

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	2
2.2.1 Media	2
2.2.2 <i>Escherichia coli</i> K12 and <i>Bacillus subtilis</i> 168	5
2.2.3 General cultivation of <i>Streptomyces</i> sp. Tü2401	5
2.2.4 Batch Fermentation of <i>Streptomyces</i> sp. Tü2401	5
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	6
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	7

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	10
2.6 Genome Analysis	10
3 Results & Discussion	12
3.1 Determination of Extraction Conditions	12
3.1.1 Comparison of Production Media	12
3.1.2 Extraction experiments	13
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	19
3.3 Dereplication	19
3.3.1 HPLC Mass Spectrometry	19
3.3.2 Trimethylsilane Derivatization and Gas Chromatography	19
3.4 Antibacterial Activity Spectrum	19
3.4.1 Activity against <i>Escherichia coli</i> K12	19
3.4.2 Activity against <i>Bacillus subtilis</i> 168	19
3.4.3 Induction of the <i>yorB</i> Reporter System	19
3.5 Bioinformatic Analysis	23
3.5.1 Phylogeny of Strain Tü2401	23
3.5.2 AntiSMASH Cluster Identification	23
Bibliography	27
A Appendix	30
A.1 HPLC Methods	30
A.2 Chromatographic Data	33

A.3 Genomic Analysis	33
A.3.1 Phylogenetic Data	33

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	Results of the <i>yorB</i> reporter system bioassay with strain Tü2401	21
3.5	Maximum likelihood tree of <i>Streptomyces</i> sp. Tü2401.	23
3.6	Structures of known enediyne natural products	25
A.1	UV-chromatogram of reverse extract fractionation with the Luna® NH ₂ column.	33
A.2	Cluster and subcluster search results for the cluster located on contig 4.	35

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	4
2.5	Mobile phase compositions used for Thin-Layer Chromatography	8
2.6	Method for ion exchange chromatography	9
3.1	Known inducers of the <i>yorB</i> reporter system	20
3.2	Bioassay results from agar-plate and standing culture extraction	22
A.1	Standard C18 screening method	30
A.2	Standard aminocolumn method	30
A.3	Aminocolumn method adapted for MS coupling	31
A.4	The standard ZIC-HILIC method	31
A.5	ZIC-HILIC method adapted for MS coupling	31
A.6	Screening method for HPLC-MS	32
A.7	Screening Method Polar-C18	32
A.8	Reverse Screening Method Polar-C18	32
A.9	Reference genomes for the construction of the phylogenetic tree	33
A.10	Single copy genomes for tree construction	34

2 Methods

2.1 Chemicals & Instruments

All chemicals and solvents were supplied by Merck, if not specified otherwise. Vendors for specific solvents are listed in Table 2.1. Water was purified prior to use by a MilliQ filtration system.

Table 2.1: Used chemicals and solvents

Supplier	Chemical
J. T. Baker	Acetonitrile Chloroform
Alfa Aesar	Methyl acetate
Fisher Chemicals	Ethyl acetate

High performance liquid chromatography (HPLC) systems were manufactured by Agilent. The components of the HPLC systems are listed in Table 2.2. Detailed specifications of used HPLC-columns are listed in Table 2.3.

2.2 Strain Cultivation

2.2.1 Media

All media were prepared by dissolving the components listed in Table 2.4 in MilliQ-H₂O and adjusting the pH with NaOH and HCl. For solid media, 2 % (w/v) agar was added. Media were sterilized by autoclaving at 121 °C and 230 kPa for 15 min. Fluid media were stored at ambient temperature, solid media at 8 °C.

Table 2.2: Components of HPLC systems

	Component	Description
Agilent 1100 Series	G1322A	Degasser
	G1311A	Quaternary Pump
	G1313A	Autosampler
	G1316A	Column Compartment
	G1315B	Diode Array Detector
Agilent 1200 Series	G1379B	Degasser
	G1312A	Binary Pump
	G1367B	Autosampler
	G1330B	Thermostat
	G1316A	Column Compartment
	G1315B	Diode Array Detector
Agilent 1260 Infinity	G4225A	Degasser
	G1312C	Binary Pump
	G1329B	Autosampler
	G1330B	Thermostat
	G1316A	Column Compartment
	G1315D	Diode Array Detector

Table 2.3: Column Specifications

Manufacturer	Line	Type	Dimensions
Merck	SeQuant®	ZIC®-HILIC 3.5 µm 100 Å	150 × 4.6 mm
Phenomenex	Luna®	NH ₂ 5 µm 100 Å	250 × 4.6 mm
	Kinetex®	Polar-C18 2.6 µm 100 Å	150 × 4.6 mm
Dr. Maisch	Nucleosil-100	C18 5 µm 100 Å	100 × 2.5 mm

Table 2.4: Media components for the cultivation of strain Tü2401. All amounts are calculated for one liter of MilliQ-H₂O. The pH was adjusted with NaOH and HCl.

