CHARLES UNIVERSITY IN PRAGUE

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Department of Analytical Chemistry

Liquid Chromatography Methods for Analysis of Actinomycete Secondary Metabolites – Potential Antibiotics

Metody kapalinové chromatografie pro analýzu sekundárních metabolitů aktinomycet – potenciálních antibiotik

Dissertation thesis

Prague, 2012 **Mgr. Zdeněk Kameník**

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The author solemnly declares that this dissertation thesis is based on his original data, all other sources are appropriately referenced and that the entire thesis has not been submitted for obtaining other university degree. The extent of author's participation in the presented research papers attached in the thesis is stated in the following text.

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The presented thesis is based on the following papers that are included as appendices:

Paper 1

Zdeněk Kameník, Jan Kopecký, Markéta Marečková, Dana Ulanova, Jitka Novotná, Stanislav Pospíšil, Jana Olšovská: HPLC-fluorescence detection method for determination of key intermediates of the lincomycin biosynthesis in fermentation broth; *Analytical and Bioanalytical Chemistry*. 2009, 393, 1779–1787.

Paper 2

Dana Ulanova, Jitka Novotná, Yvona Smutná, Zdeněk Kameník, Radek Gažák, Miroslav Šulc, Petr Sedmera, Stanislav Kadlčík, Kamila Plháčková, Jiří Janata: Mutasynthesis of lincomycin derivatives with activity against drug-resistant staphylococci; *Antimicrobial Agents and Chemotherapy*. 2010, 54 (2), 927–930.

Paper 3

Tereza Tylová, Zdeněk Kameník, Miroslav Flieger, Jana Olšovská: Comparison of LC columns packed with 2.6 μm core-shell and sub-2 μm porous particles for gradient separation of antibiotics; *Chromatographia*, 2011, 74, 19–27.

Paper 4

Jana Olšovská, Zdeněk Kameník, Tomáš Cajthaml: Hyphenated ultra high--performance liquid chromatography-nano quantity analyte detector technique for determination of compounds with low UV absorption; *Journal of Chromatography A*. 2009, 1216, 5774–5778.

Paper 5

Zdeněk Kameník, Franz Hadacek, Markéta Marečková, Dana Ulanova, Jan Kopecký, Vladimír Chobot, Kamila Plháčková, Jana Olšovská: Ultra-high-performance liquid chromatography fingerprinting method for chemical screening of metabolites in cultivation broth; *Journal of Chromatography A*. 2010, 1217, 8016–8025.

DECLARATION OF CO-AUTHORS

On behalf of the other co-authors I declare that Mgr. Zdeněk Kameník contributed substantially to **Paper 1** (his share 75%) and his participation in this paper was following: nearly all the experimental work, data evaluation, manuscript preparation, and corresponding author.

On behalf of the other co-authors I declare that Mgr. Zdeněk Kameník contributed substantially to **Paper 3** (his share 45%) and his participation was following: a half of the experimental work, data evaluation and manuscript preparation, and corresponding author.

On behalf of the other co-authors I declare that Mgr. Zdeněk Kameník contributed substantially to **Paper 4** (his share 50%) and his participation was following: all the experimental work, partially data evaluation and manuscript preparation.

On behalf of the other co-authors I declare that Mgr. Zdeněk Kameník contributed substantially to **Paper 5** (his share 80%) and his participation was following: nearly all the experimental work, data evaluation and manuscript preparation, corresponding author.

Prague, January 2012 RNDr. Jana Olšovská, Ph.D.

On behalf of the other co-authors I declare that Mgr. Zdeněk Kameník contributed substantially to **Paper 2** (his share 20%) and his participation was following: part of the experimental work and data evaluation (analytical chemistry part).

Prague, January 2012 Ing. Jiří Janata, CSc.

ABSTRACT (EN)

This dissertation thesis contains scientific results achieved in the field of analytical chemistry, particularly liquid chromatography. The major part of the results has been published in prestigious international journals in five papers. In addition to that, relevant yet unpublished results have been included as well.

