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1 Introduction

1.1 Natural products from *Streptomyces*

1.2 Hydrophilic Antibiotics

A common molecular parameter to determine hydrophilicity is $\log P$, the logarithm of the partition coefficient between octanol and water.¹ Since accurate solubility prediction is of great importance in drug design, numerous methods have been implemented to calculate $\log P$ from chemical structures.²⁻⁴

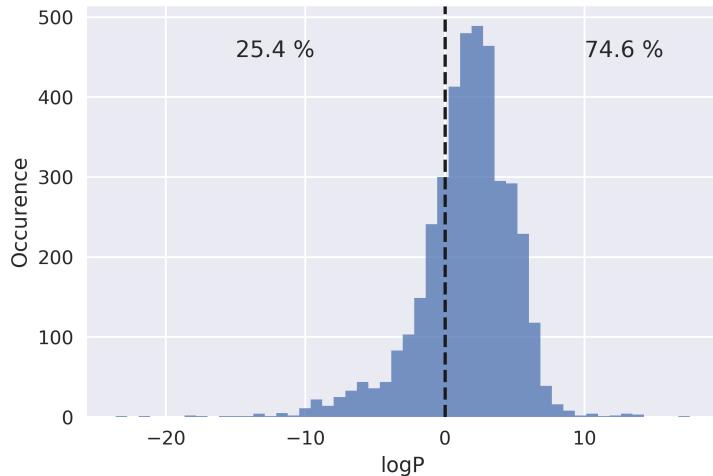


Figure 1.1: Distribution of logP in the StreptomeDB Database The molecular partition coefficient ($\log P$) was calculated for 3985 entries in the StreptomeDB 2.0 secondary metabolite database⁵ using the descriptor methods implemented in the RDKit cheminformatics suite.⁶ 74.6 % of compounds are predicted to have $\log P$ value above zero and 25.6 % equal zero or below.

1.3 Antibiotics against Gram-negative bacteria

1.4 Isolation strategies for hydrophilic natural products

1.5 Aim of this thesis

3 Results & Discussion

3.1 Determination of Extraction Conditions

The strain Tü2401 was grown in seven different complex media for either four or seven days during preliminary experiments. Culture broth supernatants of the NL 200, NL 300, NL 500 and OM displayed antibiotic activity against *E. coli* K12on plate assays. A comparison between the viable production media and cultivation times is necessary to determine the optimal production medium for the following experiments.

Initial HPLC procedures failed to separate the active compound with standard reverse-phase screening methods. The compound displayed no retention on C-18 columns with acetonitrile/water gradients, making it likely to possess strong hydrophilic properties. Additionally, the compound was not able to be extracted from the culture broth with ethyl acetate. Its high solubility in water requires the development of an adapted extraction procedure to aid the purification process.

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. During preliminary testing, the strain Tü2401 was cultivated in seven different complex media. Four of the resulting culture broth supernatants displayed antibiotic activity against the test strain *E. coli* K12. The obtained culture broth supernatants were filtrated by using 0.45 µm and 0.2 µm 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 ($\varnothing > 1$ 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