Name	pH	Component	Amount	Vendor
LB		Yeast extract	5 g	Roth
		Tryptone	10 g	Roth
		NaCl	10 g	Roth
ISP2	7.3	Yeast extract	4 g	Oxoid
		Malt extract	10 g	Thermo Fisher
NL 200	7.5	D(-)Mannitol	20 g	Merck
		Cornsteep Powder	20 g	Sigma-Aldrich
NL 300	7.5	D(-)Mannitol	20 g	Merck
		Cotton Seed	20 g	Pharmamedia
NL 410	7.0	Glucose	10 g	Roth
		Glycerol	10 g	Acros Organics
		Oatmeal	5 g	Holo Bio Hafergold
		Soymeal	10 g	Hensel
		Yeast extract	5 g	Oxoid
		Bacto Casaminoacids	5 g	Difco
		CaCO ₃	1 g	Merck
NL 500	8.0	Starch	10 g	Roth
		Glucose	10 g	Roth
		Glycerol	10 g	Acros Organics
		Fish Meal	15 g	Sigma-Aldrich
		Sea Salts	10 g	Sigma-Aldrich
OM	7.3	Oatmeal	20 g	Holo Bio Hafergold
		Trace metal mix	5 mL	
Trace metal mix		CaCl ₂ · 2 H ₂ O	3 g	
		Fe ³⁺ citrate	1 g	
		MnSO ₄ · H ₂ O	200 mg	
		ZnCl ₂	100 mg	
		CuSO ₄ · 5 H ₂ O	25 mg	
		Na ₂ B ₄ O ₇ · 10 H ₂ O	20 mg	
		CoCl ₂ · 6 H ₂ O	4 mg	
		Na ₂ MoO ₄ · 2 H ₂ O	10 mg	

2.2.2 *Escherichia coli* K12 and *Bacillus subtilis* 168

Escherichia coli K12 and *Bacillus subtilis* 168 were cultivated in LB medium (10 g peptone, 5 g yeast extract, 10 g NaCl per liter; pre-mixed by Roth) at either 37 °C (K12) or 30 °C (168). Liquid cultures were shaken at 200 rpm in flasks with baffles and spirals. Plate cultures were grown in an incubator. Pre-cultures were inoculated with cells from stored agar plates and incubated for 16 h. Main cultures were inoculated with 1 % (v/v) of pre-culture and incubated until the desired optical density at 600 nm (OD_{600}) was reached.

2.2.3 General cultivation of *Streptomyces* sp. Tü2401

Agar-plate cultures of *Streptomyces* sp. Tü2401 were grown on ISP2 medium at 29 °C for four to seven days. 100 µL of spore solution or liquid culture were used for inoculation. Liquid cultures were incubated at 27 °C in shake flasks with aluminium caps. Pre-cultures were inoculated with spores from plate-grown mycelium or 50 µL of glycerol spore-stock. They were grown for three days in 20 mL of NL 410 in 100 mL flasks. Main cultures were inoculated with 5 mL of pre-culture. They were grown in 100 mL medium in 500 mL flasks for four to seven days.

Standing cultures of *Streptomyces* sp. Tü2401 were grown in 500 mL flasks fitted with either aluminium or foam caps. 5 mL of pre-culture grown in NL 410 were used to inoculate 100 mL of ISP2 liquid medium. The cultures were grown for seven days at 27 °C.

2.2.4 Batch Fermentation of *Streptomyces* sp. Tü2401

Streptomyces sp. Tü2401 was cultivated at a ten-liter scale in a continuous stirred tank bioreactor. 500 mL of pre-culture were grown in five 500 mL shake flasks containing 100 mL of NL 410 medium without CaCO₃. The pre-cultures were inoculated from stored ISP-agar plates and grown for 72 h at 27 °C. The pre-cultures were pooled and used to inoculate 9.5 L of OM medium for fermentation. The temperature was kept at 27 °C with an airflow of 5 L min⁻¹ and a rotor speed of 200 rpm. Control samples of 15 mL were taken throughout the process at regular intervals. Fermentation was stopped after 125 h and the culture broth was harvested. Further processing is described in section 2.4.3.

2.3 Bioassays

2.3.1 Agar Diffusion Assays

Agar diffusion bioactivity assays against *E. coli* K12 and *B. subtilis* 168 were conducted on LB-agar in petri dishes. Round petri dishes ($\varnothing = 92\text{ mm}$) were filled with 20 mL of liquid agar, square dishes ($120 \times 120\text{ mm}$) were filled with 40 mL. Solidified agar plates were stored at 8°C .

400 μL (200 μL for round plates) of liquid culture at an OD_{600} of 0.3 to 0.6 and were spread on the solid agar plate with a drigalski spatula until dry. Round wells ($\varnothing = 7\text{ mm}$) were punched out of the agar and filled with 50 μL of sample. Processed plates were stored for 1 h at ambient temperature, before incubating them over night at either 30°C or 37°C .

2.3.2 *yorB* Reporter Gene Assay

The *yorB* reporter gene assays were performed by Katharina Wex of the group of Prof. Dr. Heike Brötz-Oesterhelt at the Interfaculty Institute of Microbiology and Infection Medicine Tübingen

Agar-based *yorB* reporter gene assays were performed with *Bacillus subtilis* 1S34 pHJS105-*yorB-lacZ2*.¹ 20 mL of LB-agar supplemented with 50 $\mu\text{g mL}^{-1}$ spectinomycin were inoculated with 500 μL of overnight culture and grown until reaching the stationary phase. 50 mL LB-softagar (0.7 % agarose) with 150 $\mu\text{g mL}^{-1}$ 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) was prepared and mixed with the strain to a concentration of 3×10^7 colony-forming units per milliliter. The agar was filled into a square petri dish and prepared with sample wells. 50 μL of test samples were applied and the plate was incubated at 30°C over night.

2.4 Sample Preparation and Extraction

Extracts and reverse extracts of *Streptomyces* sp. Tü2401 were generally obtained through filtrated culture broth supernatant. After cultivating the strain for 4 to 7 days, the harvested biomass was centrifuged at 9000 rpm for 20 min. The supernatant was collected and filtered through a 0.2 μm sterile filter. The filtrate was stored at 4°C .

