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## 3. Results & Discussion

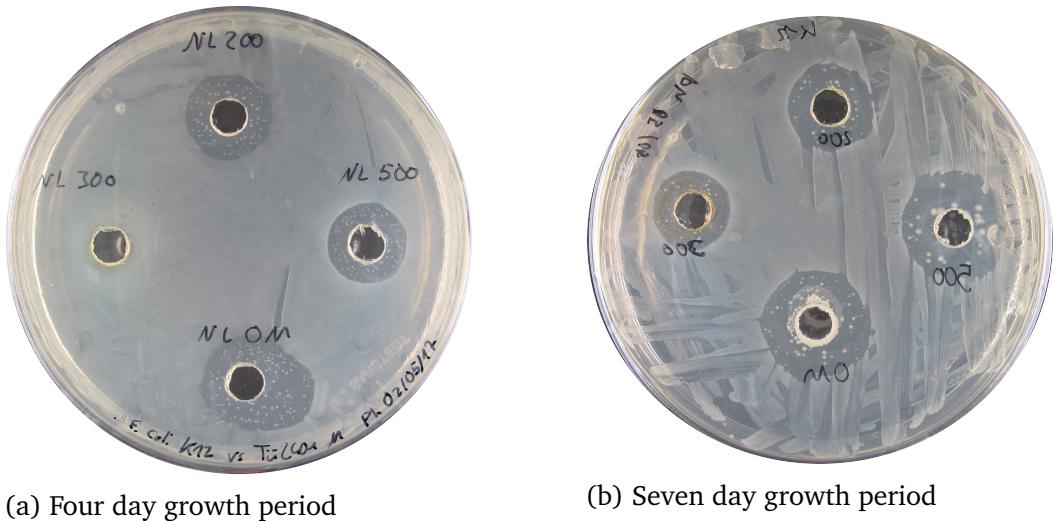
### 3.1. Determination of Extraction Conditions

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 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 caused 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)



**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.

and evaporative 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 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.

