

Contents

List of Figures	IV
List of Tables	V
Abbreviations	VI
1. Introduction	1
1.1. Natural products from <i>Streptomyces</i>	1
1.2. Hydrophilic Antibiotics	1
1.3. Antibiotics against Gram-negative bacteria	1
1.4. Isolation strategies for hydrophilic natural products	1
1.5. Aim of this thesis	1
2. Methods	2
2.1. Chemicals & Instruments	2
2.2. Strain Cultivation	4
2.2.1. Media	4
2.2.2. <i>Escherichia coli</i> K12 and <i>Bacillus subtilis</i> 168	4
2.2.3. General cultivation of <i>Streptomyces</i> sp. Tü2401	4
2.2.4. Batch Fermentation of <i>Streptomyces</i> sp. Tü2401	6
2.3. Bioassays	6
2.3.1. Agar Diffusion Assays	6
2.3.2. <i>yorB</i> Reporter Gene Assay	6
2.4. Sample Preparation and Extraction	7
2.4.1. Preparation of Culture Broth Extracts	7
2.4.2. Determination of Extraction Conditions	7
2.4.3. Processing of Fermentation Culture Broth	7
2.4.4. Agar Plate Extraction	8

2.5. Bioactivity-guided Isolation	8
2.5.1. Thin Layer Chromatography	8
2.5.2. Ion Exchange Chromatography	9
2.5.3. Trimethylsilane Derivatization and Gas Chromatography	9
2.5.4. Preparative HPLC	10
2.5.5. Analytical HPLC and Mass Spectrometry	11
2.6. Genome Analysis	11
3. Results & Discussion	12
3.1. Determination of Extraction Conditions	12
3.1.1. Comparison of Production Media	12
3.1.2. Extraction experiments	15
3.2. Chromatographic Separation	15
3.2.1. Reverse-Phase HPLC	15
3.2.2. Hydrophilic Interaction Liquid Chromatography	15
3.2.3. Ion Exchange Chromatography	17
3.2.4. Thin-Layer Chromatography	18
3.2.5. Gel-filtration	18
3.3. Dereplication	18
3.3.1. HPLC Mass Spectrometry	18
3.3.2. Trimethylsilane Derivatization and Gas Chromatography	18
3.4. Antibacterial Activity Spectrum	18
3.4.1. Activity against <i>Escherichia coli</i> K12	18
3.4.2. Activity against <i>Bacillus subtilis</i> 168	18
3.4.3. Extraction of yorB-inducing Compound	18
3.5. Species Determination and AntiSMASH Cluster Analysis	19
3.5.1. Phylogeny of Strain Tü2401	19
3.5.2. AntiSMASH Cluster Identification	19
Bibliography	25
A. Appendix	27
A.1. HPLC Methods	27
A.2. Chromatographic Data	30

A.3. Genomic Analysis	30
A.3.1. Phylogenetic Data	30

List of Figures

3.1.	K12 Bioassay results with media supernatants	13
3.2.	Chromatogram of medium supernatant screening	14
3.3.	Bioassay results of different OM medium extracts against <i>E. coli</i> K12	16
3.4.	Maximum likelihood tree of <i>Streptomyces</i> sp. Tü2401.	21
3.5.	Structures of known enediyne natural products	23
A.1.	UV-chromatogram of reverse extract fractionation with the Luna® NH ₂ column.	30
A.2.	Cluster and subcluster search results for the cluster located on contig 4.	32

List of Tables

2.1. Used chemicals and solvents	2
2.2. Components of HPLC systems	3
2.3. Column Specifications	3
2.4. Media components for the cultivation of strain Tü2401	5
2.5. Mobile phase compositions used for Thin-Layer Chromatography	8
2.6. Method for ion exchange chromatography	10
3.1. Bioassay results from agar-plate and standing culture extraction	20
A.1. Standard C18 screening method	27
A.2. Standard aminocolumn method	27
A.3. Aminocolumn method adapted for MS coupling	28
A.4. The standard ZIC-HILIC method	28
A.5. ZIC-HILIC method adapted for MS coupling	28
A.6. Screening method for HPLC-MS	29
A.7. Screening Method Polar-C18	29
A.8. Reverse Screening Method Polar-C18	29
A.9. Reference genomes for the construction of the phylogenetic tree	30
A.10. Single copy genomes for tree construction	31

3. Results & Discussion

3.1. Determination of Extraction Conditions

Preliminary experiments demonstrated, that the antibiotic compound could not be extracted with ethyl acetate. Only the medium supernatant of the complex media NL 200, NL 300, NL 500 and OM produced notable inhibition zones on plate bioactivity assays. Moreover, the compound was not retained in any matter, when the supernatant was separated by HPLC with a common C18 column. The compound is likely very hydrophilic, so that usual extraction and reverse-phase chromatography protocols are not applicable. Because of the difficulties involved in working with aqueous solvents, an extraction procedure involving organic solvents would be beneficial. Additionally, out of the four complex media, in which Tü2401 is able to produce the antibiotic, one with optimal properties has to be determined.

3.1.1. Comparison of Production Media

To determine the optimal production medium, Tü2401 was grown in each of the NL and OM media for either four or seven days. The obtained medium supernatants were filtrated by using $0.45\text{ }\mu\text{m}$ and $0.2\text{ }\mu\text{m}$ consecutively. OM supernatant proved to be the easiest to filtrate, while the NL media clogged the filters after few milliliters had passed through. Each filtrate was subjected to the standard bioassay against *Escherichia coli* K12 to determine the antibiotic activity. Pictures of the assay results are displayed in Figure 3.1.

