# Tetracenomycin M, a Novel Genetically Engineered Tetracenomycin Resulting from a Combination of Mithramycin and Tetracenomycin Biosynthetic Genes

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**Abstract:** The hybrid strain *Streptomyces glaucescens* Tü 49 (pGB 7) contains recombined genes of the tetracenomycin C (**4**) and the mithramycin (**6**) biosynthesis cluster. It was designed by the transformation of plasmid pGB 7 into the tetracenomycin producer *Streptomyces glaucescens* Tü 49. Plasmid pGB 7 carries the minimal polyketide synthase (PKS) genes of mithramycin biosynthesis (*mtmP*, *mtmK*, *mtmS*) along with its upstream (*mtmX*, which encodes a gene product of unknown function) and downstream flanking genes (the ketoreductase-coding *mtmT 1* and fragments of *mtmO 1*, which encodes an oxygenase). It was assumed that early intermediates of the well-known biosynthesis of **4**, such as tetracenomycin $F_2$ (**3**) or very similar molecules, are likely to also serve as intermediates of the biosynthesis of aureolic acid antibiotics, such as **6**. Thus, the enzymes of both parent biosynthetic pathways should be able to act on such intermediates, and several hybrid molecules were expected. Although the experiment resulted in new products, only the novel hybrid natural product tetracenomycin M (**1**), whose constitution was determined unambigously by spectroscopic methods, was obtained in larger amounts. The formation of **1** can be explained, if a combination of enzymes of both parent biosynthetic pathways is taken into consideration. When plasmid pGB 7 is transformed into *Streptomyces lividans* TK 21, that is, a strain which does not produce any secondary metabolites under our laboratory conditions, the production of SEK 15 (**2**) is observed. The latter is well known as the product of the minimal PKS of decaketides, and its exclusive production indicates that the aureolic acid antibiotics are constructed via a single decaketide chain and that the enzyme products of the flanking genes *mtmX*, *mtmT 1*, and *mtmO 1* cannot contribute in this second experiment.

**Keywords**
antibiotics · biosynthesis · gene technology · polyketides · tetracenomycins

## Introduction

The recently introduced term "combinatorial biosynthesis" refers to the design of novel hybrid or "unnatural" natural products by the manipulation of biosynthetic genes, mostly those for polyketides.[1] It is expected that combinatorial biosynthesis can be used as a strategy to produce batteries of novel natural products, and thus serve as an alternative method of generating molecular diversity.

In our studies of combinatorial biosynthesis and the design of novel polyketide antibiotics,[2] we have combined larger, multifunctional biosynthetic gene fragments[2] from a foreign polyketide producer with the biosynthetic genes of a host strain, preferably one of our well-studied wild-type strains. Other strategies are defined gene disruption and recombination experiments.[3] We have already reported that the combination of tetracenomycin or elloramycin genes with those of the urdamycin producer *Streptomyces fradiae* (the host strain) resulted in the production of two novel tetracenomycin-type antibiotics.[2a] Here we describe another novel genetically engineered tetracenomycin, tetracenomycin M (**1**), which resulted from the combination of selected genes from the mithramycin producer *S. argillaceus* with those of the tetracenomycin C producer *S. glaucescens* Tü 49.

## Results and Discussion

The hybrid strain *S. glaucescens* Tü 49 (pGB 7) was generated by heterologous expression of a 5.5 kb *Xho* I fragment from the cluster of mithramycin-production genes, cloned from

