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# Biosynthesis of Myxothiazol Z, the Ester-analog of Myxothiazol A in *Myxococcus fulvus*☆

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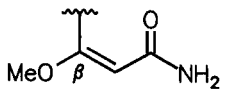
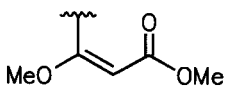
**Abstract**—Among myxobacteria only strains of *Myxococcus fulvus* are able to produce in addition to the well known myxothiazol A (**1**) an analog, myxothiazol Z (**2**), with an ester-type $\beta$-methoxyacrylate pharmacophore. Feeding experiments with labeled precursors established its biosynthesis from the amide (**1**) presumably via an *O*-methyl imidate (**3**). 

## Introduction

In our screening of myxobacteria for antifungal metabolites we frequently identify $\beta$-methoxyacrylate (MOA) inhibitors of mitochondrial respiration. The most widely distributed prototype myxothiazol A (**1**) is characterized by an amide-type pharmacophore (I), a bis-thiazole and a nonadienyl side-chain.[2] It is found in many strains of the genera *Angiococcus* and *Stigmatella*, frequently in *Myxcococcus*, and rarely in *Cystobacter* and *Corallococcus*.[3] The melithiazols are characterized by an ester-type pharmacophore (II), a mono or bisthiazole, and an ethyl or isopropyl derived side-chain.[4] So far they have only been found in the genera *Melittangium*[4] and *Cystobacter*.[5] Very rarely and exclusively in strains of *Myxococcus fulvus* we found the ester analog of myxothiazol A, which was designated myxothiazol Z (**2**). Among 64 strains only four strains produced myxothiazol Z (**2**) in addition to myxothiazol A (**1**) (Table 1). No strain was found to produce exclusively myxothiazol Z. On the other hand the melithiazols are only known with an ester-type pharmacophore II.




I




II

Myxothiazol Z (**2**) was first isolated from strain Mx f20, and its structure derived straight forward from the spectroscopic data given in the experimental part.[6] The absolute configuration of the three stereogenic centers is identical to that in myxothiazol A (**1**) according to interconversion experiments. Thus myxothiazol Z (**2**) could be synthesized *from* **1** via the iminoester **3** as well as transformed *into* **1** by treatment with aluminium amide.[7,8]

**Table 1.** Production of myxothiazol A (**1**) and myxothiazol Z (**2**) by *Myxococcus fulvus* in the presence of Amberlite XAD-16 adsorber resin (for cultivation, see Experimental)

| Strain | Myxothiazol A (**1**) (mg/l)[a] | Myxothiazol Z (**2**) (mg/l)[a] |
|---|---|---|
| Mx f333 | 11.0 | 10.0 |
| Mx f3338 | 20.0 | 14.0 |
| | 5.3[b] | 6.0[b] |
| Mx f20 | 13.0 | 3.7 |
| Mx f354 | 6.7 | 1.8 |
| Mx f364 | 13.0 | 6.8 |

[a] Determined by HPLC.
[b] In the absence of adsorber resin.

**Table 2.** Time course of appearance of labeled myxothiazol A (**1**) and myxothiazol Z (**2**) after feeding of [1-$^{14}$C]propionate and [$^{14}CH_3$]methionine (strain Mx f333/8; for cultivation conditions, see Experimental)

| Hours | [1-$^{14}$C]propionate (cpm)[a] | | [$^{14}CH_3$]methionine (cpm)[a] | |
|---|---|---|---|---|
| | **1** | **2** | **1** | **2** |
| 1 | 14 | 1 | 7 | 4 |
| 3 | 36 | 3 | 29 | 16 |
| 6 | 78 | 3 | 54 | 22 |
| 12 | 85 | 5 | 102 | 56 |

[a] Determined by TLC separation and counting.

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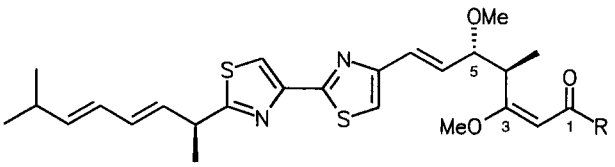



Myxothiazol A (**1**) R = $NH_2$
Myxothiazol Z (**2**) R = OMe

Recently myxothiazol Z has been described by Ahn et al. as co-metabolite KR025 of myxothiazol A in *Myxococcus fulvus*, strain JW025.[9] KR025 is presumably identical with myxothiazol Z although its optical rotation of $\alpha_D$=+152 is far off from the our value of $\alpha_D$=+79.2.

