro-2-methylpropionate by a *Xanthobacter* sp.[6] Both of these compounds require CoA activation prior to removal of the functional group with further metabolism proceeding via β-hydroxyisobutyryl-CoA. The involvement of CoA-, ATP-dependent thiokinases in the photoheterotrophic assimilation of aliphatic and aromatic compounds is well established.[9,10]

## Experimental

### *Media and culture conditions*

The medium used contained per litre: $NH_4Cl$, 1 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $NaHCO_3$, 2 g; $MgCl_2 \cdot 6H_2O$; 1 mL concentrated buffer solution (155 g $K_2HPO_4$; 85 g $NaH_2PO_4 \cdot H_2O$ per litre, pH 7.0); 10 mL of a trace element solution according to Pfennig and Lippert[11] and 1 mL of a vitamin solution.[12] The carbon source was either malate or acetate (1–2 g/L). Unless otherwise stated 3-mercapto-2-methylpropionate (*ca* 0.1 mM) was the sole sulphur source. The pH of the medium was 7.0. Cells were cultivated in serum bottles (120 mL) which were filled with medium and incubated anaerobically (flushed with oxygen free nitrogen) at 30 °C, constantly illuminated by standard tungsten light bulbs (40 W) at a distance of 15–25 cm.

*Isolation of phototrophic bacteria.* Ditch water was first dialysed (72 h against 4 × 3 L distilled water) in an attempt to reduce the sulphur concentration. The dialysate was used to inoculate serum bottles (120 mL) which were filled with medium and incubated as outlined above. Incubations with no added sulphur served to check that the cultures were indeed sulphur limited and growth of the isolated organisms was also checked using $Na_2S \cdot xH_2O$ (*ca* 0.5 mM) as sole source of sulphur. Once growth was established the bacteria were regularly subcultured and a stable population maintained. A pure culture was selected from these populations using anaerobic agar deep dilution tubes, which were incubated in sealed, nitrogen flushed, glass desiccators at 30 °C under constant illumination (40 W). Subsequently pure colonies were obtained by streaking the cultures on to nutrient agar plates which were incubated in sealed, nitrogen flushed, glass desiccators at 30 °C under constant illumination. The purity of all subsequent cultures was checked by microscopic examinations and by streaking out cultures on to nutrient agar plates which were either incubated illuminated under anaerobic conditions or aerobically at 30 °C. The pure strain was maintained on nutrient agar slopes (pregrown by incubating in sealed, nitrogen flushed, glass desiccators at 30 °C under constant illumination) stored at 4 °C.

### *Cell preparations*

Cells were harvested, washed cell preparations made and cell free extracts prepared as previously described.[6] Cells were permeabilized using Triton X-100 (0.1 % over night at –20 °C).

Toxicity of the 3-mercapto-2-methylpropionate was investigated by attempting to grow the isolate at a range of concentrations (0–0.2 mM) and monitoring growth (absorbance at 600 nm).

*Analytical techniques*

3-Mercapto-2-methylpropionate was analysed using the GC method described for the analysis of 3-chloro-2-methylpropionate.[6] Analysis of β-hydroxyisobutyrate was also performed using this method except the following conditions were employed: oven 145 °C, injector port 165 °C. detector 180 °C. Methacrylate was detected by HPLC using a reversed phase column and 2 mM octylamine/75 mM phosphate buffer, pH 7.0 as mobile phase. The method of Ingebretson and Farstad[13] was employed for the analysis of CoA derivatives.

*Chemicals*

3-Mercapto-2-methylpropionate was prepared by dissolving 11 g of 3-acetylthio-2-methylpropionate in 100 mL 1 N NaOH and stirring for 3–4 h under pH-stat conditions at pH 12. Subsequently the reaction mixture was acidified to pH 1.5 using concentrated $H_2SO_4$. The resulting 3-mercapto-2-methylpropionate was extracted with dichloroethane (2 × 75 mL). The extract was then dried by the addition of anhydrous $MgSO_4$ and subsequently evaporated by distillation. 3-Mercapto-2-methylpropionate (chemical purity >99 %) was obtained. All other chemicals were obtained from commercial sources and were of the highest purity available.

## Acknowledgements

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