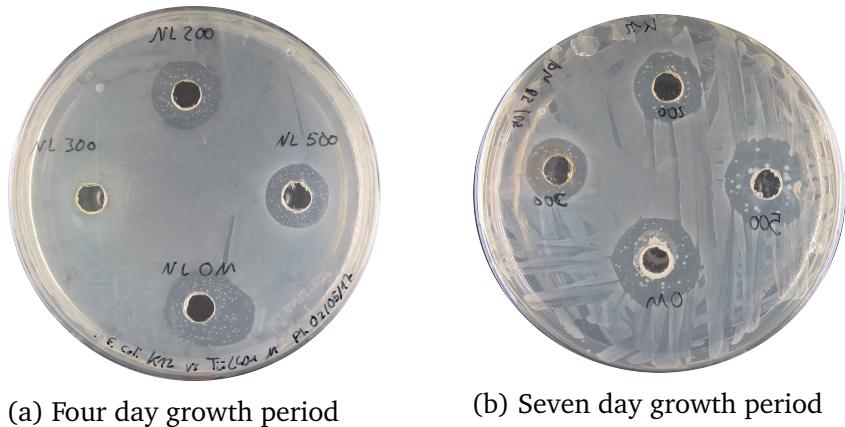


Figure 3.1: K12 Bioassay results with media supernatants *Streptomyces* sp. Tü2401 was grown for either four (a) or seven days (b) in four different complex media. The filtrated culture broth supernatant was tested against *Escherichia coli* K12. Media: (Top) NL 200 (Left) NL 300 (Right) NL 500 (Bottom)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

zones greater than 1.5 cm. When cultivated in NL 300, Tü2401 seems to take longer than four days to produce an amount of visible bioactivity. 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 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 culture broth 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.

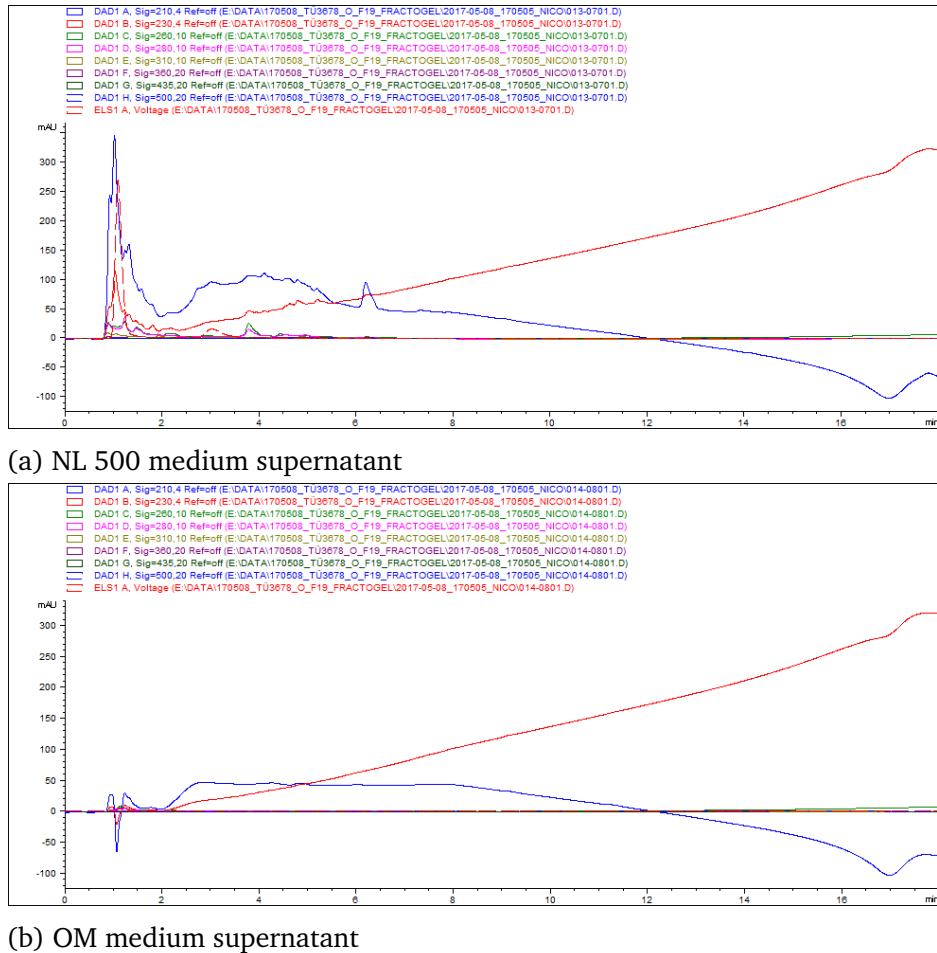


Figure 3.2: Chromatogram of medium supernatant screening. 5 μ L of culture broth supernatant were injected and separated on a C18 column. A screening gradient of 4.5 to 100 % acetonitrile was employed. UV absorption and ELSD voltage were measured.