The relative amounts of myxothiazols A and Z formed by strain Mx f333 could not be influenced significantly by variation of the culture medium, pH value, or aeration rate. By clonal selection only clones producing mixtures of **1** and **2** were obtained, and only one clone, Mx f333/8, out of 21 tested gave slightly improved yields. The fact that no wild strain or clone was identified to produce exclusively myxothiazol Z (**2**) suggests that the amide **1** is formed first in the biosynthesis and transformed later in certain strains to the ester **2**.

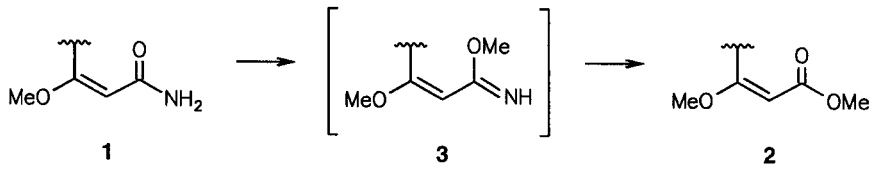
In support of this hypothesis pulse feeding experiments of clone Mx f333/8 with [1-$^{14}$C]propionate and [$^{14}CH_3$] methionine were performed (Table 2). Incorporation of propionate which is an indicator of denovo synthesis clearly favours amide **1** over ester **2**, whereas early incorporation of label from methionine into ester **2** indicates biotransformation of non-labeled **1** already present. Accordingly after feeding of [$^{13}CH_3$]methionine the [3,5-($O^{13}CH_3$)$_2$]- and [1,3,5-($O^{13}CH_3$)$_3$]-labeled myxothiazols **1** and **2** were obtained. This confirms earlier feeding experiments[10] and proves that the methyl group of the ester is introduced from methionine (Table 3, Exp. 1). The marked difference in $^{13}$C enrichments of **1** and **2** on feeding of young cultures reflects the time lag of ester formation when most of the label added was already consumed.

Feeding of labeled **1** and **2** from Exp. 1 clearly showed that, in addition to denovo synthesis, the amide **1** was transformed to ester **2** whereas the added ester **2** was recovered and the newly formed amide **1** was not labeled (Table 3, Exp. 2 and 3). This result is contrary to what is expected from an energetic point of view which requires activation of the amide group as in the chemical conversion.[6] This might be achieved by *O*-methylation to the iminoester **3** followed by hydrolysis to **2**. In support of this hypothesis [1-$O^{13}CH_3$]-labeled iminoester **3** was synthesized by methylation of **1** with [$^{13}$C]methyl iodide and silver oxide and fed to a strain Mx f333/8. After 8 h 30% was converted to myxothiazol Z (**2**) and 10% to myxothiazol A (**1**). However, this is not significantly different from 20% resp. 16% conversion by spontaneous hydrolysis in the sterile culture medium. Attempts to identify iminoester **3** as intermediate during cultivation of strain Mx f333/8 by HPLC/ESI-MS failed (Scheme 1).

**Table 3.** Feeding experiments of strain Mx f333 with $^{13}$C-labeled methionine, myxothiazol A (**1**) myxothiazol Z (**2**)

| Exp. | Compound fed | Atom % $^{13}$C | Products isolated | Atom % $^{13}$C[a] | | |
|---|---|---|---|---|---|---|
| | | | | 1-OMe | 3-OMe | 5-OMe |
| 1 | [$^{13}CH_3$]methionine | 99 | Myxothiazol A (**1**) | – | 18.2 | 18.6 |
| | | | Myxothiazol Z (**2**) | 3.2 | 3.2 | 2.9 |
| 2 | [3,5-($O^{13}CH_3$)$_2$]myxothiazol A (**1**) | 18.4[a] | Myxothiazol A (**1**) | – | 7.1 | 7.0 |
| | | | Myxothiazol Z (**2**) | 1.0 | 4.3 | 4.2 |
| 3 | [1,3,5-($O^{13}CH_3$)$_3$]myxothiazol Z (**2**) | 3.1[a] | Myxothiazol A (**1**) | – | 1.2 | 1.1 |
| | | | Myxothiazol Z (**2**) | 2.0 | 2.0 | 2.1 |

[a] Calculated from peak intensities normalized to the average intensity of all non-labeled proton bearing carbon atoms.