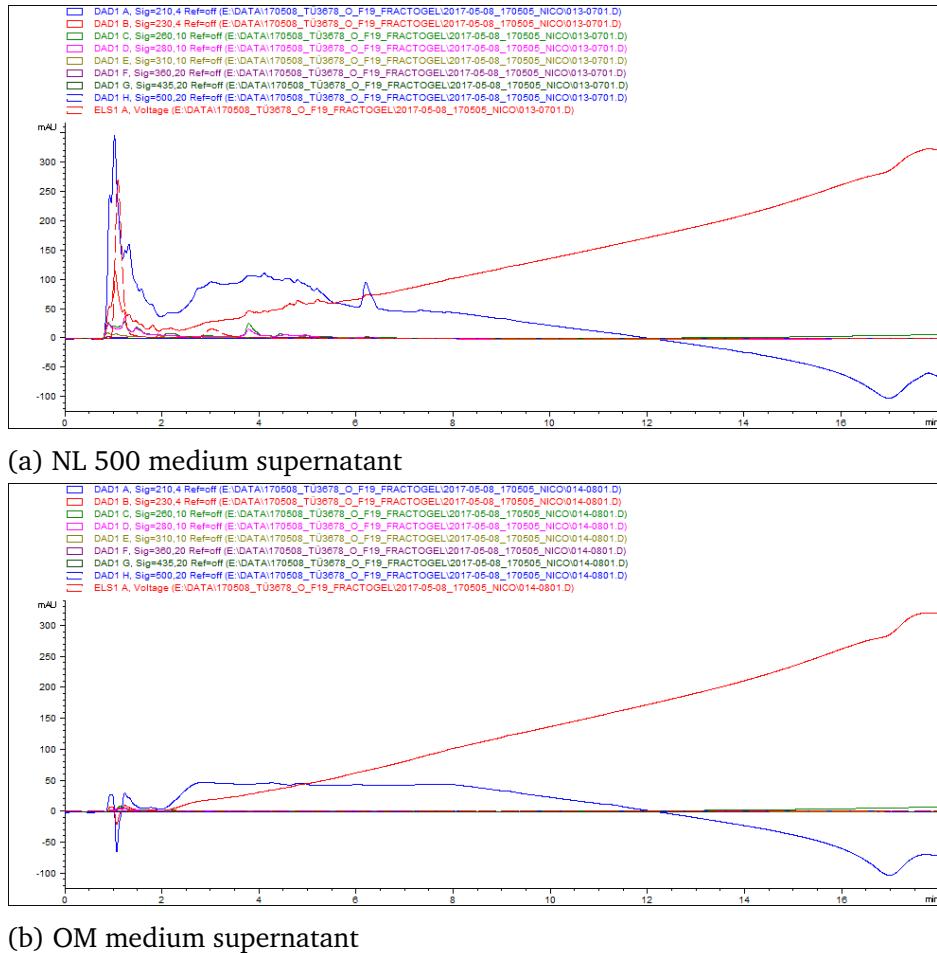


Figure 3.2.: **Chromatogram of medium supernatant screening.** 5  $\mu\text{L}$  of medium supernatant were injected and separated on a C18 column. A screening gradient of 4.5 to 100 % acetonitrile was employed. UV absorption and ELSD voltage were measured.

### 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

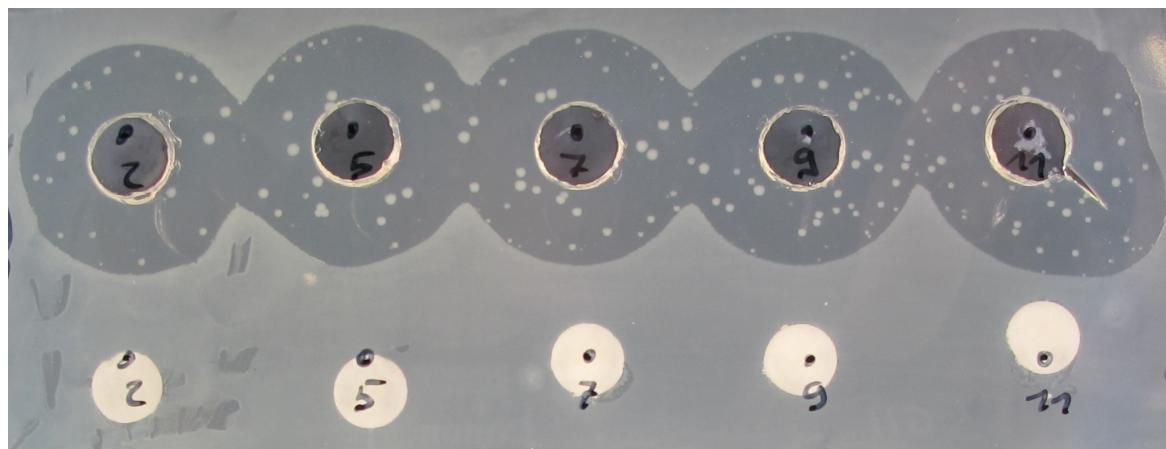
### 3.2.2. Hydrophilic Interaction Liquid Chromatography

- Short reiteration of HILIC
- Characteristics of NH<sub>2</sub> column
- Characteristics of ZIC-HILIC column