In general terms, the work presented here contributed to the concerted efforts to tackle the current lack of novel antibiotics. Specifically, high-performance liquid chromatography (HPLC) and ultra high-performance liquid chromatography (UHPLC) techniques coupled to a variety of detection systems have been employed for analysis of antibiotics and actinomycete secondary metabolites.

The first thematic part describes the development of liquid chromatography methods for analysis of lincomycin precursors, lincomycin precursor analogues, and lincomycin derivatives. The methods have been applied to study lincomycin biosynthetic pathway and obtain improved lincomycin derivatives by mutasynthesis.

The second thematic part aims at investigating alternative approaches for analysis of antibiotics. Firstly, the core-shell particle and the sub-2 μm particle chromatographic columns were compared. The core-shell particle columns compatible with HPLC proved to be a convenient alternative to the sub-2 μm particle columns compatible only with UHPLC. However, it applies only for analysis of tetracyclines under acidic conditions, not for analysis of macrolides under alkaline conditions. Secondly, the compatibility of a condensation nucleation light-scattering detector and the UHPLC system was investigated under both isocratic and gradient conditions. Also, the limits of detection for several macrolides were found to be significantly lower with this detector than with an ultraviolet detector.

The third thematic part focuses on the development and application of a universal fingerprinting method for secondary metabolites in cultivation broth of actinomycetes. The method is based on UHPLC with diode-array ultraviolet detection and provides two 3D fingerprints of secondary metabolites for a sample. The fingerprints contain physico-chemical information on the fingerprinted analytes, which can be used for further statistical evaluation.

ABSTRACT (CZ)

Tato disertační práce obsahuje vědecké výsledky dosažené v oboru analytické chemie, konkrétně kapalinové chromatografie. Většina těchto výsledků byla uveřejněna v prestižních zahraničních časopisech v pěti publikacích. Navíc obsahuje práce některé významné, dosud nepublikované výsledky.

Z obecného pohledu přispěla tato práce k současným snahám vynakládaným za účelem objevu nových antibiotik. Konkrétně byly v rámci této práce využity techniky vysokoúčinné kapalinové (HPLC) a ultra-vysokoúčinné kapalinové chromatografie (UHPLC) pro analýzu antibiotik a sekundárních metabolitů aktinomycet.

První tematický okruh popisuje vývoj metod kapalinové chromatografie pro analýzu prekurzorů linkomycinu a jejich analogů a derivátů linkomycinu. Metody byly využity pro studium biosyntézy antibiotika linkomycinu a pro získání účinnějších derivátů linkomycinu pomocí mutasyntézy.

Druhý tematický okruh je věnován alternativním přístupům pro analýzu antibiotik. Chromatografické kolony s povrchově porézními částicemi byly porovnány s kolonami obsahujícími částice menší než 2 μm. Kolony s povrchově porézními částicemi, které jsou kompatibilní s HPLC, se ukázaly být vhodnou alternativou k druhému uvedenému typu kolon, který lze využít pouze pro UHPLC. Toto zjištění však platí pouze pro analýzu tetracyklinů v kyselých chromatografických podmínkách, nikoli pro analýzu makrolidů v alkalických podmínkách. Dále bylo studováno spojení ultra-vysoko účinného kapalinového chromatografu s detektorem využívajícím technologii kondenzace a tvorby aerosolu (CNLSD). Toto spojení bylo kompatibilní jak v izokratickém, tak v gradientovém módu. Současně bylo zjištěno, že limity detekce některých makrolidů jsou ve spojení s CNLSD detektorem až třikrát nižší než v případě detekce v ultrafialové oblasti (UV).

Poslední tematický okruh se zabývá vývojem a aplikací univerzální fingerprintové metody zaměřené na analýzu sekundárních metabolitů v kultivačním médiu aktinomycet. Metoda je založená na ultra-vysokoúčinné kapalinové chromatografii ve spojení s UV detektorem diodového pole a poskytuje dva 3D fingerprinty pro každý vzorek. Tato dvojice fingerprintů obsahuje informace o fyzikálně-chemických vlastnostech jednotlivých látek, které mohou být dále využity pro statistické zpracování.