**Scheme 1.** Proposed biosynthesis of myxothiazol Z (**2**) from myxothiazol A (**1**).

A final proof of the proposed pathway can be expected from sequencing of the myxothiazol Z biosynthesis genes. According to recent results[11] the final step of myxothiazol A biosynthesis in *Stigmatella aurantiaca* is oxydative degradation of a terminal *N*-acyl amino acid residue to the carboxoamide. The ester producing strains of *Myxococcus fulvus* investigated here should have additional genes coding for a *O*-methyl transferase and a hydrolase.

## Experimental

### General methods

*Analytical TLC:* TLC aluminium sheets, silica gel Si 60

$F_{245}$, 0.2 mm (Merck); detection: UV absorption at $\lambda$=254 nm and spraying with vanillin/sulfuric acid (0.5 g vanillin in 100 ml of sulfuric acid/ethanol (8:2) and heating to 120°C). *Analytical HPLC:* YMC-C18 AQ, 5 μm, 125×2 mm, eluent: methanol/water (80:20), 0.3 ml/min; DAD detection. *Preparative HPLC:* if not specified otherwise Nucleosil 100 C-18, 7 μm, 2×25 cm, methanol/water 85:15, flow rate 17 ml/min. *NMR spectra*: Spectrometer AM-300 (Bruker, $^1$H: 300 MHz, $^{13}$C:s 75.5 MHz) or AM-400 spectrometer (Bruker, $^1$H: 400 MHz, $^{13}$C: 100 MHz), internal standard was the solvent. *UV spectra:* spectral photometer UV-2102 PC (Shimadzu). *IR spectra:* FT-IR spectrometer 20 DXB (Nicolet). *CI-mass spectra:* spectrometer MAT 95 (Finnigan). *Radioactivity:* Automatic TLC-Linear Analyser Tracemaster 20 (Berthold).

**Isolation and cultivation of strains**

All *Myxococcus fulvus* strains were isolated at the GBF according to a described procedure:[12] Mx f20 from rotting wood collected at Nuwara Elya (Sri Lanka) in 1973, Mx f333 from soil collected at Wind Gap, Pennsylvania (USA) in 1989, Mx f354 from soil in 1991 and Mx f364 from rotting wood in 1992, both samples collected on Izu Peninsula in the southwest of Tokyo (Japan; courtesy of Dr S. Yamanaka, Ajinomoto Comp.).

The culture medium IR1 consisted 0.1% soymeal, 0.4% peptone (Marcor), 0.2% technical starch, 0.1% $MgSO_4$×$7H_2O$, 0.05% $CaCl_2$×$2H_2O$, and 1.2% HEPES buffer.

For screening the strains were grown on 100 ml of IR1medium containing 2 ml of XAD-16 adsorber resin (Rohm & Haas) in 250 ml Erlenmeyer flasks on a gyratory shaker at 150 rpm and 30°C (standard conditions). Five days after inoculation the adsorber resin and cells were harvested by centrifugation and extracted batchwise with 1×10 ml of MeOH and 3×10 ml of acetone. The extract was evaporated to dryness, the residue dissolved in 2 ml of methanol and analyzed by HPLC, **1**:$t_R$=3.4 min, **2**:$t_R$=8.1 min.