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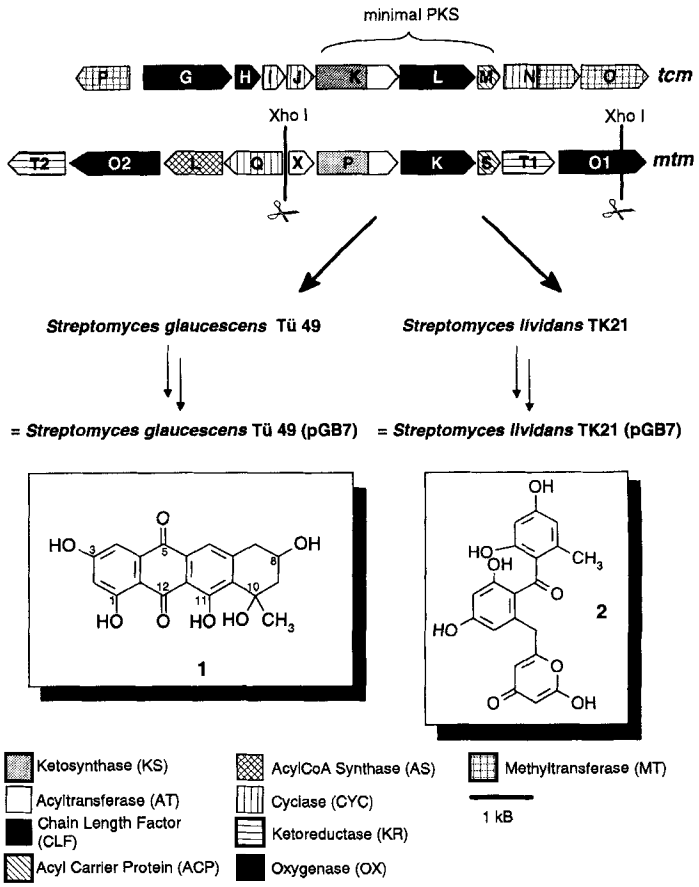
*S. argillaceus*,[4a] in the tetracenomycin producer *S. glaucescens* Tü 49. This fragment was subcloned into the *Sal*I site of pUC 19 and then transferred as a *Hind*III–*Eco*RI fragment into the same restriction sites of the bifunctional (*Streptomyces–Escherichia coli*) plasmid pWHM 3.[4b] The resulting construction (pGB 7) was introduced into *S. lividans* TK 21 and *S. glaucescens* Tü 49 by protoplast transformation. The *Xho* I fragment of *S. argillaceus*[4a] contains mainly the minimal polyketide synthase (PKS) genes, which comprise the ketosynthase/acyltransferase (KS/AT, *mtmP*), the chain-length factor (CLF, *mtmK*), and the acyl carrier protein (ACP, *mtmS*). Upstream of this minimal PKS is another gene (*mtmX*) that encodes an enzyme of unknown function, and downstream of the ACP are *mtmT1* and an incomplete gene (*mtmO1*), which encode a ketoreductase and an oxygenase, respectively (Scheme 1).[4a] When pGB 7 was introduced into *S. lividans* TK 21,[5] the decaketide SEK 15[6] (**2**) was produced in high yields (ca. 50 mg $L^{-1}$). This result confirms[7] our earlier hypothesis[8] that aureolic acid antibiotics such as mithramycin (**6**) or chromomycin are biosynthesized via a single decaketide chain, and not via two or more ketide chains.[9] Therefore, we assume that production of SEK 15 (**2**) in *S. lividans* TK 21 (pGB 7) resulted only from the expression of the *mtm* PKS genes, and that *mtmX*, *mtmT1*, and *mtmO1* did not contribute to this result. An additional experiment in which *S. lividans* TK 21 was transformed with the the same *mtm* gene set as in pGB 7, but with the omission of *mtmX*, also resulted in the production of SEK 15 (**2**).

**Abstract in German:** *Der Hybridstamm Streptomyces glaucescens Tü49 (pGB7) enthält rekombinante Gene aus dem Tetracenomycin-C(**4**)- und dem Mithramycin(**6**)-Biosynthesegencluster. Er wurde durch Transformation von Plasmid pGB7 in den Tetracenomycinproduzenten Streptomyces glaucescens Tü49 hergestellt. Plasmid pGB7 enthält die Minimal-Polyketidsynthase(PKS)-Gene der Mithramycin-Biosynthese (mtmP, mtmK, mtmS), die stromaufwärts durch mtmX, das ein Enzym unbekannter Funktion codiert, und stromabwärts durch mtmT1 sowie einen Großteil von mtmO1 flankiert sind. Die letztgenannten Gene codieren eine Ketoreduktase bzw. eine Oxygenase. Es wurde vermutet, daß frühe Intermediate der gut untersuchten Biosynthese von **4**, z. B. Tetracenomycin $F_2$ (**3**) oder ähnliche Verbindungen, auch als Intermediate der Biosynthese von Aureolsäure-Antibiotica wie **6** eine Rolle spielen. Daher sollten die Enzyme beider Elternbiosynthesewege solche Intermediate umsetzen können und zu etlichen Hybridprodukten führen. Tatsächlich lieferte das Experiment neue Produkte, aber nur eines in größerer Ausbeute: den neuen Hybridnaturstoff Tetracenomycin M (**1**), dessen Konstitution spektroskopisch zweifelsfrei aufgeklärt wurde. Die Bildung von **1** kann mit der Kombination von Enzymen beider Elternbiosynthesen erklärt werden. Wenn Plasmid pGB7 in Streptomyces lividans TK21 transformiert wird, d. h. in einen Stamm, der unter Laborbedingungen gewöhnlich keine Sekundärmetabolite produziert, entsteht SEK15 **2**, ein bekanntes Produkt der Minimal-PKS von Decaketiden. Daraus läßt sich schließen, daß Aureolsäure-Antibiotica nur über eine Decaketidkette biosynthetisiert werden und daß in diesem zweiten Experiment die Enzyme der flankierenden Gene mtmX, mtmT1 und mtmO1 keinen Beitrag leisten.*