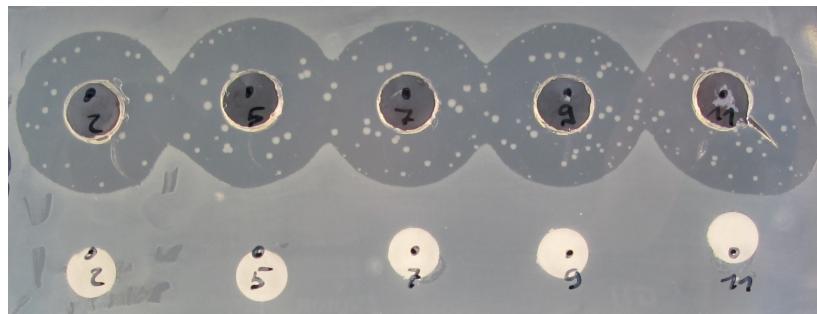
3.1.2 Extraction experiments

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 point of zero charge could pass by pH adjustment. Through reduced charge, the water-solubility could be lowered, enabling the extraction with an organic solvent.

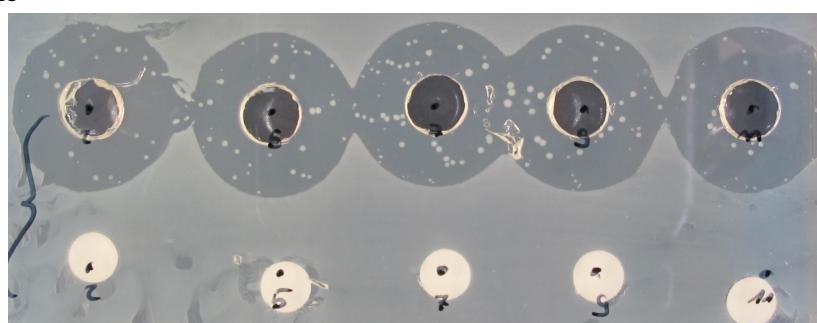
OM culture broth supernatant was divided into three groups of five aliquots. The pH of each aliquot was adjusted to either 2, 5, 7, 9 or 11. Each group was then extracted with either ethyl acetate, methyl acetate or ethyl formate. Both phases were separated and tested for bioactivity against *E. coli*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

The results of the diffusion-assay show, that the organic phases do not contain any antibiotic activity, regardless of the solvent or pH. All aqueous phases, however, produce an inhibition zone of about 1.6 cm, equal to the unextracted supernatant. The antibiotic compound could therefore not be extracted with either ethyl acetate, methyl acetate or ethyl formate. It may contain a high amount of polar but uncharged groups or be zwitterionic, ensuring similar hydrophilicity over a wide pH range. If the structure does possess a point of zero charge, its pH range may be too small to have been reached with the broad steps used. A repetition of the experiment with pH steps of 1 or 0.5 and a wider range of 1 to 14 could be applied to further explore the existence of a zero-charge point, that enables organic extraction.

The similar size of inhibition zones across all tested pH values also implies a resistance to degradation of the active compound over the range of 2–11. A notable trait of all inhibition zones caused by either pure broth supernatant or aqueous extraction phases is the presence of resistant colonies within. Since they grow seemingly random across the whole zone surface and not just the edges, concentration dependent resistance can be ruled out. It is more likely, that a resistant subpopulation of *E. coli* K12 is present in the glycerol stocks used to inoculate the test strain. The resistant subpopulation and the nonresistant strain must contain differences in antibiotic resistance genes. Genome sequencing of both strains with subsequent comparison of resistance genes could test this hypothesis and lead to valuable insights to the mode of action. From there on, the class of responsible compound classes could be narrowed down, enabling the development of



(a) Ethyl acetate



(b) Methyl acetate



(c) Ethyl formate

Figure 3.3: Bioassay results of different OM culture broth 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.

more specialized isolation procedures.