2.4.1 Preparation of Culture Broth Extracts

Culture broth filtrate extracts were prepared by adding an equal amount of solvent to the filtrate and shaking the mixture for 30 min. The phases were separated by centrifugation at 4000 rpm for 10 min. Both phases were collected separately and stored at 4 °C. Samples were concentrated by drying under vacuum at either 40 °C (ethyl acetate, methyl acetate) or 60 °C (butanol, water) and resuspending them in a fifth of the initial volume. Organic phases were resuspended in methanol, aqueous phases in water.

2.4.2 Determination of Extraction Conditions

Three sets of five 15 mL falcon tubes were filled with 5 mL filtered Tü2401 culture broth. For each set, the pH of the samples was adjusted to 2, 5, 7, 9 or 11 with NaOH and HCl. Each set was extracted with either ethyl acetate, methyl acetate or ethyl formate and both phases were collected. Each phase was tested for bioactivity against *E. coli* K12.

2.4.3 Processing of Fermentation Culture Broth

The harvested fermentation broth was supplemented with diatomaceous earth and filtered through Pall T 1500 filter plates (relative retention range 10 - 30 µm). The remaining filter cake was discarded and the filtrate transferred to a stirring bucket. Two liters of ethyl acetate were added to the filtrate and stirred for 30 min. After phase-separation, the organic phase was collected and the aqueous phase extracted again. The process was repeated five times. Both phases were collected separately and concentrated in a rotary evaporator at 40 °C. The concentrated aqueous phase was frozen at –20 °C and lyophilized. The organic concentrate was stored at 8 °C.

2.4.4 Agar Plate Extraction

Standard ISP2 agar plates were ground with a blender and extracted with an equal volume of butanol or ethyl acetate for 1 h. The mixture was centrifuged at 4000 rpm for 1 h and the supernatant collected. The remaining slurry was resuspended in the same amount of water, centrifuged at 4000 rpm for 1 h and the supernatant collected.

Special ISP2 agar plates with low-melting-point agarose (LMPA) were prepared by substituting the 2 % (w/v) agar of regular plates with 4 % (w/v) LMPA. 75 mL of LMPA

agar plates were melted in Schott-flasks at 70 °C and extracted with either butanol or ethyl acetate for 30 min. The organic phase was collected and the remaining agar extracted again with 50 mL of water. All collected organic extracts were dried at 40 °C (ethyl acetate) or 60 °C (butanol) and resuspended in 1 mL methanol.

2.5 Bioactivity-guided Isolation

2.5.1 Thin Layer Chromatography

Thin layer Chromatography was performed with reverse extracts of Tü2401 on TLC Silica Gel 60 F₂₅₄ plates by Merck. Aqueous samples were applied by pipetting 1 µL at a time and letting the plate dry until the next application. The TLC chambers were filled up to 1 cm with solvent and incubated for 12 h. The plates were run until either 75 % of the plate had been soaked or 2 h had passed. The solvents used as mobile phases are listed in Table 2.5.

Table 2.5: Mobile phase compositions used for Thin-Layer Chromatography

Solvent	Ratio (v/v)
Acetonitrile / Water	85:15
Butanol / Acetic acid / Water	14:3:2 and 42:10:7
Butanol / Ethanol / Water	3:2:1
Ethyl acetate / 2-Propanol / Water	6:3:1

The working orcin staining solution was prepared by mixing two storage solutions, solution A and B, at a ratio of 10:1 (v/v). Solution A contained 1 % (w/v) Fe(III)Cl₃ in 10 % sulfuric acid, solution B contained 6 % (w/v) Orcin in ethanol. The plates were sprayed with the working solution and treated with a heat gun for a few seconds.

Preparative samples were obtained by scraping the silica off the unstained plate and collecting it in reaction tubes. The samples were then extracted with 1 mL methanol, vortexed and sonicated for 30 min. After centrifugation at 14,000 rpm for 5 min, the supernatant was transferred to a new tube. The extraction was performed twice. The methanolic samples were dried at 30 °C and resuspended in an amount of water equal to the sample initially applied on the TLC plate.

2.5.2 Ion Exchange Chromatography

Ion exchange chromatography was performed with both a strong anion (Diaion SA11A, 20-50 mesh, Cl⁻ form) and a strong cation exchange resin (Dowex 50WX4, 100-200 mesh, Na⁺ form). Three solutions were used for all operations: An acidic solution (1 % (v/v) formic acid, pH 2), a neutral solution (MilliQ-H₂O, pH 7) and a basic solution (2 % (v/v) ammonium hydroxide, pH 11). Prior to column preparation, both resins were swollen for 24 h. The anion exchange resin (AnX) was swollen in the basic buffer and the cation exchange resin (CatX) in the basic one. 12.5 mm diameter glass columns were filled with resin up to a bed height of 10 cm (AnX) or 9.5 cm (CatX). Both columns were operated at a constant flow of 2.5 mL min⁻¹. All method steps are listed in Table 2.6. The pH of the applied sample was adjusted to pH 2 (CatX) or pH 11 (AnX) with NaOH and HCl. The flow-through of each step was collected and stored at 4 °C.

Table 2.6: **Method for ion exchange chromatography.** pH values and relative volume of the solutions used for ion exchange chromatography with both strong anion exchange (AnX) and cation exchange (CatX) resins. Both resins were loaded with 1 mL of sample.

Step	AnX pH	CatX pH	Column Volumes
Equilibration	11	2	2
Wash 1	7	7	1
Sample application	11	2	*
Wash 2	11	2	1
Wash 3	7	7	1
Elution	2	11	5

2.5.3 Trimethylsilane Derivatization and Gas Chromatography

The derivatization and gas chromatography (GC) measurements were performed by Dr. Dorothee Wistuba of the mass spectrometry department at the institute of organic chemistry in Tübingen.