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LIST OF SYMBOLS AND ABBREVIATIONS

A	Area under the chromatographic peak
3D	Three dimensional
APCI	Atmosphere pressure chemical ionization
ASCII	American standard code for information interchange
BEH	Bridged ethylene hybrid
BuLIN	4´-Butyl-4´-depropyllincomycin
BuPL	4-Butyl-L-proline
c	Analyte concentration [$\mu g\ ml^{-1}$]
CID	Collision-induced dissociation
CNLSD	Condensation nucleation light-scattering detector
DAD	Diode-array detector
DNA	Deoxyribonucleic acid
ELSD	Evaporative light-scattering detector
ESI	Electrospray ionization
HILIC	Hydrophilic interaction chromatography
HLB	Hydrophilic-lipophilic balanced copolymer
HPLC	High-performance liquid chromatography
i.d.	Inner diameter
LC	Liquid chromatography
LIN	Lincomycin A
m/z	Mass-to-charge ratio
MAX	Mixed-mode anion-exchange sorbent
MCX	Mixed-mode cation-exchange sorbent
MS	Mass spectrometry
MTL	Methylthiolincosamide
NDL	*N*-Demethyllincomycin
NMR	Nuclear magnetic resonance
NQAD™	Nano quantity analyte detector
OSMAC	One-Strain-Many-Compounds approach
PeLIN	4´-Pentyl-4´-depropyllincomycin
PePL	4-Pentyl-L-proline
PPL	4-Propyl-L-proline
R^2	determination coefficient
RSD	Relative standard deviation
SPE	Solid-phase extraction
UHPLC	Ultra high-performance liquid chromatography
UV	Ultraviolet
WT	Wild type strain (natural strain)
YT	4-(3-carboxy-3-oxo-propenyl)-2,3-dihydro-1*H*-pyrrole-2-carboxylic acid

1 INTRODUCTION

Antibiotics are the most powerful tool for the treatment of infections caused by bacteria or fungi. Therefore, the increasing number of pathogenic strains resistant to different types of antibiotics is a major medical problem today.[1] Given the resistance of pathogenic strains, often multiple, and new types of infections, there is a constant need of searching and developing of new antimicrobial agents.[2] The bacteria actinomycetes, particularly the genus *Streptomyces* sp., proved to be the most potent source of natural antibiotics. The majority of antibiotics including those actinomycetes-derived were discovered in the "Golden Age" of antibiotics, in the 1940s-1960s. Since then, the rate of novel antibiotics has declined dramatically.[3] Surprisingly, actinomycetes still represent a tremendous reservoir of yet unknown antibiotics as evidenced by Watve *et al.*[4] However, to exploit this source of antibiotics, improved strategies have to be deployed. Current strategies applied to overcome the lack of novel antibiotics include collection of actinomycete strains from extreme habitats, the OSMAC (One-Strain-Many-Compounds) approach, culture--independent methods, genome mining approach, mutasythesis, and combinatorial biosynthesis. For these strategies, it is essential to employ modern methods of analytical chemistry. In fact, analytical chemistry methods are even mandatory for secondary metabolite screening, antibiotic isolation, and biosynthetic pathway study. Also, they are essential for preparation of unnatural compounds by means of mutasynthesis or combinatorial biosynthesis. Therefore, to obtain novel antibiotics, it is crucial to focus on the development of analytical methods, particularly liquid chromatography techniques, which are efficient and universal enough for the respective type of analytes.