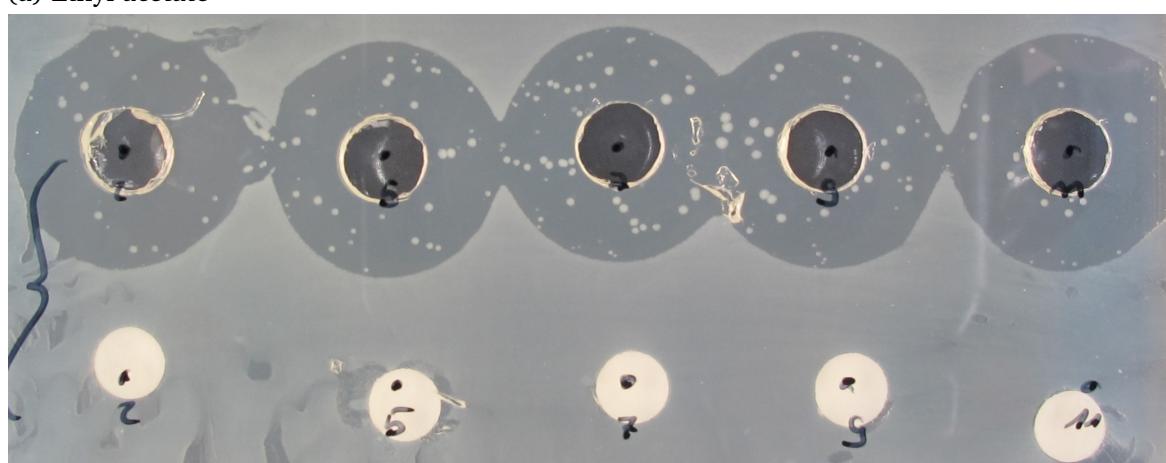
The Luna® NH<sub>2</sub> by Phenomenex features a silica matrix modified with 3-aminopropyl groups. The used model had a particle size of 3.5 µm and a pore size of 100 Å. The dimensions were 4.6 × 250 mm.

#### Luna NH<sub>2</sub> Column

Separation via the Luna® NH<sub>2</sub> 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<sup>-1</sup>. 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.<sup>1-4</sup> 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.<sup>5</sup> 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 Måansson *et al.* was devised.<sup>5</sup> 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.<sup>6</sup> 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.<sup>7</sup> Further characterization with greater natural product libraries strengthened this theory.<sup>8,9</sup> *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):

Table 3.1.: **Known inducers of the *yorB* reporter system.** Substances with known modes of action identified as inducers of the *yorB* reporter system in *Bacillus subtilis*.<sup>8,9</sup> 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

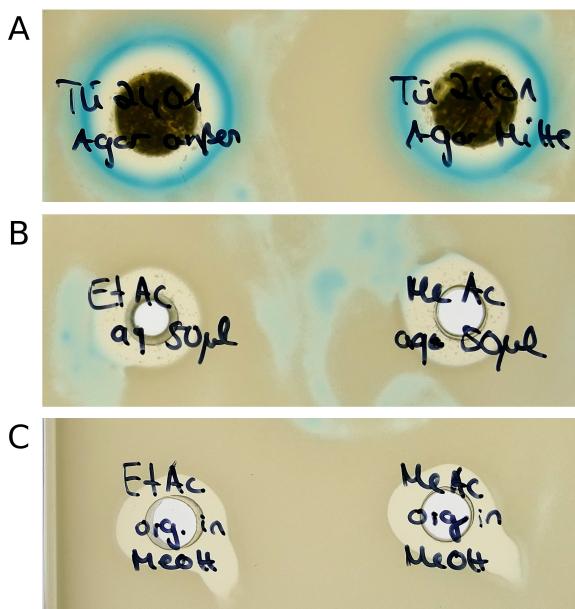


Figure 3.4.: Results of the *yorB* reporter system bioassay with agar stamps of *Tü2401*body

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 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 .