Dried HPLC fractions were suspended in a mixture of 460 µL N,O-Bis(trimethylsilyl)-trifluoroacetamide and 40 µL pyridine, before heating them to 110 °C. After derivatization, samples were dried with nitrogen gas and redissolved in dichloromethane. The derivatized samples were analyzed with a Hewlett Packard (HP) 6890 GC-system coupled

to a HP 5973 mass selective detector. The Agilent DB5 column measured 13 m × 0.25 mm with a film thickness of 0.1 µm. Helium was used as the carrier gas.

2.5.4 Preparative HPLC

Preparative HPLC was performed on either the Agilent 1100 Series or Agilent 1260 Infinity instrument coupled to an Agilent G1346C fraction collector. Coupling of a Sedex Model 85 evaporative light scattering detector (ELSD) was achieved by mounting a 1:5-splitter after the UV-Detector. 4/5 of the flow was directed to the fraction collector, the remaining 1/5 to the ELSD. The ELSD was operated at a temperature of 40 °C and with nitrogen gas at a pressure of 3.2 bar. Detailed information about the used columns and methods can be found in Table 2.3 and the appendix. The obtained data was analyzed with the Agilent Chemstation (Version B.04.03). All samples were centrifuged at 14,000 rpm, before transferring the supernatant to HPLC-vials. All fractions were collected by timeslices of 1 min, stored at 4 °C to 8 °C, and subsequently dried at 40 °C. The samples were resuspended in an amount of water equal to the amount of injected sample and stored at 4 °C to 8 °C. Detailed method descriptions are found in the appendix at section A.1.

2.5.5 Analytical HPLC and Mass Spectrometry

For mass spectrometry, an Agilent 1200 series HPLC system was coupled to an Agilent 6330 IonTrap LC-MS mass spectrometer. It features electrospray ionization with alternating positive and negative modes. The instrument was controlled with 6300 Series Trap Control (Version 6.1) and data was analyzed using DataAnalysis for 6300 Series Ion Trap LC-MS (Version 3.4). Measuring parameters are detailed for each method in section A.1.

2.6 Genome Analysis

The taxonomic analysis was performed by Mohammad Alanjary of the group of Prof. Dr. Nadine Ziemert at the Interfaculty Institute of Microbiology and Infection Medicine in Tübingen

The phylogenetic tree was constructed by using 50 sequenced *Streptomyces* genomes (Table A.9) as reference. From these genomes and the concatenated contigs of *Streptomyces* sp. Tü2401, 50 single copy genes were identified by The gene sequences were

aligned using the MAFFT tool (version 7) and refined using trimAl (version 1.2).²⁻⁴ The maximum-likelihood tree was constructed with RAxML (version 8).⁵ The average nucleotide identity was calculated with the JSpeciesWS web tool.⁶ The final tree was visualized using Interactive tree of life (iTOL) v3.⁷

All five contigs were uploaded individually to the Antibiotics & Secondary Metabolite Analysis Shell (AntiSMASH) webserver.⁸⁻¹⁰ Clusters were identified with standard settings and ClusterFinder enabled.

3 Results & Discussion

3.1 Determination of Extraction Conditions

Preliminary experiments demonstrated, that the antibiotic compound could not be extracted with ethyl acetate. Only culture broth 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 reverse-phase method. 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 culture broth 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, whereas the others clogged the filters after a few milli liters. 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 caused notable ($\emptyset > 1\text{ cm}$) 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 evaporative light scattering detector (ELSD). A C18 column was used in combination

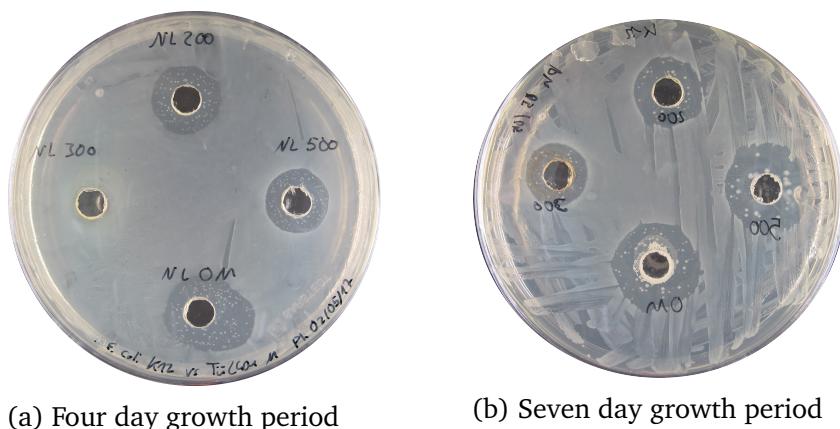


Figure 3.1: K12 Bioassay results with media supernatants *Streptomyces* sp. Tü2401 was grown for either four (a) or seven days (b) in four different complex media. The filtrated medium supernatant was tested against *Escherichia coli* K12. Media: (Top) NL 200 (Left) NL 300 (Right) NL 500 (Bottom) OM.

with a 4.5 % to 100 % acetonitrile 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 the injection peak is present. The OM supernatant seems to predominantly contain compounds, that can not be separated with a reverse-phase screening gradient on a C18 column. Since the antibiotic compound of interest showed no retention under similar conditions, the use of OM as a production medium would result in 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.