Scheme 1. Parts of the mithramycin (*mtm*) biosynthetic gene cluster. Plasmid pBG 7 containing the *mtmXPKST1O1* genes from the mithramycin producer *S. argillaceus* was transformed into *S. lividans* and *S. glaucescens*. The former experiment caused the production of SEK 15 (**2**), and the latter the production of the new hybrid antibiotic tetracenomycin M (**1**). The complete biosynthetic gene cluster of tetracenomycin C (**4**) biosynthesis (*tcm*) is shown for comparison and for the sake of clarity (see text).

Since we have proposed that the biosynthesis of the aureolic acid antibiotics involves the same or similar intermediates as found for the biosynthesis of tetracenomycin C (**4**),[10, 11] one might expect to observe an effect of the *mtmX*, *mtmT1*, or *mtmO1* genes on the intermediates of tetracenomycin C biosynthesis upon introduction of pGB 7 into *S. glaucescens* Tü 49. Furthermore, the TcmJ and TcmN cyclases[11a] could prevent the formation of SEK 15 (**2**).

Indeed, fermentation of the hybrid strain *S. glaucescens* Tü 49 (pGB 7) yielded a new orange compound in large amounts. This compound is not produced by either of the parent bacterial strains *S. argillaceus* and *S. glaucescens* Tü 49, as was shown by careful TLC and HPLC analysis. Additional minor new products were not further analyzed, owing to their low yields. The molecular formula $C_{19}H_{16}O_7$ of the new orange compound was confirmed by FAB ([$M^+$] peak)and EI ([$M^+ - H$] peak) mass spectra. The NMR spectra (Table 1) showed all the expected signals, for example, three aromatic signals (a singlet at $\delta = 7.35$; two doublets at $\delta = 6.57$ and 7.08, with the typical *meta* coupling constant of 2.5 Hz), the $CH_2$-CH-$CH_2$ fragment of ring D (H,H COSY experiment), and all five OH signals. Two of the latter appear in the offset region, an indication of hydrogen bonding to an adjacent carbonyl group. The $^{13}C$ NMR spectrum shows signals for all 19 carbon atoms: one $CH_3$, two



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Table 1. $^1H$ and $^{13}C$ NMR data ($B_0 = 7.05$ T) of tetracenomycin M (**1**) ($\delta$ in ppm relative to internal TMS, multiplicities of the $^{13}C$ NMR spectra from the APT (attached proton test) experiment, [D$_6$]DMSO).

| Position | $^{13}C$ signal [a] | $^1H$ signal [b] ($J$ in Hz) |
|---|---|---|
| 1 | 164.4, s | – |
| 2 | 108.0, d | 6.57, d (2.5), 1H |
| 3 | 165.9, s | – |
| 4 | 108.8, d | 7.08, d (2.5), 1H |
| 4a | 135.0, s | – |
| 5 | 181.1, s | – |
| 5a | 130.8, s | – |
| 6 | 120.4, d | 7.35, s, 1H |
| 6a | 145.0, s | – |
| 7 | 40.7, t | 2.63, dd (16, 9), 1H; 3.02, dd (16, 4), 1H |
| 8 | 62.1, d | 3.97 m, 1H |
| 9 | 49.4, t | 1.71, d (13), 1H [c]; 2.06, d (13), 1H |
| 10 | 70.5, s | – |
| 10-CH$_3$ | 29.2, q | 1.72, s, 3H |
| 10a | 136.3, s | – |
| 11 | 161.6, s | – |
| 11a | 113.3, s | – |
| 12 | 189.8, s | – |
| 12a | 108.9, s | – |

[a] The carbons were further assigned from the long-range C,H couplings (HMBC spectrum, see Figure 1). [b] OH signals: 4.10, 4.85 (2), 12.00, 13.10; all broad singlets, exchangeable by $D_2O$. [c] Partially obscured.

$CH_2$, one aliphatic CH, three aromatic CH, and twelve quarternary, including the typical quinone carbonyls ($\delta = 181.1$ and 189.8, the latter chelated) of the anthraquinone chromophore, the presence of which is also indicated by the UV/Vis spectrum.[12] These structural elements were combined unambigously to give formula **1** by means of the observed long-range C–H couplings in the HMBC spectrum (Figure 1).