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 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.⁷⁻¹⁰ 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 2–11.¹¹ 44 % of compounds contained an acidic functionality, 17 % a basic one, and 9 % both. The high prevalence of charged groups in natural products combined with the hydrophilic nature of the antibiotic compound make the presence of basic or acidic functionalities, such as amino- or carboxyl-groups, likely. This can be utilized for purification purposes via ion-exchange chromatography (IEC).

Ion exchange chromatography (IEC) utilizes charged functional groups bound to an insoluble resin matrix.¹² Charged analytes are separated by the equilibrium between themselves and the counterions associated with the matrix. Since the charge of natural products largely depends on pH and pK_a , the choice of buffers, resins and counterions has to be tailored to each experiment. Through optimised conditions for stationary and mobile phase, selective “catch and release” purification methods can be designed.

IEC has a wide range of applications. It is used in wastewater treatment, hydrometallurgy and chromatography.¹³ Biological macromolecules such as nucleic acids, carbohydrates and proteins are also readily separated with this method.^{12,14,15} It is also applicable for ionizable small molecules and can aid in their purification from complex sources.^{12,16,17}

As of now the molecular properties of the antibiotic compound remain unclear. An explorative IEC method similar to the approach of Måansson *et al.* was devised to test the applicability of this technique.¹¹ 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.¹⁸ 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.¹⁹ Further characterization with greater natural product libraries strengthened this theory.^{20,21} *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*.^{20,21} Producing organisms are examples featured in the PubChem database and 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. verticillus</i>	5360373
Myxin	DNA strand breaking	Fungi	72510
Phleomycin	DNA strand breaking	<i>S. verticillus</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
Desmethylsiberiromycin	DNA binding	<i>Streptomyces</i> spp.	6437361
Porfiromycin	DNA crosslinking	<i>S. arduus</i>	13116

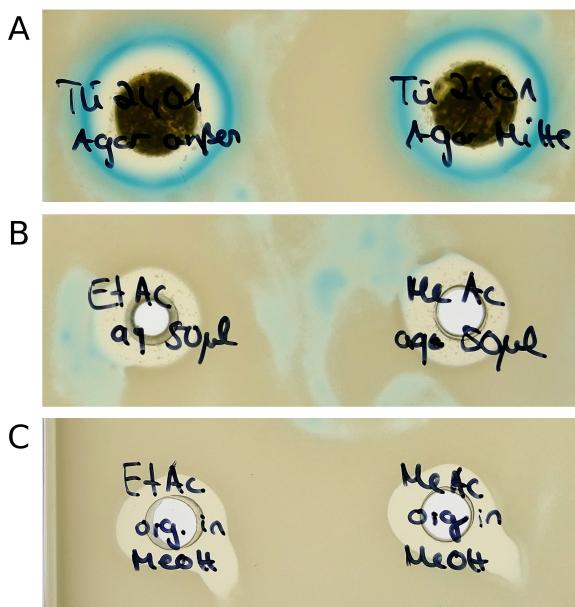


Figure 3.4: Results of the *yorB* reporter system bioassay with strain Tü2401 Foto of the bioassay plate taken one day after incubation.

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

The genome of *Streptomyces* sp. Tü2401 was fully sequenced and obtained in five contigs. It encompasses 8,139,204 base pairs with a GC content of 71.4 %. 4627 open-reading frames were identified by automatic annotation. A brief summary of each contig is shown in Table 3.3.

Analysis of the whole-genome sequence might lead to valuable insights into the phylogeny of the strain and its potential for secondary metabolite production.

3.5.1 Phylogeny of Strain Tü2401

50 single-copy household genes were identified in the Tü2401 genome and 50 reference genomes of fully sequenced *Streptomyces* strains. The multiple sequence alignment of these genes was used to construct the maximum-likelihood tree shown in Figure 3.5.

The closest relative to Tü2401 is *Streptomyces mediolani* NBRC15427. The first members of this species were isolated 1969 in Milan and investigated for carotenoid production.^{22,23} Publications in regard to secondary metabolite are sparse. (–)-8-O-methyltetrangomycin is mentioned to be synthesized by strain AC37; an angucycline with antibiotic activity against *Listeria monocytogenes* and cytotoxic properties.^{24,25}

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*

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			-	-

Table 3.3: Contig summary of the sequenced *Streptomyces* sp. Tü2401 genome.