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

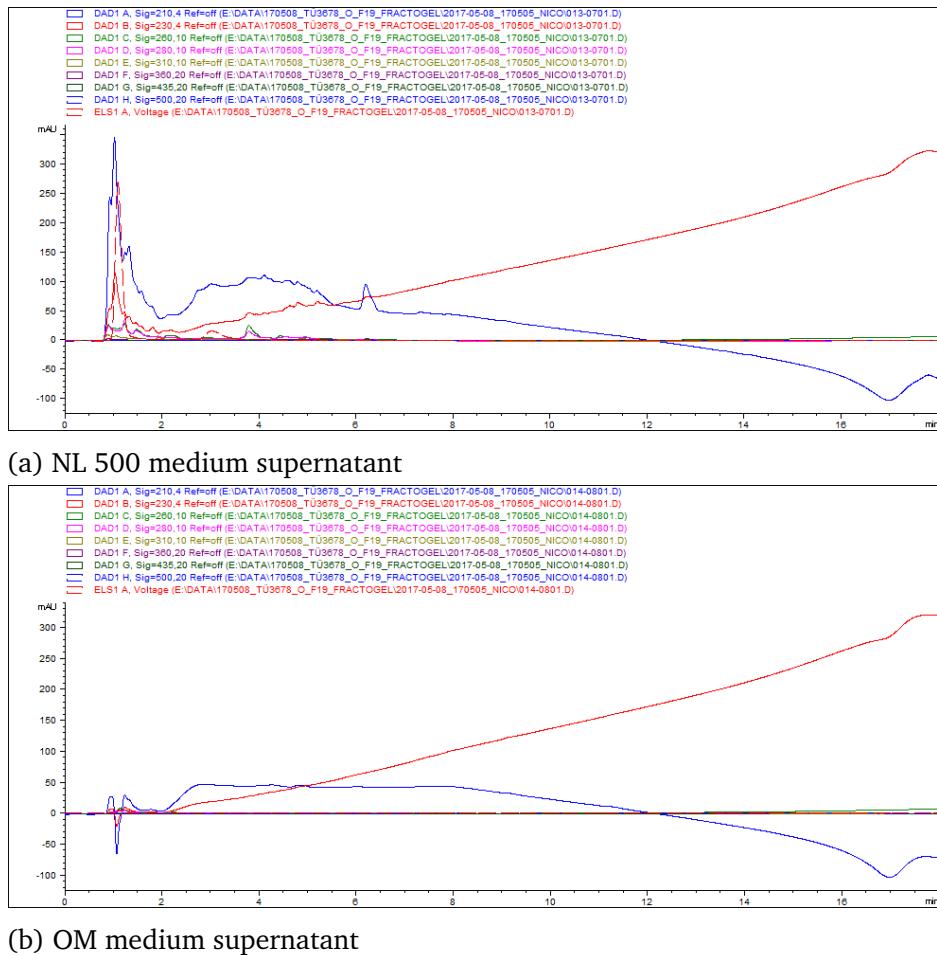


Figure 3.2: Chromatogram of medium supernatant screening. 5 μ L of medium supernatant were injected and separated on a C18 column. A screening gradient of 4.5 to 100 % acetonitrile was employed. UV absorption and ELSD voltage were measured.

are charged at certain pH-ranges. If the compound does too, the isoelectric point could be passed 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 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