### 3.5. Bioinformatic Analysis

#### 3.5.1. Phylogeny of Strain Tü2401

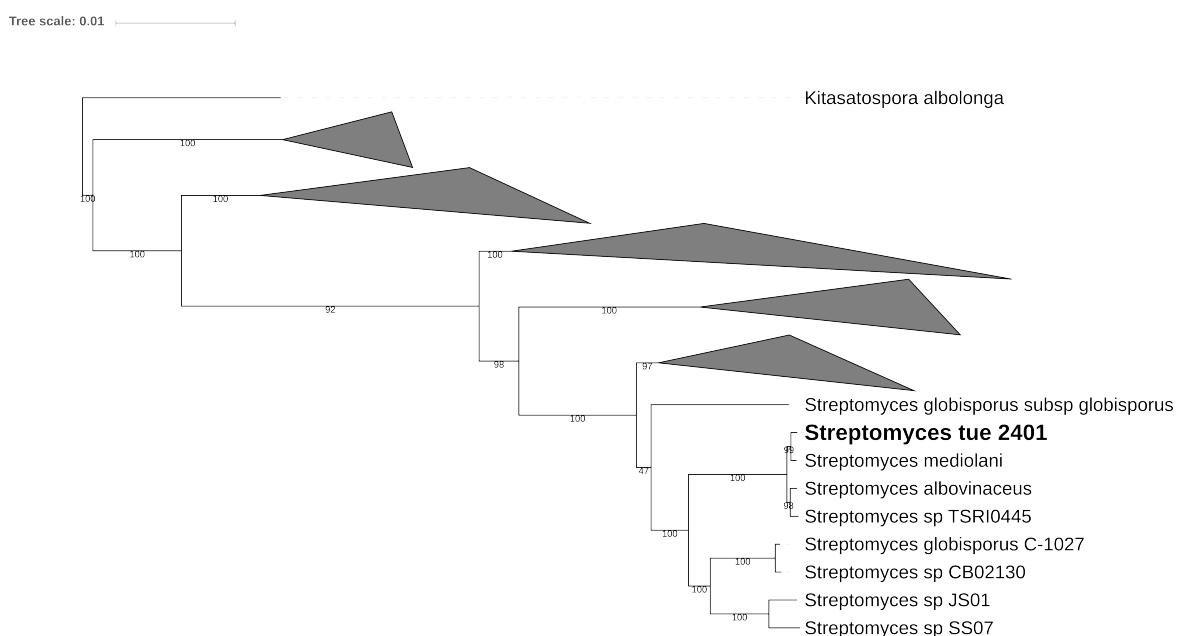


Figure 3.5.: Maximum likelihood tree of *Streptomyces* sp. Tü2401. The tree was constructed using a multiple-sequence alignment of 50 single-copy genes across 50 *Streptomyces* reference genomes. The node belonging to *Streptomyces* sp. Tü2401 is highlighted with bold text. Only the nine most closely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

**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			-	-

### 3.5.2. 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.<sup>10–13</sup> 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  $\gamma_1^I$ , esperamicin A<sub>1</sub> and dynemicin A.<sup>10</sup> Enediynes are potent cytotoxic agents because of their ability to induce DNA double-strand breaks.<sup>14</sup> Electronic rearrangement of the carbocycle produces a benzenoid diradical, which abstracts hydrogen atoms from the DNA-backbone. The resulting 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.<sup>10</sup> The nine-membered chromophore of C-1027, which was isolated from *Streptomyces globisporus* C-1027, is bound noncovalently to an 110 amino acid apoprotein.<sup>12,15–22</sup> The isolated chromophore has been shown to be very unstable, whereas the holo C-1027 did not lose activity under the same conditions.<sup>18–20</sup> The apoprotein binds specifically to the C-1027 chromophore, supposedly by hydrophobic pocket, which binds the benzoxazine side chain.<sup>19,23</sup> The enediyne antibiotics neocarzinostatin and maduropeptin, which were also isolated from actinomycetes, feature highly specific and protective apoproteins as well.<sup>12</sup>