Base pair count, automatically identified open-reading frames (ORFs) and GC-content is listed for each contig sorted by size. Total values were calculated from the summary of individual records.

Contig	Base pairs	ORFs	GC-content [%]
1	3 481 134	3221	71.6
2	2 271 151	2062	71.7
3	2 103 347	1953	71.2
4	159 117	135	69.1
5	124 275	145	70.6
Total	8 139 204	4627	71.4

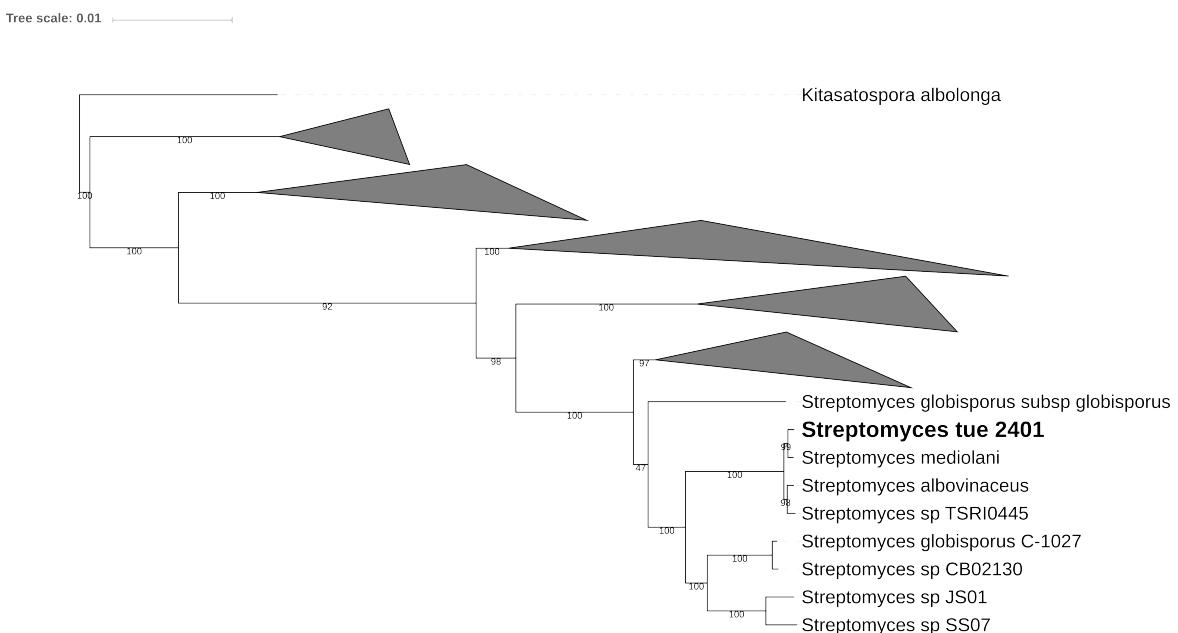


Figure 3.5: Maximum likelihood tree of *Streptomyces* sp. Tü2401. The tree was constructed using a multiple-sequence alignment of 50 single-copy genes across 50 *Streptomyces* reference genomes. The node belonging to *Streptomyces* sp. Tü2401 is highlighted with bold text. Only the nine most closely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

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.^{26–29} 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.²⁶ Enediynes are potent cytotoxic agents because of their ability to induce DNA double-strand breaks.³⁰ 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.²⁶ The nine-membered chromophore of C-1027, which was isolated from *Streptomyces globisporus* C-1027, is bound noncovalently to an 110 amino acid apoprotein.^{28,31–38} The isolated chromophore has been shown to be very unstable, whereas the holo C-1027 did not lose activity under the same conditions.^{34–36} The apoprotein binds specifically to the C-1027 chromophore, supposedly by hydrophobic pocket, which binds the benzoxazine side chain.^{35,39} The enediyne antibiotics neocarzinostatin and maduropeptin, which were also isolated from actinomycetes, feature highly specific and protective apoproteins as well.²⁸

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.²¹ 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.