This dissertation thesis begins with a theoretical background, which offers a broad context to the topic. It is divided into two parts. The first part attempts to outline the topic from the perspective of microbiology and genetics as well as introduce the analytes of interest – secondary metabolites of actinomycetes (chapter 2). The second part deals with the perspective of analytical chemistry in the topic by introducing the liquid chromatography techniques (chapter 3). Next, the objectives of the work (chapter 4), the instrumentation and the experimental procedures for the unpublished results (chapter 5) are described. Further, the results

and discussion section gives a brief summary of the published results and provides information on the unpublished results in more detail (chapter 6). The supplementary data section reveals chemical structures of selected actinomycete secondary metabolites and their UV spectra. For more specific introductory parts and literature reviews, description of the experimental procedures and detailed information on the results and discussion, see the relevant papers published by the author, attached as the appendices.

2 ANTIBIOTICS FROM NATURE SOURCES

This chapter aims to introduce the origin of antibiotics, define them, outline the research concerned with the discovery of novel antibiotics and characterize the antibiotic producers.

2.1 Secondary Metabolites, Antibiotics, and Their Producers

Before defining antibiotics and revealing their producers, we have to start with a more general term – secondary metabolites. What are these metabolites, where do they come from and why do they exist? Organisms, predominantly plants, fungi and bacteria, produce and excrete a broad spectrum of weird chemicals as illustrated in Fig 2.1. These mostly low-molecular-weight chemicals are referred as to secondary metabolites. Compared to primary metabolites (*i.e.* amino acids, fatty acids, carbohydrates, nucleotides, proteins, lipids, polysaccharides, DNA), secondary metabolites occur in significantly higher diversity and their role, which is discussed below, is much more mysterious.

2.1.1 Secondary Metabolites in Nature

A universal explanation why secondary metabolites are actually produced and what their function is has been intensely discussed since the beginning of the 20th century when scientists finally began to explore this issue. Some of the most important hypotheses reviewed in the literature[5-12] are outlined as follows. The suggestion that secondary metabolites represent an alternative to livers and kidneys and serve as detoxification or waste products was denied. Other ideas considered were that these compounds are relics of previous specific use (a functional or metabolic role), or that they are produced only “by chance” due to random mutations. Also, because of their biological activity, some secondary metabolites have been seen as chemical warfare or more generally as tools for chemical interactions between organisms. This led to the development of the chemical co-evolution concept claiming that interactions between organisms mediated by chemicals could represent a selective force in the evolution.

Within the scope of this text, it is sufficient to define secondary metabolites as compounds that are not directly involved in the normal growth, development, or reproduction of an organism (contrary to primary metabolites), but provide other benefits to the producing organism required for its survival in the environment. In other words, secondary metabolites are dispensable for sustaining life but indispensable for long term survival.