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

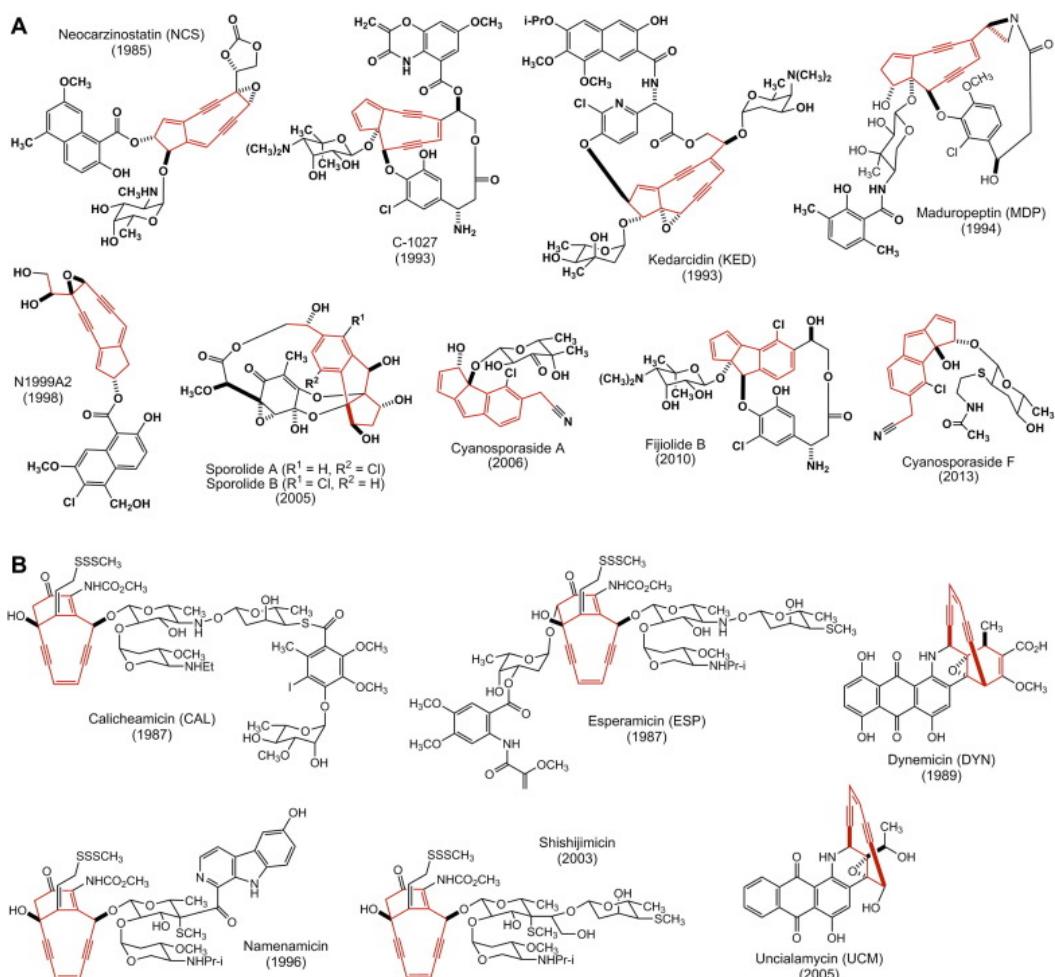


Figure 3.6.: **Structures of known enediyne natural products.** Enediyne cores are highlighted in red. (A) Compounds with nine-membered rings. (B) Compounds with ten-membered rings. The year of structure confirmation is displayed in parentheses. The sporolides, cyanosporolids and fijiolides do not contain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

breaks and crosslinks are reported to induce the system, though none of them belong to the family of endiyne antibiotics.<sup>9</sup> 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 ?? 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 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.<sup>21</sup> The active chromophore could be extracted from the apoprotein with methanol at 0 °C.<sup>19</sup> 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 supernatant. 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.<sup>24</sup>

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.<sup>10,25</sup> 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 <sup>-1</sup>
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 <sup>-1</sup>
Temperature	25 °C
Injection Volume	50 µL

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

<b>Parameter</b>	<b>Value</b>
Column	Luna NH <sub>2</sub> 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 <sup>-1</sup>
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**

<b>Parameter</b>	<b>Value</b>
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 <sup>-1</sup>
Temperature	25 °C
Injection Volume	50 µL

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

<b>Parameter</b>	<b>Value</b>
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 <sup>-1</sup>
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**

<b>Parameter</b>	<b>Value</b>
Column	Nucleosil-100 5 µm 150×3 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 <sup>-1</sup>
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**

<b>Parameter</b>	<b>Value</b>
Column	Kinetex Polar-C18 2.6 µm 150×4.6 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 <sup>-1</sup>
Temperature	50 °C
Injection Volume	50 µL

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

<b>Parameter</b>	<b>Value</b>
Column	Kinetex Polar-C18 2.6 µm 150×4.6 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 <sup>-1</sup>
Temperature	50 °C
Injection Volume	50 µL