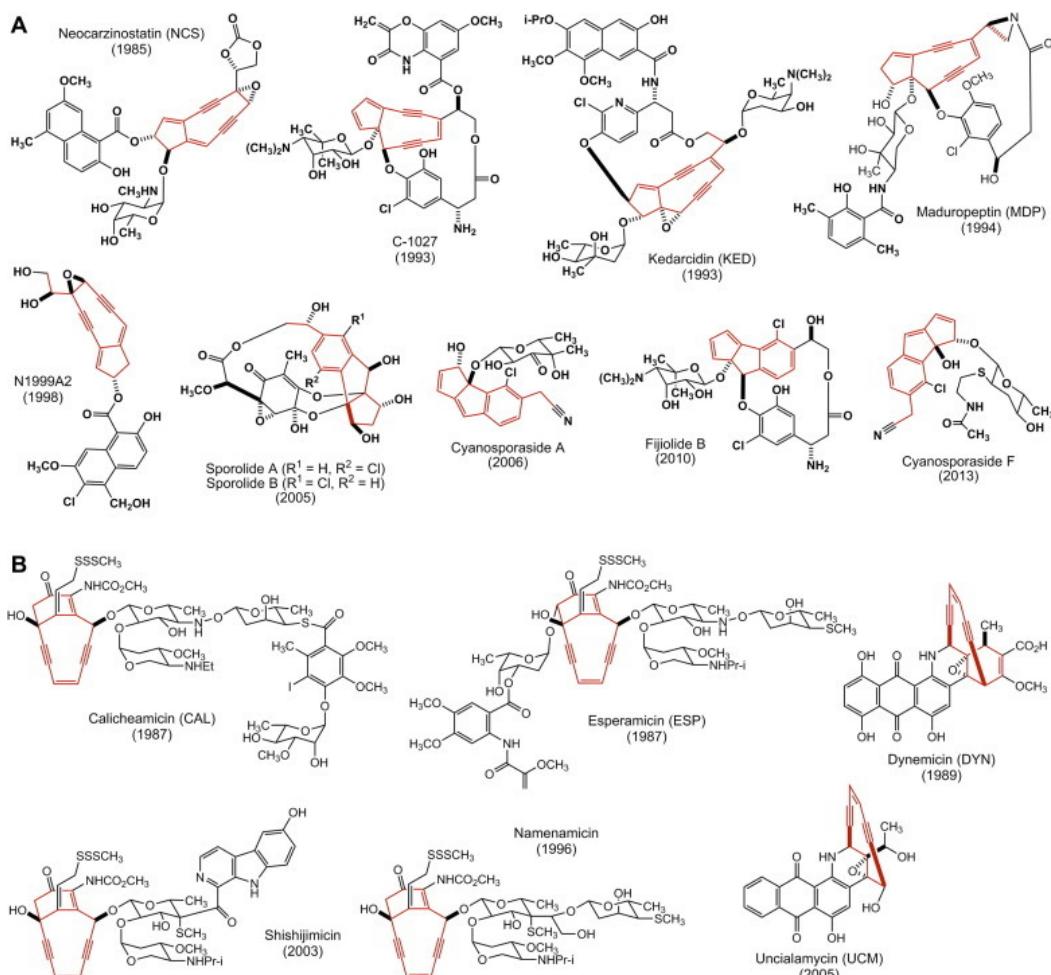


Figure 3.6: **Structures of known enediyne natural products.** Enediyne cores are highlighted in red. (A) Compounds with nine-membered rings. (B) Compounds with ten-membered rings. The year of structure confirmation is displayed in parentheses. The sporolides, cyanosporolids and fijiolides do not contain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

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.³⁷ The active chromophore could be extracted from the apoprotein with methanol at 0 °C.³⁵ 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.⁴⁰

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.^{26,41} Isolation of a new endiyne compound could hold a strong promise for the discovery of a new anticancer drug lead structure.