**Production of myxothiazol A (1) and myxothiazol Z (2)**

A 100 l bioreactor (Giovanola) equipped with a flat-blade stirrer containing 65 l of the culture medium IR1 (without HEPES buffer), XAD-16 (Rohm & Haas) 1.5; Tegosipon (Goldschmidt) 0.0015; initial pH: 7.2) was inoculated with 5 l of a 3 day old preculture grown in IR1 medium without XAD and Tegosipon on a gyratory shaker (150 rpm, 30°C). The fermentation was run for 5 days at 30°C with an aeration rate of 14 l/min and a stirrer speed of 150 rpm. The pH was automatically maintained below 7.4 with 5% $H_2SO_4$. After 5 days of cultivation the adsorber resin was harvested by passing the culture broth through a process filter (0.12 $m^2$) of 210 μm mesh size. The adsorber resin was transferred to a short chromatography column and eluted with 6 l of methanol at a rate of 2 l/h. The eluate was concentrated and extracted with three volumes of ethyl acetate in three batches. Evaporation of the organic layer gave 10.4 g of an oily residue. For separation a column with 100 g silica gel (Merck Si 100, 60–100 μm, $\phi$ 5.5 cm) was conditioned with *t*-butylmethylether (BME)/*n*-heptane 1:2, the sample was applied to the column and eluted with BME/*n*-hexane 1:1 to give a crude fraction of 4.8 g containing myxothiazol Z (**2**) followed by elution with BME and BME/methanol 20:1 to give a fraction of 0.62 g containing myxothiazol A (**1**). The myxothiazol Z fraction was further purified by chromatography on Sephadex LH 20 (acetone) followed by MPLC on RP-18 (HD-Sil 30–60 μm, methanol/water 85:15) to give 246 mg (**2**). The myxothiazol A fraction was purified similarly by MPLC on RP-18 with methanol/water 80:20 to give 290 mg (**1**).

**Myxothiazol Z (2).** Colourless oil. TLC: $R_f$=0.61; dichloromethane/methanol 95:5; blue–gray coloration with vanillin–sulfuric acid reagent. $^1$H NMR ($CDCl_3$): $\delta$=4.95 (s, 2-H), 4.16 (dq, *J*=7.6, 7.0 Hz, 4-H), 3.80 (tbr, *J*=ca. 7.4 Hz, 5-H), 6.40 (dd, *J*=15.4, 7.2 Hz, 6-H), 6.56 (d, *J*=15.4 Hz, 7-H), 7.07 (s, 9-H), 7.84 (s, 12-H), 3.93 (dq, *J*=7.0 Hz, 14-H), 5.78 (dd, *J*=15.0, 7.0 Hz, 15-H), 6.16 (ddd, *J*=15.0, 10.2, 0.9 Hz, 16-H), 6.02 (ddd, *J*=15.2, 10.2, 1.2 Hz, 17-H), 5.68 (dd, *J*=15.2, 7.0 Hz, 18-H), 2.33 (dqq, *J*=7.0, 6.7, 6.7 Hz, 19-H), 1.00 (d, *J*=6.7 Hz, 20-$H_3$, and 19-$CH_3$), 1.20 (d, *J*=6.9 Hz, 4-$CH_3$), 1.54 (d, *J*=6.7 Hz, 14-$CH_3$), 3.59 (s, 3-$OCH_3$), 3.22 (s, 1-$OCH_3$), 3.65 (s, 5-$OCH_3$). $^{13}$C NMR ($CDH_3$): Within ±0.1 ppm as reported by Ahn et al.[9] Optical rotation (methanol): $\alpha_D^{22}$=+79.2 (*c*=1.4). UV (methanol): $\lambda_{max}$(log $\epsilon$)=232 (4.63), 312 (3.99). IR (KBr): 1711, 1624 $cm^{-1}$. DCI-MS (*i*-butane); *m/z* (%): 503 $[M+H]^+$ (100). $C_{26}$ $H_{35}$ $N_2$ $O_4$ $S_2$ $[M+H]^+$: Calcd 503.2038; found 503.2018 (HR-DCMS).

**Myxothiazol A (1).** Colorless needles (diethyl ether/heptane), mp 79°C. TLC (dichloromethane/methanol 95:5): $R_f$=0.19.

**Feeding of [1-$^{14}$C]propionate and [$^{14}CH_4$]methionine**

50 ml cultures of strain Mx f333/8 in IR1 medium were supplemented with 100 μCi [1-$^{14}$C]sodium propionate and 10 μCi [$^{14}CH_3$]methionine on the third day, and kept under standard cultivation conditions. After 1, 3, 6 and 12 h samples of 10 ml were taken, and the wet cell mass collected by centrifugation. The cell mass was extracted with 2 ml of methanol and three times with 2 ml of acetone. The extract evaporated to dryness, and the residue dissolved in 100 μl of methanol. According to scintillation counting each of these samples showed the activity of $7\times10^4\pm10^3$ cpm. Separation of 5 μl portions by TLC on with (1) ethyl acetate and (2) dichloromethane/methanol 95:5 up to $R_f$=0.6 gave separate bands for **1** ($R_f$=0.3) and **2** ($R_f$=0.75). The radioactivity of the bands was determined with a TLC analyser. The cpm's given in Table 2 represent 5% of a sample.