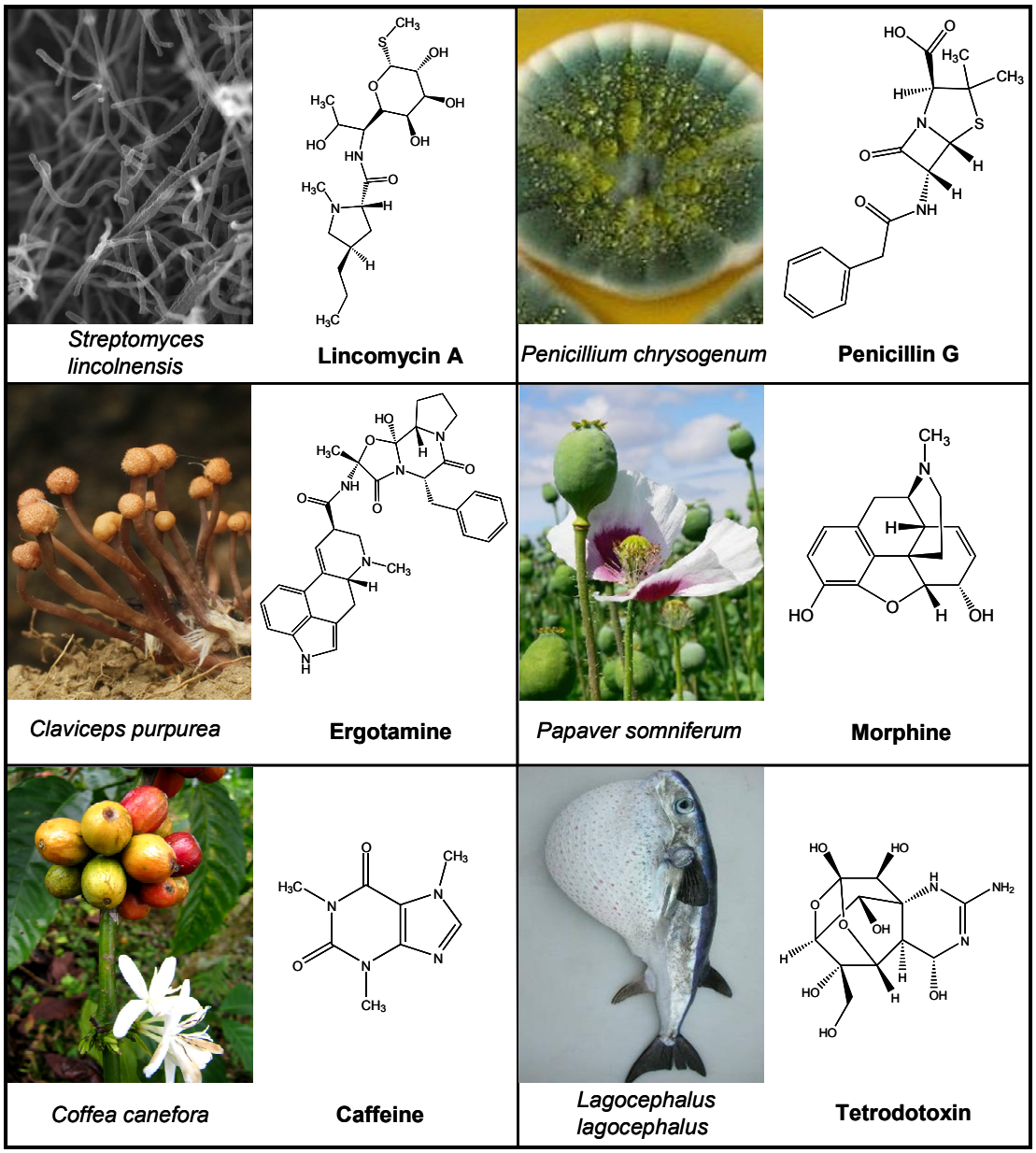

Fig 2.1 Examples of secondary metabolites and their producers.[13-16]

2.1.2 Secondary Metabolites as Antibiotics

A great number of secondary metabolites have been utilized by man. Particularly biologically active secondary metabolites performing antibacterial, antifungal, antiviral, and antitumor activities have been employed for drug development. Compounds with antibacterial (and not necessarily only) properties are called antibiotics and have been widely applied in medicine for treatment of infections caused by pathogenic bacteria.

2.1.3 Actinomycetes as Antibiotic Producers

Actinomycetes, and particularly the genus *Streptomyces*, are renowned as producers of antibiotics, many of which have been developed into successful drugs. Almost a half of the described antibiotics originate from actinomycetes.[17] Among them, erythromycin, tetracycline and their semi-synthetic derivatives as well as chloramphenicol, novobiocin, vancomycin and others used to belong or still belong to important antibiotics in clinical use.

2.2 Current Problems in the Antibiotic Issue

The society has encountered two fundamental problems concerning the antibiotic issue. Firstly, it is the increase of resistance of pathogenic bacteria to current antibiotics; and secondly, it is the decrease of novel antibiotics recently discovered. If we fail to resolve these problems, the consequences may be serious. Without new and effective antibiotics, but with increasing resistance, the society could return to the conditions of a pre-antibiotic era, when a simple lung infection could kill a child, or when doctors could not fight meningitis.[18]