Most samples cause notable ($\varnothing > 1\text{ cm}$) zones of inhibition in the agar diffusion assay. NL 500 after seven days and OM in both cases possess the greatest activity with inhibition zones greater than 1.5 cm. When cultivated in NL 300, Tü2401 seems to take longer than four days to synthesize the compound. NL 500 and OM supernatants were separated via an Agilent 1200 HPLC system equipped with a diode array detector (DAD) and electric

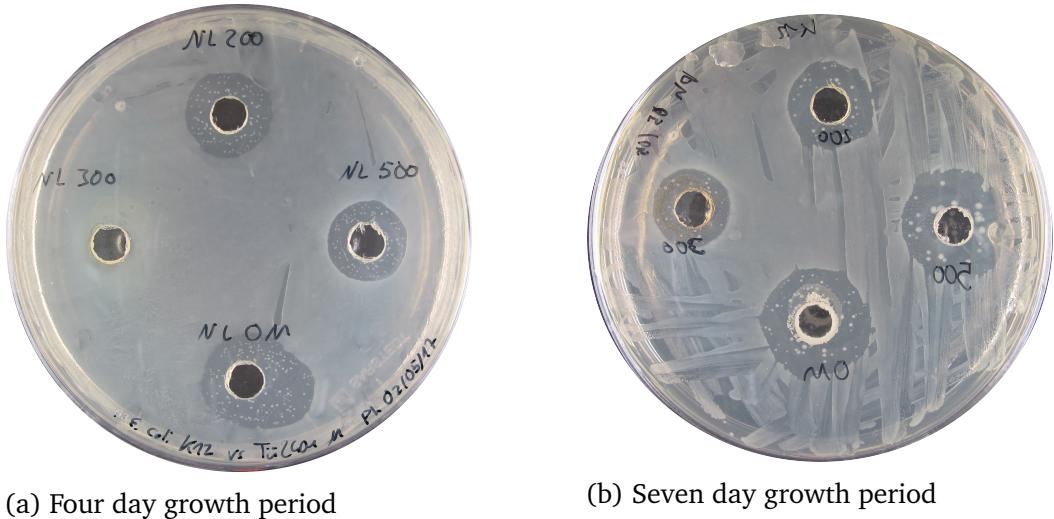


Figure 3.1.: K12 Bioassay results with media supernatants *Streptomyces* sp. Tü2401 was grown for either four (a) or seven days (b) in four different complex media. The filtrated medium supernatant was tested against *Escherichia coli* K12. Media: (Top) NL 200 (Left) NL 300 (Right) NL 500 (Bottom) OM.

light scattering detector (ELSD). A C18 column was used in combination with a 4.5 % to 100 % acetonitril screening gradient (see Table A.1). The chromatograms are shown in Figure

The screening chromatograms show, that most compounds in the medium supernatants are rather hydrophilic. No UV absorption or ELSD voltage peaks can be observed after 7 min. In the case of OM, only one peak is present at the time of injection. The OM supernatant seems to predominantly contain compounds, that can not be separated with a reverse-phase screening gradient on a C18 column. The hydrophilic, antibiotic compound of interest showed no retention under similar conditions. The use of OM as a production medium would therefore lead to fewer impurities in the hydrophobic spectrum. Combined with the visible antibacterial activity of the supernatant against *E. coli* K12 after only four days and the ease of preparation and filtration, OM was chosen as the default production medium for further experiments.

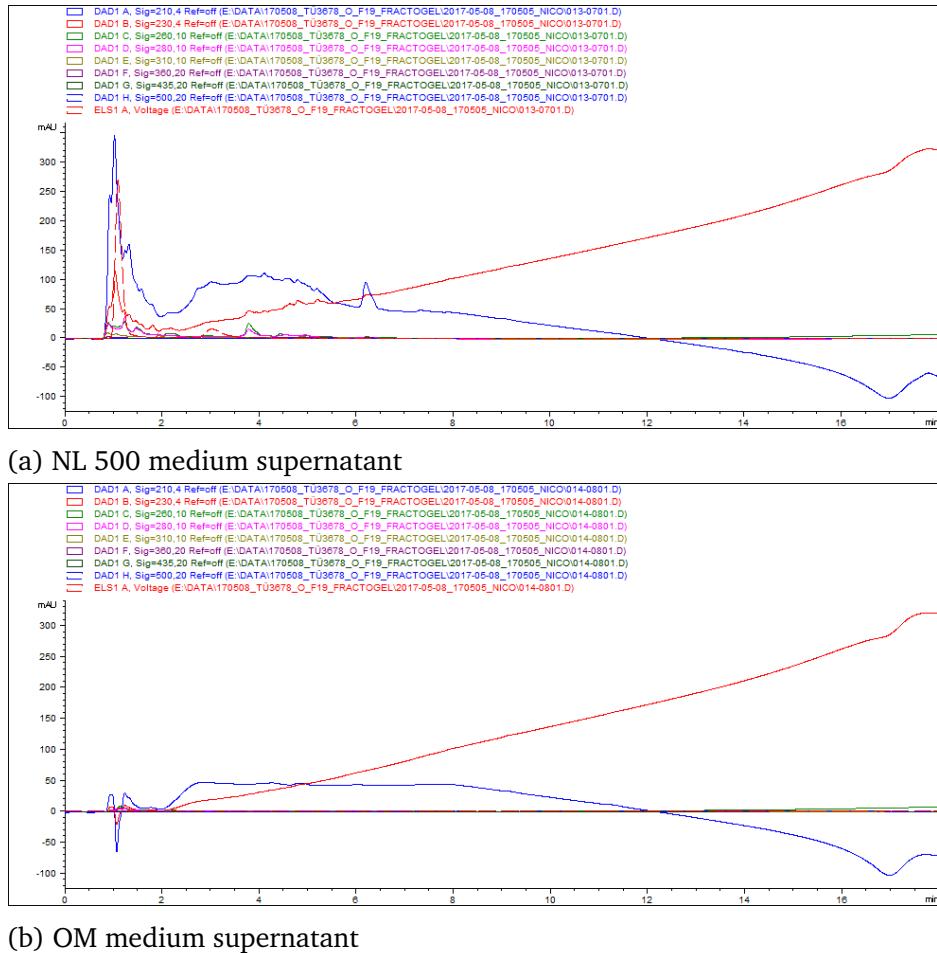


Figure 3.2.: **Chromatogram of medium supernatant screening.** 5 μL of medium supernatant were injected and separated on a C18 column. A screening gradient of 4.5 to 100 % acetonitrile was employed. UV absorption and ELSD voltage were measured.