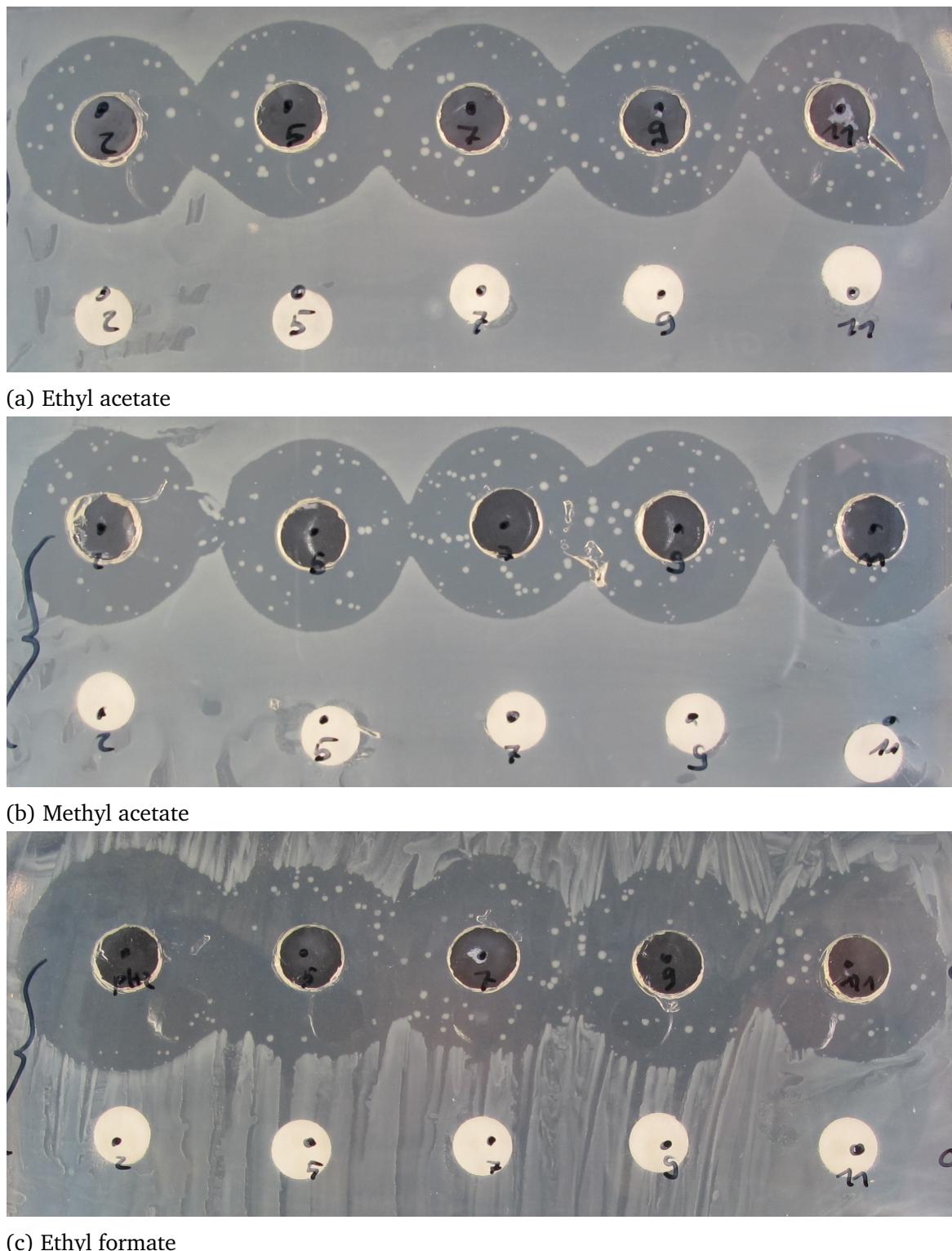


Figure 3.3: Bioassay results of different OM medium extracts against *E. coli* K12
Filtered supernatant of OM cultures of *Streptomyces* sp. Tü2401 were extracted with either (a) ethyl acetate, (b) methyl acetate or (c) ethyl formate. Sample-pH was adjusted prior to either 2, 5, 7, 9 or 11 (left to right). 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.

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.¹¹⁻¹⁴ 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, some of 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 2–11.¹⁵ 44 % contained an acidic functionality, 17 % a basic one, and 9 % both. It is likely, that the hydrophilic antibacterial compound also contains basic or acidic functional groups such as amino- or carboxyl-groups. The presence of charged functionalities in a molecule can be utilized for separation via ion-exchange chromatography (IEC).

Since as of now, not much is known about the water-soluble antibacterial compound, an explorative IEC method similar to the approach of Månnsson *et al.* was devised.¹⁵ Two ion-exchange resins with orthogonal selective properties were employed to test if the compound contains acidic or basic functionalities. Both feature "strong" ion exchange groups, which retain their charge over a broad pH-range and are immobilized on a divinylbenzene matrix. The strong cation exchanger (SCX), Dowex 50WX4, contains negatively charged sulfonic acids, while the strong anion exchanger (SAX), Diaion SA11A, contains positively charged quarternary ammonium groups. The sample would be adjusted to an extreme pH such as 2 or 11, where most ionizable groups should be charged, and

loaded onto the column. Uncharged contaminants were washed away with a buffer at the sample pH and one with pH 7. The elution buffer had the opposite pH of the sample pH, supposedly removing the charge and washing the compound off the column. In case of the SAX this means that the sample was adjusted to pH 11, where acidic functionalities such as carboxyl-groups are likely negatively charged. At pH 7 the charge should still be retained, therefore removing impurities by washing with pH 7 and 11. At pH 2 however, a carboxylic acid is likely protonated. The loss of charge greatly reduces the affinity to the SAX and the compound should be eluted with the buffer. For the SCX the same principle applies, but with the pH ranges switched to allow for retention and elution of basic compounds.

1 mL of filtrated culture broth supernatant was adjusted with NaOH and HCl to the appropriate pH and used as the sample for this experiment. The detailed method descriptions are found in section 2.5.2. All loading, washing and elution fractions were collected, concentrated and subjected to the standard bioassay against *E. coli* K12.

The assay plate belonging to the SAX fractions revealed bioactivity to be present in the first three out of five elution fractions. The loading and washing fractions were inactive. The water-soluble compound is able to form an ionic bond with sulfonic acid at pH 11 and pH 7, which is broken at pH 2. This indicates the presence of one or more functional groups with a $pK_a < 7$, which are most likely carboxyl groups. As such, the compound might contain organic acid moieties.

The tested SCX fractions exhibited no visible bioactivity at all. This could be due to an exceptionally strong bond of the compound to the cationic resin, which is still present at pH 11. A strongly basic alkaloid or amine with a $pK_a > 11$ for the protonated form would not have been eluted at the used pH. The only other possibility would be a reaction with the SCX matrix, either deactivating the compound or binding it irreversibly. Degradation at extreme pH values is unlikely, since the same buffers were used for the SAX column. Additionally, the compound has been observed to be exceptionally stable at pH 2–11 in water. Samples stored at 8 °C and various pH levels have been tested for bioactivity several months after generation and displayed no noticeable loss in activity.

3.2.4 Thin-Layer Chromatography

A classic method for separation of sugars is thin-layer chromatography (TLC).

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 Induction of the *yorB* Reporter System

Tü2401 displayed positive results with the *yorB* reporter system when grown on ISP2 plates. The *yorB* gene has been observed to be upregulated in *Bacillus subtilis* when the strain was treated with quinolones.¹⁶ Construction of a luciferase reporter system led to the hypothesis, that induction is due to DNA damage caused by inhibition of the Topoisomerase II alpha subunit.¹⁷ Further characterization with greater natural product libraries strengthened this theory.^{1,18} *yorB* induction was achieved by a variety of compounds, which damage DNA directly or interfere with its replication. They are listed in Table 3.1. Most of the inducers are produced by *Streptomyces* species, indicating an overarching potential of this family to produce DNA damaging substances.