**[*O*$^{13}CH_3$]-labeled myxothiazol A (1) and myxothiazol Z (2).** Three shaking flasks with each 900 ml of a medium consisting of 1% probion, 0.5% starch, 0.05% $MgSO_4$×$10H_2O$, 0.05% $CaCl_2$×$2H_2O$, 1.2% of HEPES buffer, and 18 ml of Amberlite XAD-16 adsorber resin were inoculated with *Myxococcus fulvus*, strain Mx f333. At the second and third day each was fed with 38 mg [$^{13}CH_3$]methionin (99 % $^{13}$C). At the fourth day the adsorber resin was collected with a sieve, washed with water and eluted with 400 ml of methanol in a column of 3 cm

diameter. The extract was concentrated, diluted with 150 ml water and extracted three times with 100 ml of ethyl acetate. The organic layer was evaporated to give 0.38 g of crude extract which was separated as described above to yield 45 mg of myxothiazol A (**1**) and 7 mg of myxothiazol Z (**2**). For $^{13}C$ content see Table 3.

**Feeding of [*O*$^{13}$CH$_3$]myxothiazol A (1).** A 300 ml shaking flask culture of strain Mx f333/8 in the medium described above was fed with 6 mg of [O$^{13}$CH$_3$]-(**1**) dissolved in 1 ml methanol 2 days after inoculation. After days the culture was consecutively extracted with 100 ml and 2×50 ml of ethyl acetate. The organic layer was dried with magnesium sulfate and evaporated to 50 mg crude extract. After chromatography on Sephadex LH 20 with acetone gave 11 mg of myxothiazol A (**1**) and 8 mg of myxothiazol Z (**2**) with a purity of approximately 90%. For $^{13}C$ content see Table 2.

**Feeding of [*O*$^{13}$CH$_3$]myxothiazol Z (2).** According to the procedure described above 6 mg of [O$^{13}$CH$_3$]-(**2**) was fed and 10 mg of myxothiazol A (**1**) and 11 mg of myxothiazol Z (**2**) were isolated. For $^{13}C$ content see Table 2.

**[1-*O*$^{13}$CH$_3$]iminoester (3).** 50 mg of myxothiazol A (**1**), 1 g of [$^{13}$C]methyl iodide (99% $^{13}C$) and 0.5 g of silver oxide were stirred in a closed vial for 30 h at 70°C. The excess of methyl iodide was recovered by distillation, and the product mixture was separated by preparative HPLC on RP-18 (Eurosil Bioselect C 18, 10 μm, 16×250 mm, 70:30 acetonitrile/ammonium acetate buffer, 50 mmol, pH 7.0, gradient to 95:5) into 8.3 mg of iminoester **3**[8], 10.9 mg of *N*-methyl myxothiazol A[8] ($^1$H NMR in $CDCl_3$: $\delta$=2.80 dd, $J$=137.0, 5.0 Hz, NH$^{13}$CH$_3$), 5.4 mg of myxothiazol nitrile[8] and educt (**1**).

**Feeding of [1-*O*$^{13}$CH$_3$]iminoester (3).** 4 mg samples of (**3**) dissolved in 100 μl of methanol were added to a 50 ml shaking flask culture of strain Mx f333/8 on the third day after inoculation and 50 ml of sterile culture medium. After 8 h the culture and the reference were extracted exhaustively with ethyl acetate. The combined organic phases were evaporated to dryness and analysed for **1**, **2** and **3** content by HPLC (Nucleosil C-18, 5 μm, 2×125 mm, acetonitrile 10 mmol ammonium acetate buffer gradient from 66:34 to 95:5 in 15 min, then isoratic, $t_R$: **1**=5.8 min, *N*-methyl myxothiazol A=6.5 min, myxothiazol nitrile=9.0 min, **2**=8.4 min, **3**=18.5 min).

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