3.1.2. Extraction experiments

Three organic solvents, which are immiscible with water, were tested for extraction of the antibiotic compound. Ethyl acetate, methyl acetate and ethyl formate. Additionally, the supernatant was adjusted to five different pH values ranging from 2 to 11. A hydrophilic molecule is likely to contain functional groups like amines or carboxylic acids, which are charged at certain pH-ranges. If the compound does too, the isoelectric point could pass by pH adjustment. Through reduced charge, the water-solubility could be lowered, enabling the extraction with an organic solvent.

OM medium supernatant was divided into three groups of five aliquots. The pH of each aliquot was adjusted to either 2, 5, 7, 9 or 11. Each group was then extracted with either ethyl acetate, methyl acetate or ethyl formate. Both phases were separated and tested for bioactivity against *E. coli* K12.

3.2. Chromatographic Separation

3.2.1. Reverse-Phase HPLC

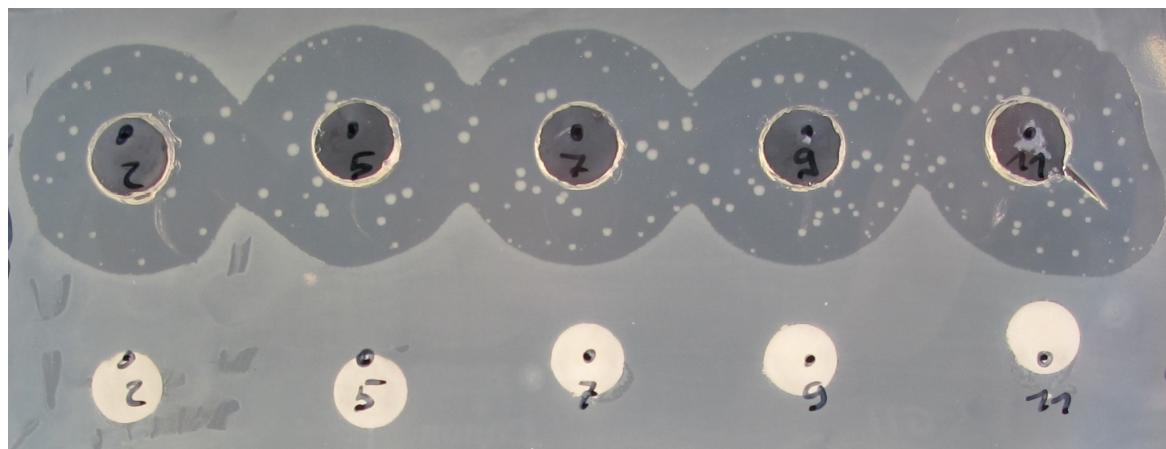
3.2.2. Hydrophilic Interaction Liquid Chromatography

- Short reiteration of HILIC
- Characteristics of NH₂ column
- Characteristics of ZIC-HILIC column

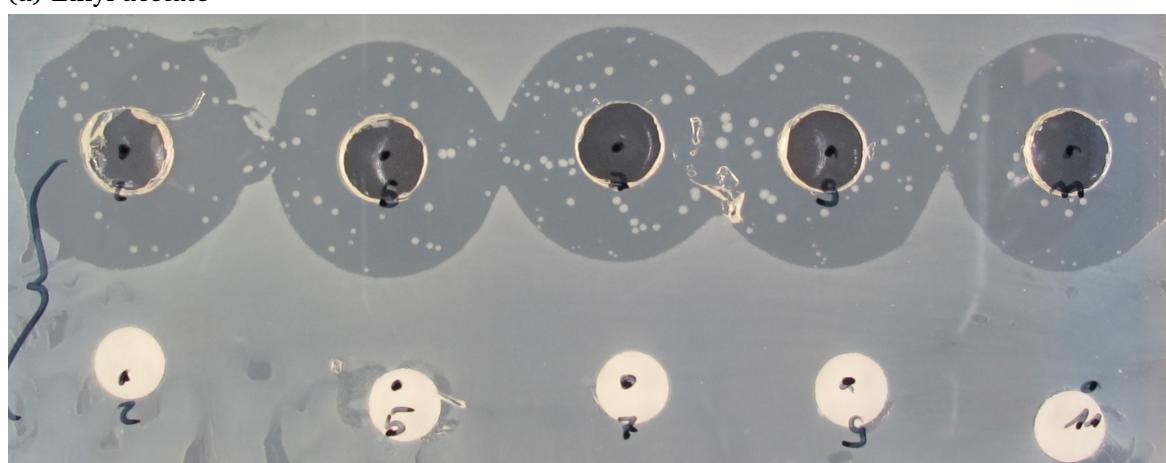
The Luna® NH₂ by Phenomenex features a silica matrix modified with 3-aminopropyl groups. The used model had a particle size of 3.5 µm and a pore size of 100 Å. The dimensions were 4.6 × 250 mm.