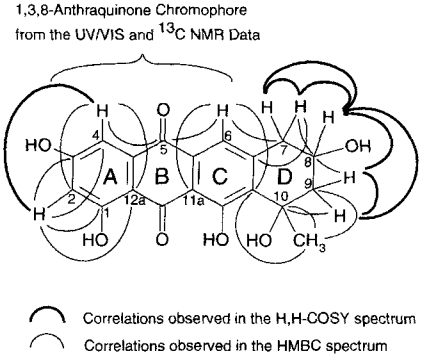
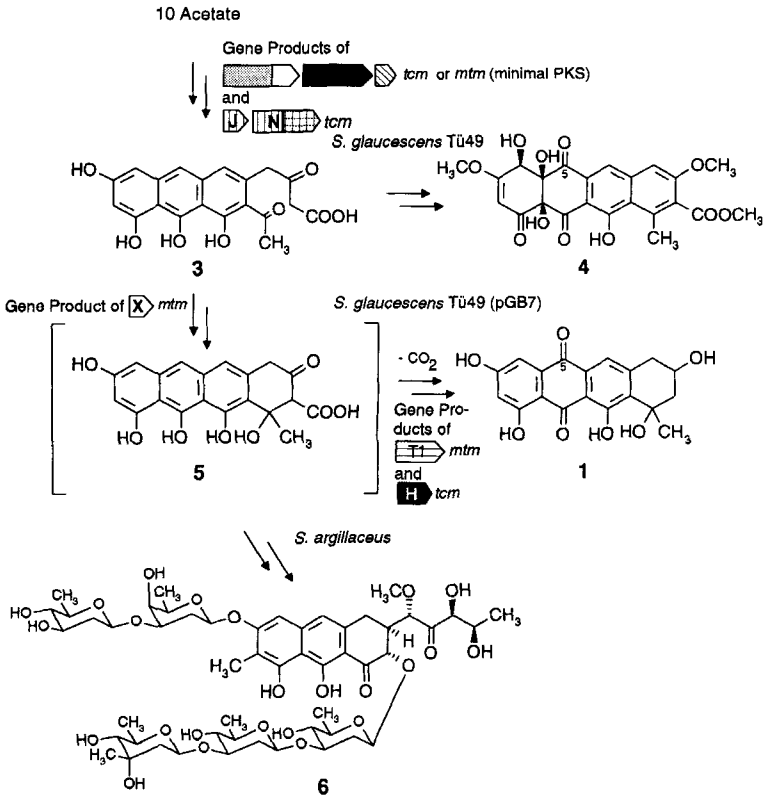



Figure 1. Structure elucidation of tetracenomycin M (**1**): observed direct H—H couplings (H,H COSY) and $^nJ_{C,H}$ long-range couplings (HMBC) in the NMR spectra. The 1,3,8-trihydroxyanthraquinone chromophore (rings A–C) follows from the $^{13}C$ NMR data and the UV/Vis spectrum.

The formation of tetracenomycin M (**1**) can be explained as the result of mixing of the two parent biosynthetic pathways by heterologous expression of pGB7 into the tetracenomycin C producer *S. glaucescens* Tü49 (Scheme 2). Tetracenomycin F$_2$ (**3**),[10a] the product of the *tcm* PKS, may also be an intermediate in the biosynthesis of mithramycin (**6**) and, therefore, a substrate of the enzymes produced by the *mtmX* and/or *mtmT1* genes. The two biosynthetic pathways may branch at the point




Scheme 2. Possible biosynthetic sequence leading to mithramycin (**6**) via the intermediates tetracenomycin F$_2$ (**3**) and **5**. Thus, the formation of the hybrid antibiotic tetracenomycin M (**1**), produced only by the recombinant strain *S. glaucescens* Tü49 (pGB7), may result from the combination of genes of the tetracenomycin (*tcm*) and those from the mithramycin (*mtm*) biosynthesis. The structure of tetracenomycin M (**1**) gives evidence for intermediate **5** in mithramycin biosynthesis as well as for the function of the *mtmX* gene product.

of closure of the fourth ring: in the biosynthesis of tetracenomycin C (**4**), this step is calalyzed by TcmI cyclase[10c] to give the aromatic product tetracenomycin F$_1$,[10a,c] which then is oxidized to the quinone tetracenomycin D$_3$ by TcmH oxygenase.[10,11] In this case the fourth ring is closed by an intramolecular aldol condensation, but in the biosynthesis of mithramycin (**6**), this fourth ring can be closed by the intramolecular aldol addition reaction that generates the tertiary alcohol functionality in **1** and the putative intermediate **5**. This step is most likely catalyzed by the product of *mtmX*, which therefore should be a cyclase (Scheme 2).[13] It is likely that other enzymes of the tetracenomycin C pathway in the hybrid strain *S. glaucescens* Tü49 (pGB7) act on intermediate **5**, and oxidize (through TcmH) and decarboxylate it to give **1**.[14a] Reduction of the C8 carbonyl group to the secondary alcohol must be catalyzed either by the ketoreductase MtmT1 or an unknown reductase from *S. glaucescens* (Scheme 2).[14b]