2.2.1 Resistance to Antibiotics

In contrast to other drugs, antibiotics can start to lose their efficacy immediately after their clinical use begins. This is because bacterial pathogens are able to develop resistance to antibiotics.[19] In fact, the rapidly growing number of antibiotic resistant bacteria, driven mainly by misuse of antibiotics, represents one of the most serious

public health problems nowadays.[2] As an example, Fig. 2.2 depicts the increasing rate of species resistant to three antibiotics: the most advanced β-lactam, methicillin; the antibiotic of last resort vancomycin; and synthetic fluorquinolones.[20] Moreover, in 2010 a recent worldwide threat appeared when the 'super bug' *Klebsiella pneumoniae* resistant to the majority of antibiotics emerged in India, Pakistan, and the UK.[21]

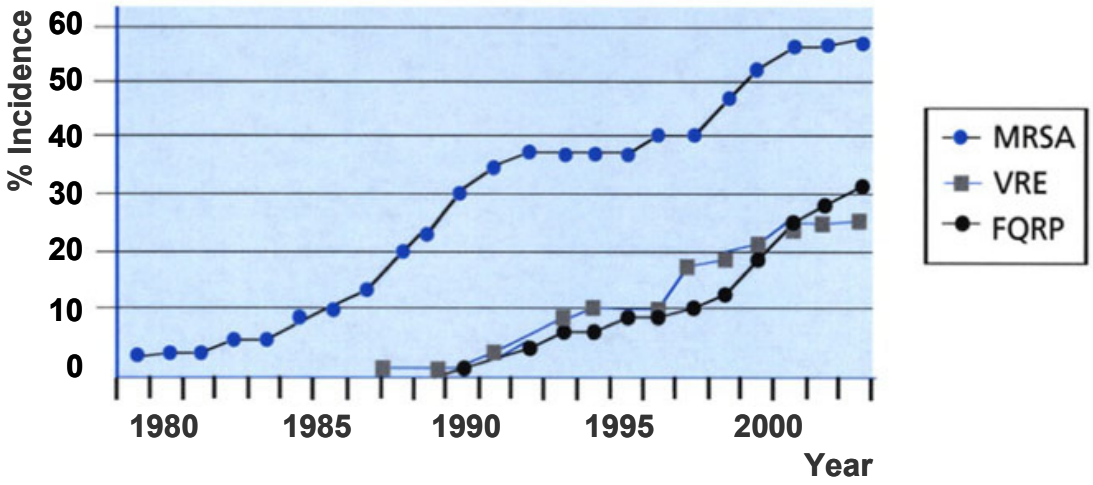

Fig. 2.2 Increasing resistance to antibiotics.[22] MRSA – methicillin-resistant *Staphylococcus aureus*; VRE – vancomycin-resistant *Enteroccoci*; FQRP – fluoroquinolone-resistant *Pseudomonas aeruginosa*.

2.2.2 Lack of Novel Antibiotics

The rate of new antibiotic discovery has recently declined dramatically and this trend is continuing (see Fig. 2.3).[3] This is primarily caused by economic, medical, social, and political factors[23]. But also, the traditional source of antibiotics, actinomycetes, appears to have been exploited because predominantly known compounds have recently been rediscovered[24-26]. However, M. G. Watve *et al.* estimated that only about 3% of the compounds produced by *Streptomyces* have been described so far, suggesting that this genus still represents a promising source.[4] R. H. Baltz explained this phenomenon by the idea that the already discovered antibiotics occur in high frequencies compared to those not yet discovered, which are very rare.[27] It presents a challenge for current and future attempts to obtain novel antibiotics. And only deployment of improved or novel strategies can be fruitful because all the easy--to-find antibiotics have already been found.