Luna NH₂ Column

Separation via the Luna® NH₂ column was performed with an isocratic method (see A.2). The mobile phase consisted of 80 % acetonitrile and 20 % water at a flow of 2.0 mL min⁻¹. Both solvents contained 0.1 % formic acid as a modifier. 50 µL of filtrated reverse extract at pH 11 were injected and fractions collected every minute. The fractions were subjected



(a) Ethyl acetate



(b) Methyl acetate



(c) Ethyl formate

Figure 3.3.: Bioassay results of different OM medium extracts against *E. coli* K12

Filtered supernatant of OM cultures of *Streptomyces* sp. Tü2401 were extracted with either (a) ethyl acetate, (b) methyl acetate or (c) ethyl formate. Sample-pH was adjusted prior to either 2, 5, 7, 9 or 11 (left to right). Both phases were tested separately. Upper row consists of 50 µL aqueous phase pipetted directly into wells. Lower row consists of 50 µL organic phase pipetted onto a sterile filter plate and placed on the agar.

to the standard agar-diffusion bioassay against *E. coli* K12. The chromatogram is shown in

With this method, the water-soluble compound could be separated from the injection peak. Fractions 5, 6 and 7 produced noticeable zones of inhibition, correlating to elution between 4 and 7 min. With the injection peak eluting at 1.5 min, a relative retention of 2.5 to 5.5 min could be achieved. The majority seems to have eluted between 3.5 and 5.5 min, since the inhibition zones of fractions 6 and 7 were the largest with a diameter of 1.7 cm. Fraction 5 only produced a diameter of 1.2 cm. In the UV-chromatogram, two baseline-separated peaks with distinct spectra were detected in the timeframe correlated to bioactivity. The first was detected at 5 min, the second at 6 min. The UV-spectra indicate that two bioactive compounds with similar retention times have eluted right between the fraction collector timeframes. A single compound should have resulted in the detection of a rather broad peak with long fronting. However, if the compound does not possess a UV-chromophore, an additional method of detection is needed.

An evaporative light scattering detector (ELSD) can be used to detect analytes without chromophores, as long as they are less volatile than the solvent.¹⁻⁴ The detector can be coupled to a standard HPLC system and provides additional analytical data. To eliminate elution differences between different systems, both the ELSD and the fraction collector were attached to the same system behind the UV-detector. Since ELS-detection is inherently destructive, a splitter was used to divide the flow. One fifth was directed to the ELSD, the rest to the fraction collector. With both

3.2.3. Ion Exchange Chromatography

Water-soluble compounds contain polar functional groups, which at certain pH ranges can even be charged. A pK_a value comparison of natural products in the Antibase2008 database revealed, that the majority of entries might be charged at pH 2–11.⁵ 44 % contained an acidic functionality, 17 % a basic one, and 9 % both.

3.2.4. Thin-Layer Chromatography

3.2.5. Gel-filtration

3.3. Dereplication

3.3.1. HPLC Mass Spectrometry

3.3.2. Trimethylsilane Derivatization and Gas Chromatography

3.4. Antibacterial Activity Spectrum

3.4.1. Activity against *Escherichia coli* K12

3.4.2. Activity against *Bacillus subtilis* 168

3.4.3. Extraction of yorB-inducing Compound

Tü2401 displayed positive results in the yorB-Assay when grown on ISP2 plates. However, all previously generated samples from liquid cultures only showed antibacterial activity. Three cultivation strategies were developed to induce production of the putative compound 2 (PC2):

1. Standing cultures could allow the formation of aerial mycelium at the medium surface. Thus, enabling the synthesis of PC2 and allowing extraction of the liquid medium. Two 500 mL flasks were each filled with 100 mL of liquid ISP2 medium and inoculated with 1 mL of a one-week old NL 410 shake culture. One flask was sealed with an ordinary aluminium cap (AC), the other one with an air-permeable foam cap (FC). Both flasks were cultivated for four days, before the medium was centrifuged and the supernatant filtrated. 50 mL aliquots of each flask were extracted with either BuOH or EtAc, and both phases were collected separately. The organic phases were dried at 40 °C and solved in 1 mL MeOH. Both phases of each flask were subjected to the yorB-Assay.
2. ISP2 agar plates were previously known to enable synthesis of PC2 and could be extracted with prior breakup. Ten round ISP2 agar plates were inoculated with

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100 µL of a one-week old NL 410 shake culture and incubated for four days. One half of the plates was extracted with BuOH, the other half was extracted with EtAc.

3. ISP2 agar plates could also be prepared with low-melting-point agarose (LMPA). This would allow melting of the plates at lower temperatures. Thus, allowing for easier extraction with reduced risk of thermal decomposition during the process. Six ISP2 LMPA agar plates were inoculated with 100 µL of a week-old NL 410 shake culture and incubated for four days. The plates were then melted at 70 °C and extracted with BuOH and EtAc.

All generated extracts and their respective aqueous phases were tested for bioactivity. The standard bioassay was used to determine the antibiotic activity against *E. coli* K12 and *B. subtilis* 168, while the yorB-Assay was used to test for the mode of action. Additionally, agar stamps of grown ISP2 and ISP2 LMPA plates were tested in the yorB-Assay. A summary of the results is shown in .

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3.5. Species Determination and AntiSMASH Cluster Analysis

3.5.1. Phylogeny of Strain Tü2401

3.5.2. AntiSMASH Cluster Identification

One cluster was detected on contig four and identified as a Type1-PKS-NRPS hybrid. It shows a 95 % cluster identity to the C-1027 biosynthetic gene cluster from *Streptomyces globisporus* C-1027 (MiBiG accession no. BGC0000965). Additionally, three homologous subclusters with 100 % identity were identified, which are associated with the synthesis of C-1027, Neocarzinostatin and Maduropeptin enediynes (see Figure A.2). The presence of this cluster could indicate, that the strain Tü2401 is capable of producing a compound similar to enediyne antibiotics.