3.6 Fermentation of *Streptomyces* sp. Tü2401

During the course of the thesis, *Streptomyces* sp. Tü2401 has been cultivated in 50–500 ml shake flasks without baffles. Culture volumes from 10 to 100 ml could be reached with this approach, leading to roughly 21 mg/ml biomass and 2 mg/ml reverse extract (see Section XXX). This yields of about 200 mg reverse extract per 500 ml flask, of which the content of antibiotic could be very small. Great amounts of extract, which are necessary for further purification and structure elucidation, are very labor intensive to achieve this way. This problem could be solved by cultivating of *Streptomyces* sp. Tü2401 at a greater scale, providing a larger amount of biomass in a labor-efficient manner. Additionally, fermentation would further investigate the growth conditions, under which the active antibiotic is produced. These can vary heavily between shake flasks and stirred bioreactors, especially in regard to oxygen levels and perturbation.⁴²

The strain was fermented in OM medium at a 10 L scale in a continuous stirred tank bioreactor. The temperature was kept at 27 °C with 5 L min⁻¹ of airflow. Samples were drawn every 24 h and the culture broth supernatant subjected to the standard antibiotic assay against *E. coli* K12. The pH only decreased marginally throughout fermentation, decreasing from the initial 6.4 to the final 6.0. After 125 h the peak diameter of inhibition zone on agar plates had been observed and the culture was harvested. Cells and insoluble matter were removed by filtration, followed by extraction with ethyl acetate. Both phases were collected and concentrated by evaporation and lyophilization. The processing steps and final yields are shown in Figure 3.7. The filtrate and extraction phases were subjected to the antibiotic assay against *E. coli* K12 and *B. subtilis* 168. Both the filtrate and aqueous phase were active against *E. coli* and *B. subtilis* with inhibition zones comparable to samples from shake flasks. The organic phase was inactive.

The experiment demonstrated, that *Streptomyces* sp. Tü2401 is not only able to be cultivated in stirred bioreactors at a 10 L scale, but is also able to synthesize the hydrophilic antibiotic under these conditions. This enables larger cultivation scales to obtain greater amounts of the active compound for purification and structure determination. When combined with sufficient quantization methods for the active compound, further optimization of culture conditions can be performed to maximize yields. The filtrate and extracts still need to be subjected to the *yorB*-assay to determine, whether the putative DNA-binding compound is synthesized in this setup.

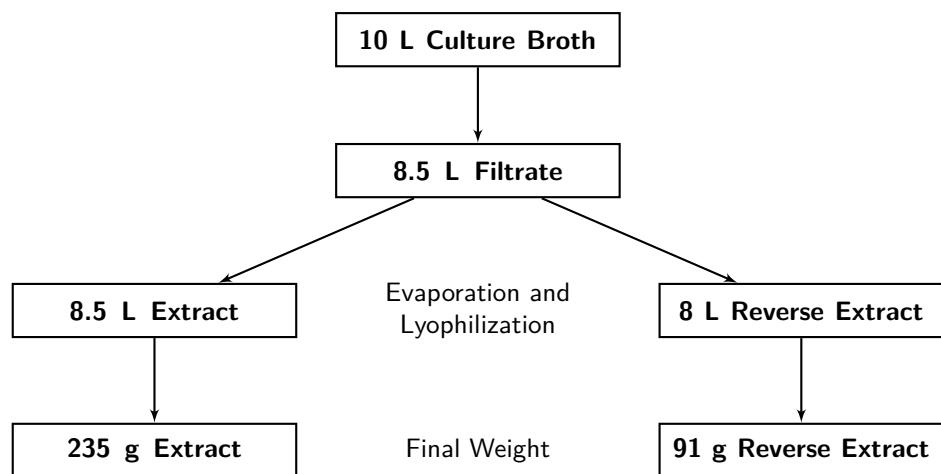


Figure 3.7: Yield Diagram of *Streptomyces* sp. Tü2401 Fermentation.

Volumes of each processing step and final dry mass yields after fermentation of *Streptomyces* sp. Tü2401 in a 10 L stirred bioreactor.

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