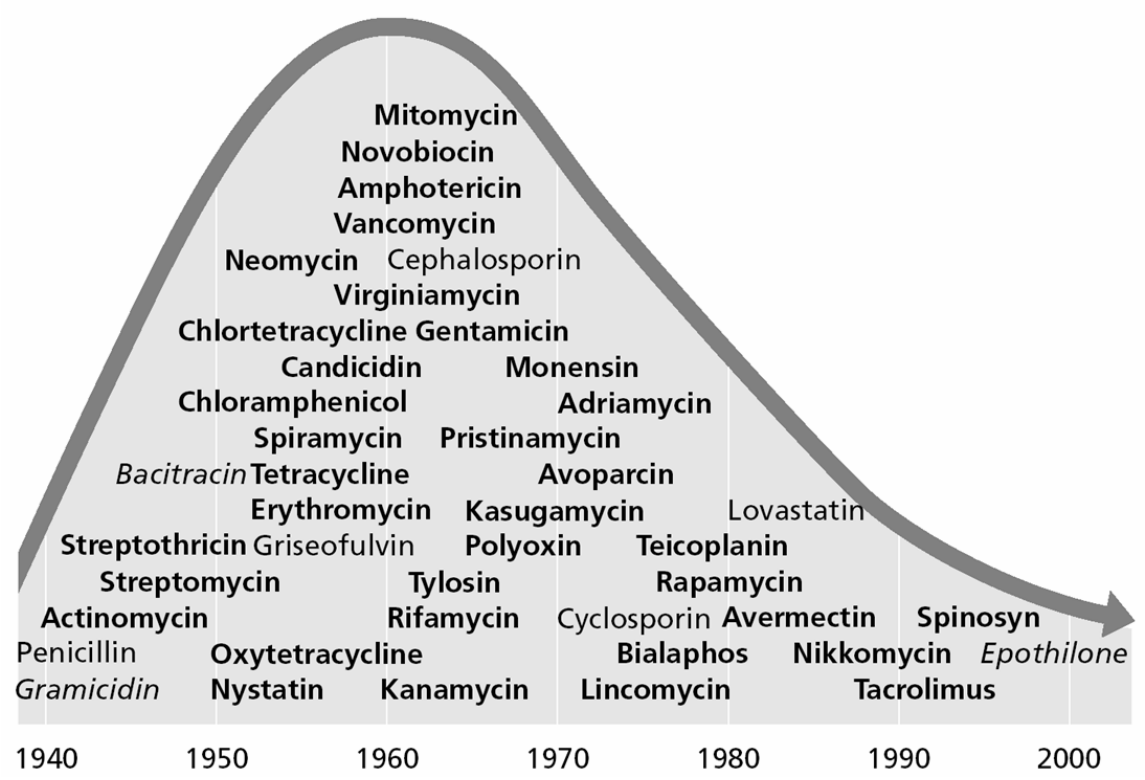

Fig. 2.3 Decreasing number of novel antibiotics.[28] Bolded antibiotics are actinomycete secondary metabolites.

2.3 Current Strategies for Obtaining Novel Antibiotics

This section summarizes the most important strategies recently deployed for discovery or preparation of novel antibiotics based on microbial products.

2.3.1 Collection of Strains

To collect the microorganisms, it is crucial to choose an appropriate site in order to eliminate uninteresting strains producing already known compounds. Diversity of the species within and among the collecting sites should be considered in particular. For these reasons, collecting environmental samples for isolation of interesting microorganisms producing promising secondary metabolites should not be performed randomly or without defined strategies.[26] Specifically, extreme habitats such as the deep subsurface, the deep sea, and sites that have extreme temperature, salinity or pH may generate novel microorganisms and therefore provide the potential for novel secondary metabolites.[26,29]

2.3.2 Cultivation Conditions and OSMAC Approach

Although, the genome of actinomycetes contains on average between 25 and 35 biosynthetic gene clusters[30] coding for production of 25 to 35 different secondary metabolites, only a few of them are produced under standard laboratory conditions. Interestingly, the production of individual secondary metabolites depends highly on the cultivation conditions. Hence, the modification of cultivation broth composition and pH, temperature, time period, shaker speed, aeration, etc. can trigger the production of secondary metabolites not observed before. Along these lines, the OSMAC (One-Strain-Many-Compounds) approach aims at revealing all secondary metabolites that a strain is capable of producing.[31] Another interesting strategy is co--cultivation of two or more strains in one flask where the production of secondary metabolites is induced by nutritional competition of the strains.[32, 33]