Enediyne natural products are a class of cytotoxic bacterial compounds, which cause extensive DNA-damage.⁶⁻⁹ 11 different enediyne natural products are known, all of which feature either a bicyclo[7.3.0]dodecadienediyne core inside a nine-membered ring or a bicyclo[7.3.1]tridecadiyne core inside a ten-membered ring (Figure 3.5). The

Table 3.1.: Bioassay results from agar-plate and standing culture extraction. Samples were screened for antibiotic activity against *E. coli* K12 and *B. subtilis* 168 and for promotor induction in the yorB-Assay. Samples from standing cultures with foam cap (FC) or aluminium cap (AC), ISP2 agar plates (ISP2) and ISP2 agar plates with low-melting-point agarose (LMPA). Samples were extracted with butanol (BuOH) or ethyl acetate (EtAc) and tested alongside their respective aqueous phases (aq.). Legend: - No activity; + / ++ / +++ antibiotic activity with inhibition zone of 1 / 1.0-1.5 / >1.5 cm; n.e. result non-evaluable

Sample	Antibacterial			positive yorB
	<i>E. coli</i>	<i>B. subtilis</i>	yorB	
FC BuOH	-	-	-	-
FC BuOH aq.	-	-	-	-
FC EtAc	-	+++	++	-
FC EtAc aq.	-	-	-	-
AC BuOH	-	-	-	-
AC BuOH aq.	-	-	-	-
AC EtAc	-	-	+	-
AC EtAc aq.	-	-	-	-
ISP2 BuOH	-	n.e.	+++	-
ISP2 BuOH aq.	-	+	-	-
ISP2 EtAc	-	++	-	-
ISP2 EtAc aq.	+	n.e.	-	+
ISP2 plaque			++	++
LMPA BuOH	+	n.e.	+++	-
LMPA BuOH aq.	-	n.e.	-	-
LMPA EtAc	-	n.e.	+++	-
LMPA EtAc aq.	-	n.e.	-	-
LMPA plaque			-	-

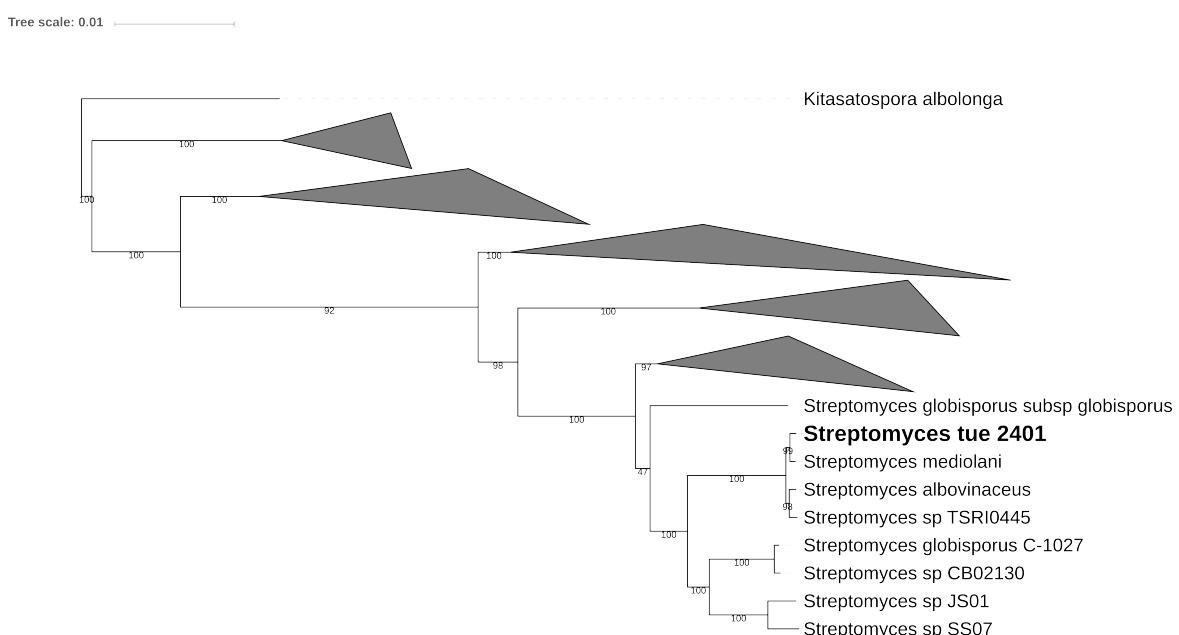


Figure 3.4.: Maximum likelihood tree of *Streptomyces* sp. Tü2401. The tree was constructed using a multiple-sequence alignment of 50 single-copy genes across 50 *Streptomyces* reference genomes. The node belonging to *Streptomyces* sp. Tü2401 is highlighted with bold text. Only the nine most closely related nodes and the outgroup are shown. Dark triangles represented hidden, collapsed nodes.