Tetracenomycin M (**1**) is a linear tetracyclic molecule that is not only a novel hybrid antibiotic but, in the context of the biosynthesis of mithramycin (**6**), is also a shunt product branching off from an early intermediate. Together with premithramycinone,[15] another recently discovered tetracyclic precursor or shunt product of the biosynthesis of **6**, tetracenomycin M (**1**) supports the hypothesis of such a tetracyclic intermediate in the biosynthesis of aureolic acid antibiotics.[8] In addition, the results described here also provide information about the function of MtmX.

Tetracenomycin M (**1**) is also another example supporting the belief that there is a high chance of creating new molecules by combining selected biosynthetic genes. In the present case, a major contribution came again[16] from an oxygenase, since it is likely that the TcmH oxygenase was able to act on an unnatural intermediate, probably compound **5**, which is very similar to the corresponding natural product, Tetracenomycin $F_1$. The use of large gene fragments for the construction of hybrid molecules[2] has again proved to be a fruitful approach in combinatorial biosynthesis.

## Experimental Section

**Bacterial strains, growth conditions, and DNA manipulation:** The mithramycin producer *S. argillaceus* ATCC 12956 was used as DNA donor. The tetracenomycin producer *S. glaucescens* Tü49 and *S. lividans* TK 21 were used as hosts. *Escherichia coli* TG1 rec0 was used as host for subcloning. The recombinant strain *S. glaucencens* (pGB7) was grown on agar plates at 28 °C (medium: 22 g $L^{-1}$ agar, 10 g $L^{-1}$ malt extract, 4 g $L^{-1}$ glucose, 4 g $L^{-1}$ yeast extract, 1 g $L^{-1}$ $CaCO_3$, 50 μg $mL^{-1}$ thiostreptone), *S. lividans* TK 21 was grown on R5 agar plates.[17] Plasmids pWHM3[4b] and pUC19 were used for subcloning. Conditions for DNA manipulation, protoplast generation and transformation were in accordance with standard procedures.[17] For the production of **1**, *S. glaucescens* (pGB7) was grown in triply baffled 250 mL Erlenmeyer flasks (each containing 100 mL culture liquid) in a rotary shaker (type GFL 3033) with a liquid medium (R2YENG)[18] in the presence of 25 μg $mL^{-1}$ thiostreptone for 120 h at 30 °C and 220 rpm.

**Isolation of tetracenomycin M (1)**: The culture was precipitated by addition of Celite, extracted twice with ethyl acetate at pH 7, and evaporated to dryness. The ethyl acetate residue was dissolved in $CH_2Cl_2$ and further purified on silica gel (silica gel SI 60, Merck, Germany). The silica gel column was washed with $CH_2Cl_2$, and the compounds were eluted with $CH_2Cl_2$/MeOH (9/1). The fractions containing tetracenomycin M were pooled, evaporated to dryness, and further purified on Sephadex LH-20 (1. acetone; 2. MeOH).

**Characterization of tetracenomycin M (1)**: For instruments and NMR methods, see ref. [2a]. $\alpha_{20}^{D}$ −32 ($c$ = 0.001 in MeOH); $R_f$ = 0.23 ($CH_2Cl_2$/MeOH, 18/1); UV (MeOH and MeOH/HCl): $\lambda_{max}$ ($\varepsilon$) = 440 (5900), 291 (10500), 270 (10400), 252 sh (9300), 223 (17800); UV (MeOH/NaOH): $\lambda_{max}$ ($\varepsilon$) = 509 (4400), 311 (10300); 253 (9000), 234 (11200), 219 (13300) nm; IR (KBr): $\tilde{\nu}$ = 3426, 2920, 2343, 1618 $cm^{-1}$; MS (FAB): $m/z$ = 356 [$M^+$]; MS (EI): $m/z$ (%) = 355 (100) [$M^+ - 1$], 338 (18) [$M^+ - H_2O$], 320 (100) [$M^+ - 2H_2O$]; HRMS for $C_{19}H_{14}O_6$: calcd 338.0790, found 338.0790; HRMS for $C_{19}H_{12}O_5$: calcd 320.0684, found 320.0686; NMR data: see Table 1.

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