## A.2. Chromatographic Data

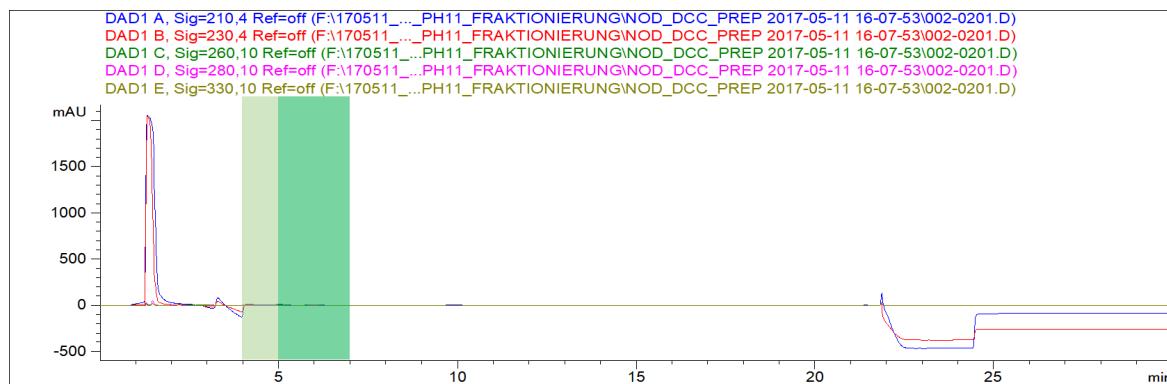


Figure A.1.: UV-chromatogram of reverse extract fractionation with the Luna<sup>®</sup> NH<sub>2</sub> column. testi

## A.3. Genomic Analysis

### A.3.1. Phylogenetic Data

Table including the reference genomes

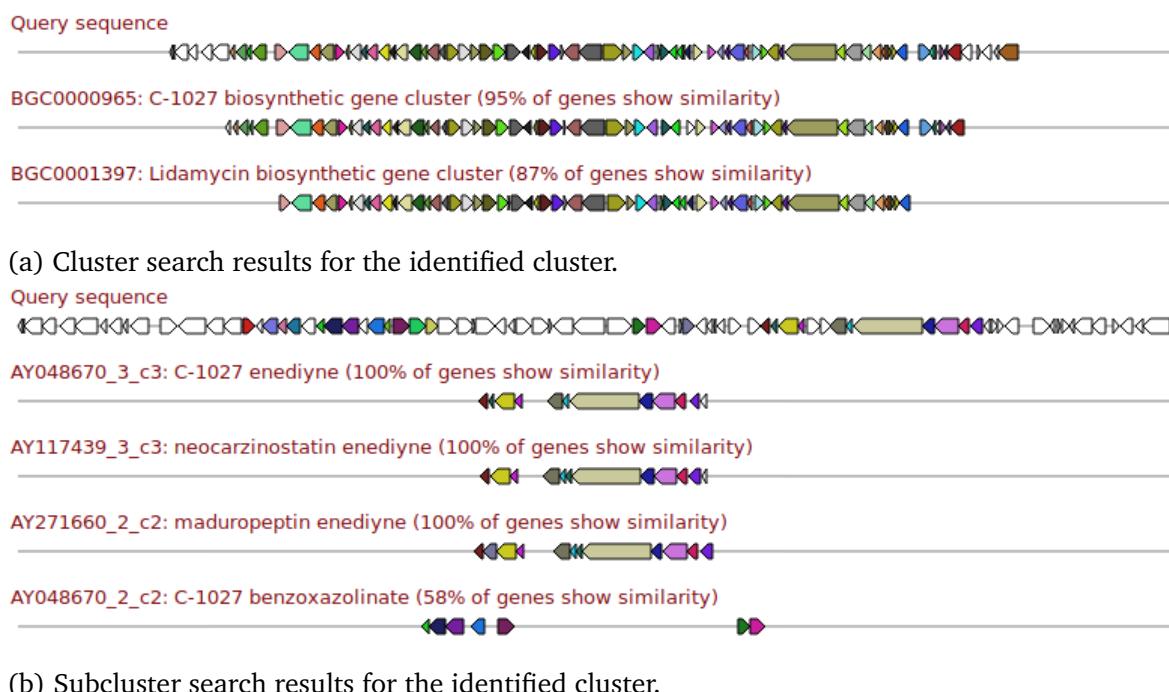
Table A.9.: Reference genomes for the construction of the phylogenetic tree

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.