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

Table 3.1: Known inducers of the *yorB* reporter system. Substances with known modes of action identified as inductors of the *yorB* reporter system in *Bacillus subtilis*.^{1,18} Producing organisms are examples featured in the PubChem database and are not exclusive. *Streptomyces* species names were abbreviated.

Name	Mode of Action	Organism	PubChem ID
8-Hydroxyquinolone	RNA polymerase inhibition		
Juglone	RNA polymerase inhibition	<i>Carya illinonensis</i>	3806
Novobiocin	DNA gyrase inhibition	<i>S. niveus</i>	54675769
Alternariol monomethylether	DNA strand breaking	<i>Alternaria</i> sp.	5360741
Bleomycin	DNA strand breaking	<i>S. verticilllus</i>	5360373
Myxin	DNA strand breaking	Fungi	72510
Phleomycin	DNA strand breaking	<i>S. verticilllus</i>	72511
Streptonigrin	DNA strand breaking	<i>S. flocculus</i>	5351165
Chromomycin	DNA intercalator	<i>S. griseus</i>	5351560
Daunorubicin	DNA intercalator	<i>S. peucetius</i>	30323
Doxorubicin	DNA intercalator	<i>S. peucetius</i>	31703
Anthramycin	DNA binding	<i>S. refuineus</i>	5311005
Desmethyllethersibiromycin	DNA binding	<i>Streptomyces</i> spp.	6437361
Porfiromycin	DNA crosslinking	<i>S. arduus</i>	13116

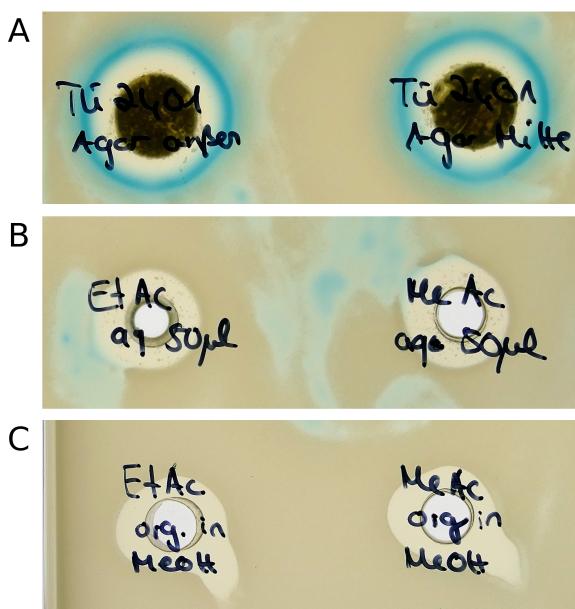


Figure 3.4: Results of the *yorB* reporter system bioassay with strain Tü2401 Foto of the bioassay plate taken one day after incubation.

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 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 .

Table 3.2: 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			-	-

3.5 Bioinformatic Analysis

3.5.1 Phylogeny of Strain Tü2401

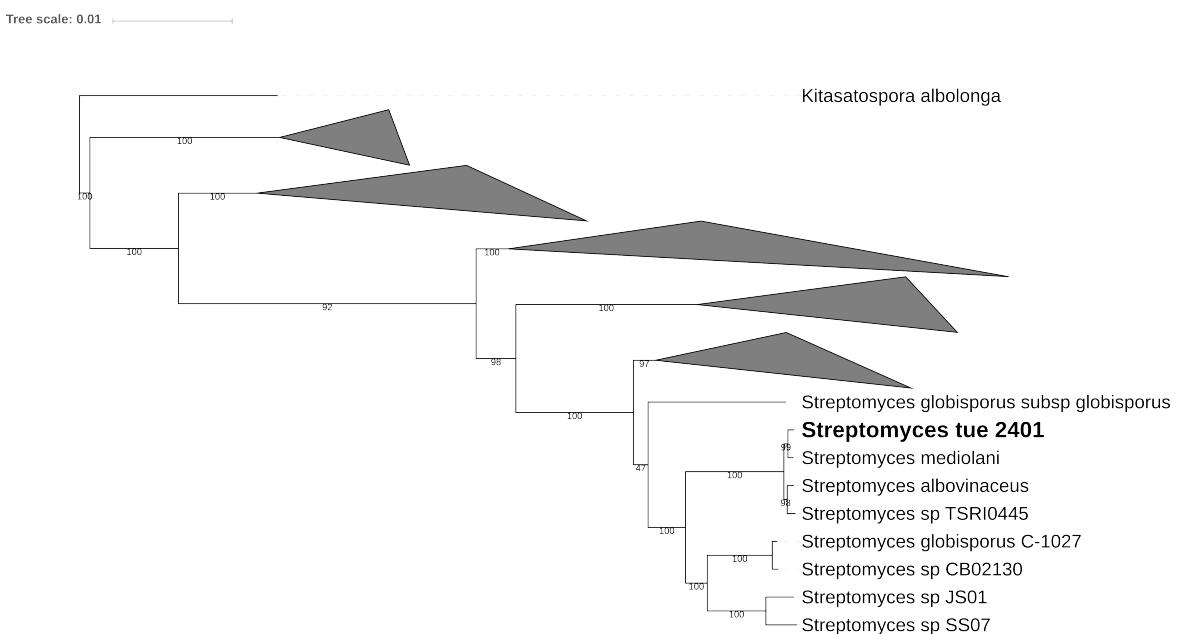


Figure 3.5: Maximum likelihood tree of *Streptomyces* sp. Tü2401. The tree was constructed using a multiple-sequence alignment of 50 single-copy genes across 50 *Streptomyces* reference genomes. The node belonging to *Streptomyces* sp. Tü2401 is highlighted with bold text. Only the nine most closely related nodes and the outgroup are shown. Dark triangles represented hidden, collapsed nodes.