9-membered family includes neocarzinostatin, C-1027 and and maduropeptin. The 10-membered family includes calicheamicin γ_1^I , esperamicin A₁ and dynemicin A.⁶ Enediynes are potent cytotoxic agents because of their ability to induce DNA double-strand breaks.¹⁰ Electronic rearrangement of the carbocycle produces a benzenoid diradical, which abstracts hydrogen atoms from the DNA-backbone. The resulting radicals cause inter-strand crosslinks or react with molecular oxygen. While the ten-membered enediyne compounds were isolated as free-standing chromophores, most of the compounds in the nine-membered family were isolated in conjunction with a protective apoprotein.⁶ The nine-membered chromophore of C-1027, which was isolated from *Streptomyces globisporus* C-1027, is bound noncovalently to an 110 amino acid apoprotein.^{8,11-18} The isolated chromophore has been shown to be very unstable, whereas the holo C-1027 did not lose activity under the same conditions.¹⁴⁻¹⁶ The apoprotein binds specifically to the C-1027 chromophore, supposedly by hydrophobic pocket, which binds the benzoxazine side chain.^{15,19} The enediyne antibiotics neocarzinostatin and maduropeptin, which were also isolated from actinomycetes, feature highly specific and protective apoproteins as well.⁸

The homologies of the identified cluster could be an indicator, that the strain Tü2401 is capable of synthesizing an enediyne antibiotic with a nine-membered core and a corresponding apoprotein. The potent DNA-strand-breaking capabilities of this compound-class could induce the *yorB* reporter system of *Bacillus subtilis* 1S34 pHJS105-*yorB-lacZ2* in the *yorB*-induction assay. A number of compounds, which cause DNA double strand breaks and crosslinks are reported to induce the system, though none of them belong to the family of endiyne antibiotics.²⁰ Whether this is due to inactivity or to this family not having been tested in the assay is unclear, since the compound library is not publicly accessible. Nevertheless, an endiyne compound could be responsible for the induction.

The assay in Section 3.4.3 showed, that the *yorB* inducing compound is produced when the strain is grown on an ISP2 agar plate, but it could not be extracted via ethyl acetate or butanol. Only very low activity was retained in the aqueous phase. If the compound is indeed an endiyne, this loss of activity could be due to the high instability of the chromophore. The apoprotein could have been detached during the extraction and concentration process, which also included high temperatures of 40 to 60 °C. Combined with an incubation time of several days between extraction and assay, this could have led to the degradation of the chromophore below the sensitivity threshold. The high temperatures and long storage times also apply to the numerous HPLC-fractioning samples

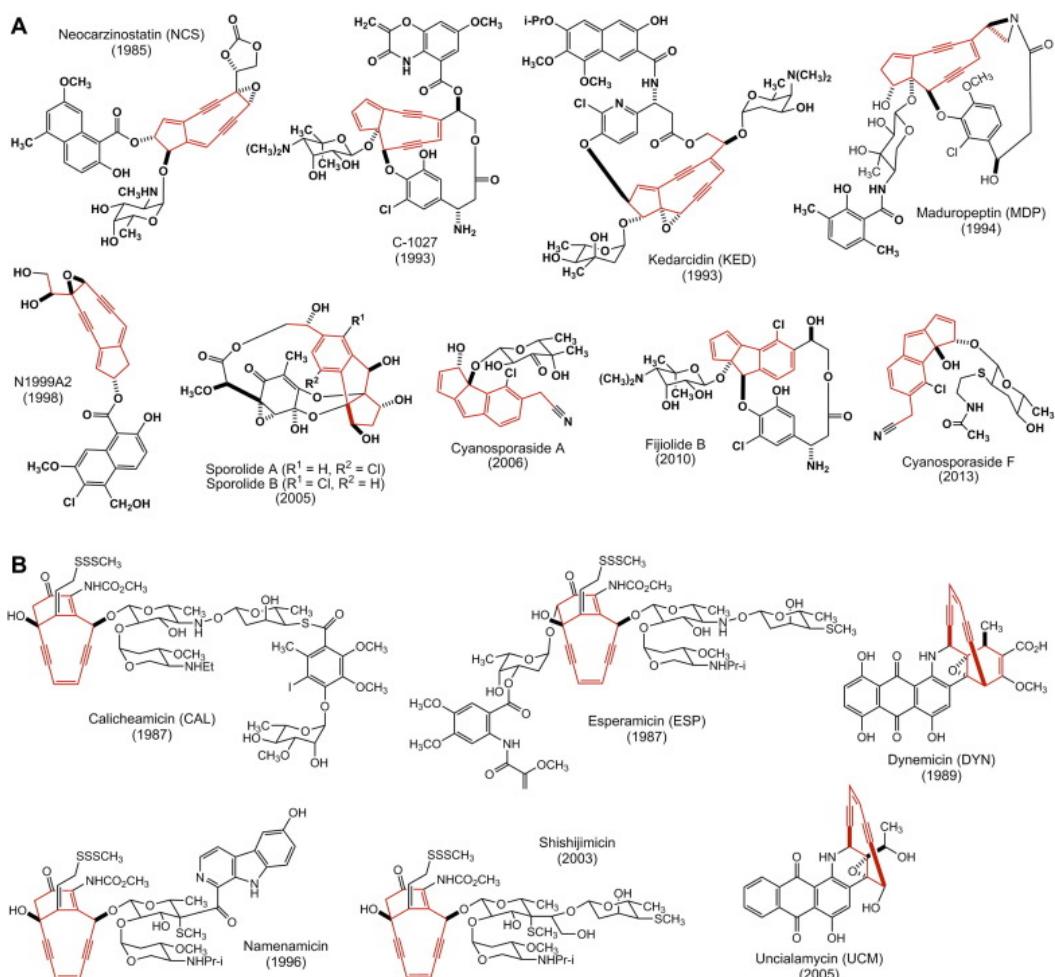


Figure 3.5.: **Structures of known enediyne natural products.** Enediyne cores are highlighted in red. (A) Compounds with nine-membered rings. (B) Compounds with ten-membered rings. The year of structure confirmation is displayed in parentheses. The sporolides, cyanosporolids and fijiolides do not contain an endiyene core, but are proposed to be derived from enediyne precursors. Reprinted from Shen *et al.* (2015) Copyright 2014 Elsevier Ltd.

subjected to the *yorB*-induction assay.