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.^{19–22} 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 3.6). The 9-membered family includes neocarzinostatin, C-1027 and maduropeptin. The 10-membered family includes calicheamicin γ_1^I , esperamicin A₁ and dynemicin A.¹⁹ Enediynes are potent cytotoxic agents because of their ability to induce DNA double-strand breaks.²³ Electronic rearrangement of the carbocycle produces a benzenoid diradical, which abstracts hydrogen atoms from the DNA-backbone. The consulting radicals cause interstrand 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.¹⁹ The nine-membered chromophore of C-1027, which was isolated from *Streptomyces globisporus* C-1027, is bound noncovalently to an 110 amino acid apoprotein.^{21,24–31} The isolated chromophore has been shown to be very unstable, whereas the holo C-1027 did not lose activity under the same conditions.^{27–29} The apoprotein binds specifically to the C-1027 chromophore, supposedly by hydrophobic pocket, which binds the benzoxazine side chain.^{28,32} The enediyne antibiotics neocarzinostatin and maduropeptin, which were also isolated from actinomycetes, feature highly specific and protective apoproteins as well.²¹

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 endiye antibiotics.¹ 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 endiye compound could be responsible for the induction.

The assay in Section ?? 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 endiye, this loss of activity could be due to the high instability of the chromophore. The apoprotein could have been detached during the extraction and

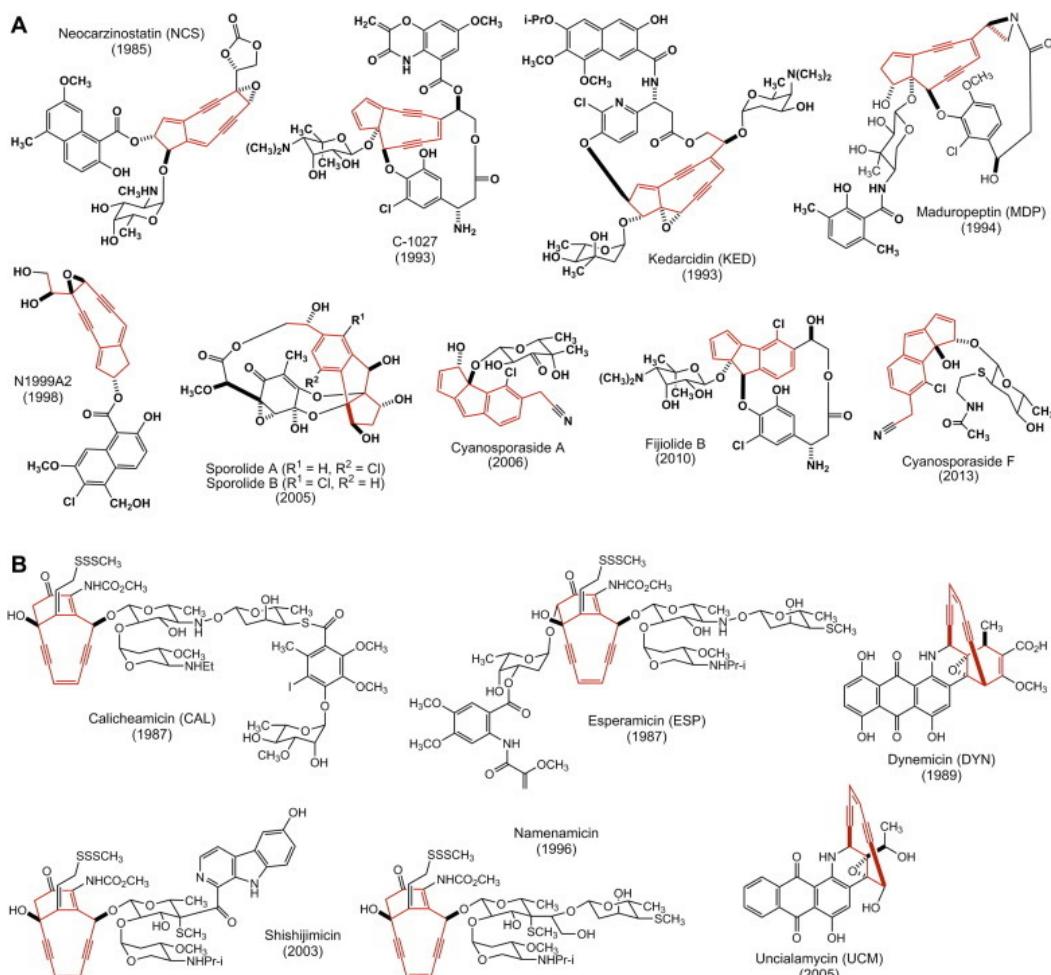


Figure 3.6: **Structures of known enediyne natural products.** Enediyne cores are highlighted in red. (A) Compounds with nine-membered rings. (B) Compounds with ten-membered rings. The year of structure confirmation is displayed in parentheses. The sporolides, cyanosporolids and fijiolides do not contain an endiyene core, but are proposed to be derived from enediyne precursors. Adapted from Shen *et al.* (2015) Copyright 2014 Elsevier Ltd.

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 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.³⁰ The active chromophore could be extracted from the apoprotein with methanol at 0 °C.²⁸ 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 endiyene-apoprotein complex should be stable enough for routine testing of culture broth and its xsupernatant. The only alternative to optimizing production conditions would be heterologous expression of the biosynthetic cluster. For the endiyene compound family though, this has, as of now, only been partly achieved for the nine-membered endiyene neocarzinostatin.³³

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.^{19,34} 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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