To verify this assumption, the putative endiyne compound has to be extracted by an adapted protocol, dereplicated and subjected to the assay in pure form. The C-1027 antibiotic protein from *S. globisporus* was precipitated from the medium supernatant by the addition of ammonium sulfate and purified by dialysis and column chromatography.¹⁷ The active chromophore could be extracted from the apoprotein with methanol at 0 °C.¹⁵ However, as of now, untreated medium supernatant samples of the strain Tü2401 did not induce the *yorB*-reporter system. The cultivation in liquid OM medium is probably not sufficient for production of the putative endiyene compound and the protocol would have to be adapted for the extraction from ISP2 agar plates. To circumvent this, other cultivation media could be employed and tested for *yorB*-induction. The holo endiyne-apoprotein complex should be stable enough for routine testing of media and media supernatants. The only alternative to optimizing production conditions would be heterologous expression of the biosynthetic cluster. For the endiyne compound family though, this has, as of now, only been partly achieved for the nine-membered endiyne neocarzinostatin.²¹

The isolation of the putative endiyne antibiotic would be a very promising target. Only eleven compounds of this class are known to this date, yet several members are in use or development as anticancer drugs with promising results.^{6,22} Isolation of a new endiyne compound could hold a strong promise for the discovery of a new anticancer drug lead structure.

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A. Appendix

A.1. HPLC Methods

Vielleicht wenn hier text steht

Table A.1.: Standard C18 screening method

Parameter	Value
Column	Nucleosil-100 C18 5 µm 150×3 mm
Solvents	A: Water + 0.1 % Formic acid B: Acetonitrile + 0.1 % Formic acid
Method	Gradient 5 - 100 % B for 15 min Plateau 100 % B for 3 min
Flow	0.85 mL min ⁻¹
Temperature	25 °C
Injection Volume	50 µL

Table A.2.: Standard aminocolumn method

Parameter	Value
Column	Luna NH2 5 µm 250×4.6 mm
Solvents	A: Water + 0.1 % Formic acid B: Acetonitrile + 0.1 % Formic acid
Method	Isocratic 80 % B for 20 min + 100 % A for 10 min
Flow	2 mL min ⁻¹
Temperature	25 °C
Injection Volume	50 µL

Table A.3.: Aminocolumn method adapted for MS coupling

Parameter	Value
Column	Luna NH ₂ 5 µm 250×4.6 mm
Solvents	A: Water + 0.1 % Formic acid B: Acetonitrile + 0.1 % Formic acid
Method	Isocratic 80 % B for 60 min.
Flow	0.5 mL min ⁻¹
Temperature	40 °C
Injection Volume	50 µL
Capillary Voltage	3500 V
Injector Temperature	350 °C
Target mass	400 m/z

Table A.4.: The standard ZIC-HILIC method

Parameter	Value
Column	ZIC-HILIC 3.5 µm 150×4.6 mm
Solvents	A: 10 mM Ammonium acetate B: Acetonitrile
Method	Isocratic 80 % B for 45 min.
Flow	0.8 mL min ⁻¹
Temperature	25 °C
Injection Volume	50 µL

Table A.5.: ZIC-HILIC method adapted for MS coupling

Parameter	Value
Column	ZIC-HILIC 3.5 µm 150×4.6 mm
Solvents	A: 10 mM Ammonium acetate B: Acetonitrile
Method	Isocratic 80 % B for 60 min.
Flow	0.5 mL min ⁻¹
Temperature	40 °C
Injection Volume	50 µL
Capillary Voltage	3500 V
Injector Temperature	350 °C
Target mass	400 m/z

Table A.6.: Screening method for HPLC-MS

Parameter	Value
Column	Nucleosil-100 5 μm 150 \times 3 mm
Solvents	A: Water + 0.1 % Formic acid B: Acetonitrile + 0.06 % Formic acid
Method	Gradient 0 - 100 % B for 15 min Plateau 100 % B for 2 min
Flow	0.4 mL min ⁻¹
Temperature	40 °C
Injection Volume	2.5 μL
Capillary Voltage	3500 V
Injector Temperature	350 °C
Target mass	400 m/z

Table A.7.: Screening Method Polar-C18

Parameter	Value
Column	Kinetex Polar-C18 2.6 μm 150 \times 4.6 mm
Solvents	A: Water + 0.1 % Formic acid B: Acetonitrile + 0.1 % Formic acid
Method	Gradient 5 - 100 % B for 20 min Plateau 100 % B for 6 min
Flow	1.2 mL min ⁻¹
Temperature	50 °C
Injection Volume	50 μL

Table A.8.: Reverse Screening Method Polar-C18

Parameter	Value
Column	Kinetex Polar-C18 2.6 μm 150 \times 4.6 mm
Solvents	A: Water + 0.1 % Formic acid B: Acetonitrile + 0.1 % Formic acid
Method	Gradient 100 - 5 % B for 20 min Plateau 100 % B for 6 min
Flow	1.2 mL min ⁻¹
Temperature	50 °C
Injection Volume	50 μL

A.2. Chromatographic Data

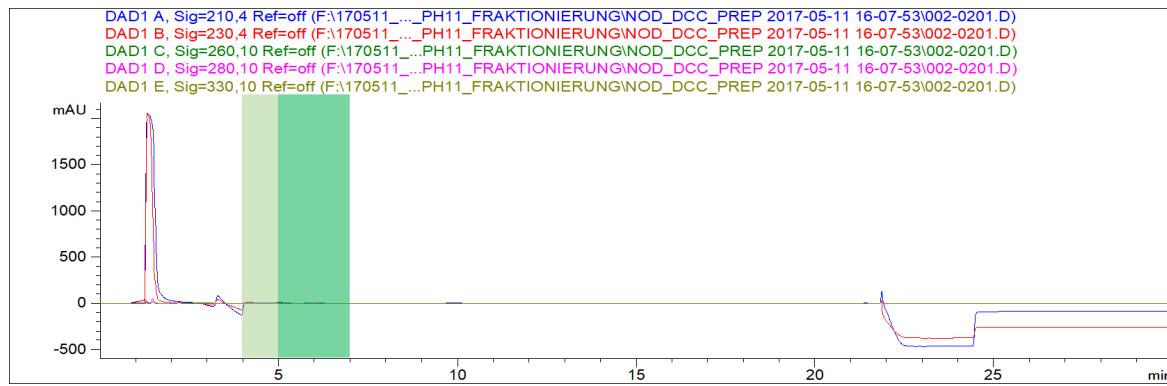


Figure A.1.: UV-chromatogram of reverse extract fractionation with the Luna[®] NH₂ column. testi

A.3. Genomic Analysis

A.3.1. Phylogenetic Data

Table including the reference genomes

Table A.9.: Reference genomes for the construction of the phylogenetic tree

1	2	3
1	2	3

Table including the identified single-copy genes

Table A.10.: Single copy genomes for tree construction

TIGR ID	Description
00008	translation initiation factor IF-1
00033	chorismate synthase
00060	ribosomal protein uL18
00062	ribosomal protein bL27
00118	acetolactate synthase, large subunit, biosynthetic type
00151	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
00171	3-isopropylmalate dehydratase, small subunit
00302	phosphoribosylformylglycinamidine synthase, purS protein
00355	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
00382	ATP-dependent Clp protease, ATP-binding subunit ClpX
00431	tRNA pseudouridine(55) synthetase
00484	translation elongation factor G
00615	recombination protein RecR
00631	exinuclease ABC subunit B
00708	cob(I)yrinic acid a,c-diamide adenosyltransferase
00962	ATP synthase F1, alpha subunit
00981	ribosomal protein uS12
01009	ribosomal protein uS3
01021	ribosomal protein uS5
01022	ribosomal protein bL36
01024	ribosomal protein bL19
01029	ribosomal protein uS7
01030	ribosomal protein bL34
01032	ribosomal protein bL20
01039	ATP synthase F1, beta subunit
01044	ribosomal protein uL22
01049	ribosomal protein uS10
01050	ribosomal protein uS19
01067	ribosomal protein uL14
01083	endonuclease III
01134	amidophorphoribosyltransferase
01162	phosphoribosylaminoimidazole carboxylase, catalytic subunit
01164	ribosomal protein uL16
01169	ribosomal protein uL1
01171	ribosomal protein uL2
01393	elongation factor 4
01980	FeS assembly protein SufB
02013	DNA-directed RNA polymerase, beta subunit
02027	DNA-directed RNA polymerase, alpha subunit
02156	phenylacetate-CoA oxygenase, PaaG subunit
02157	phenylacetate-CoA oxygenase, PaaH subunit
02952	RNA polymerase sigma-70 factor
03188	phosphoribosyl-ATP diphosphatase
03450	inositol 1-phosphate synthase
03631	ribosomal protein uS13
03632	ribosomal protein uS11

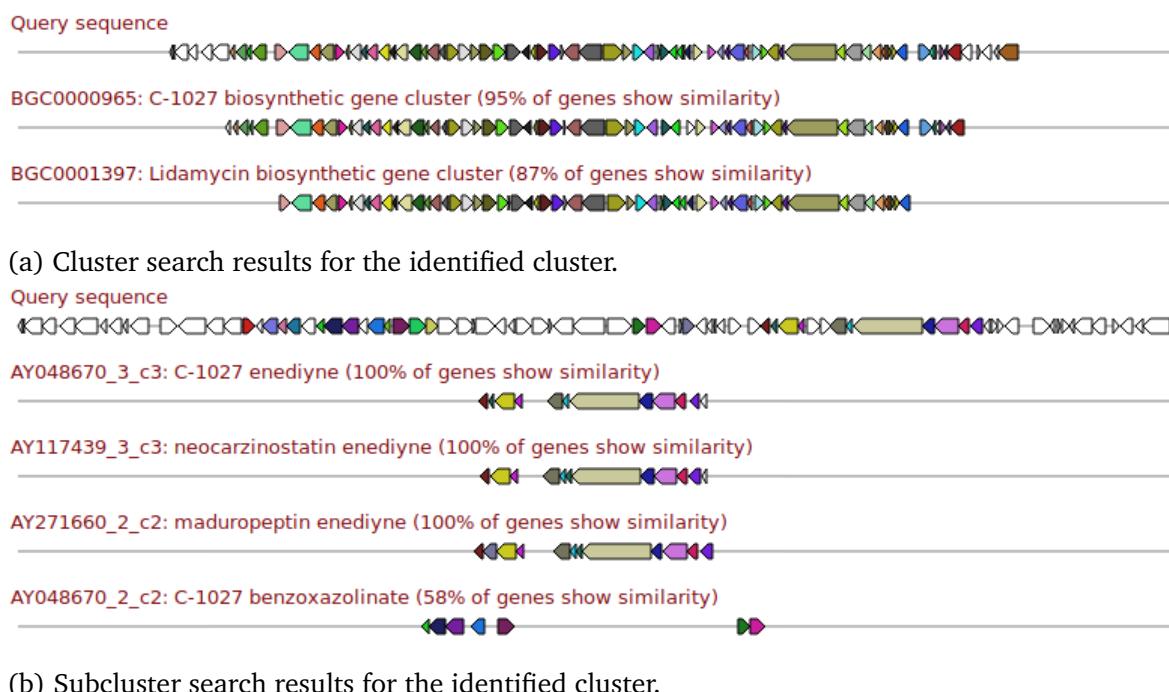


Figure A.2.: Cluster and subcluster search results for the cluster located on contig 4. The 160 kb contig was submitted to AntiSMASH with the ClusterFinder option. Only the search results with the highest similarities are shown.