2.3.3 Culture-Independent Methods

In one study, only 0.1% of soil bacteria could be cultured under laboratory conditions.[34] Since the majority of bacteria from environmental samples are unculturable, a large unexplored reservoir of novel strains and compounds is missed.[35] Therefore, culture-independent methods appear to be a great alternative. DNA from microorganisms including clusters for production of secondary metabolites can be isolated directly from nature samples and the DNA can be then cloned into suitable recipients. Both, the gene function (metabolite production) and the gene sequence is subsequently explored.[36]

2.3.4 Sequence Analysis and Genome Mining

With recent advances in genome sequencing, a huge quantity of DNA sequence data accumulates in publicly accessible databases. More than 1700 complete microbial genome sequences are available[37] including several *Streptomyces* species, *e.g. S. coelicolor, S. avermetilis*, and *S. griseus*. In these well known producers, several cryptic gene clusters corresponding to yet unknown secondary metabolites have been revealed by exploring the data.[38-40] Moreover, the genetic information is used for *in sillico* prediction of the type of secondary metabolite or even its structure

by genome mining method.[41] Additionally, analysis of the genetic information may reveal the reason why the compound has not been produced and may suggest a solution, for example to apply the OSMAC approach or positively affect regulation of the production by genetic methods.

2.3.5 Mutasynthesis and Combinatorial Biosynthesis

Mutasynthesis and combinatorial biosynthesis use chemical, molecular biological and gene engineering methods for preparation of novel compounds. Mutasynthesis is based on the fact that the substrate specifity of biosynthetic enzymes is often relaxed.[11] Fig. 2.4a illustrates natural biosynthesis of an antibiotic from two precursors, while Fig. 2.4b illustrates precursor-directed biosynthesis. In the latter case, a chemically synthesized analogue of one antibiotic precursor is added to the culture of a strain producing the antibiotic. As a result, a mixture of the antibiotic and its derivative is produced.[42] On the contrary, Fig. 2.4c illustrates the mutasynthetic approach where a mutant instead of a natural producer is cultivated. The mutant lacks a specific gene(s) and is consequently defective in biosynthesis of one precursor. Therefore, adding the analogue of the precursor to the culture medium of the mutant results in biosynthesis of the antibiotic derivative only.[43]

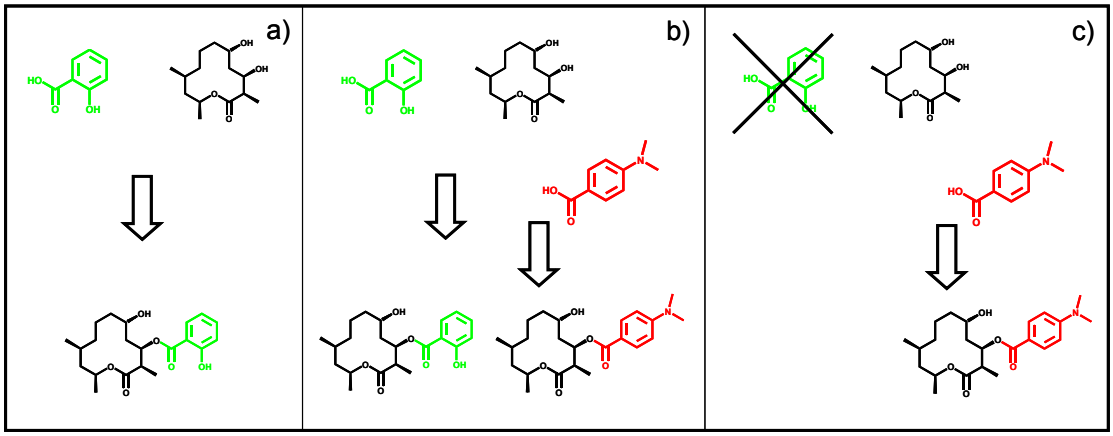

Fig. 2.4 Semi-synthetic methods for preparation of antibiotic derivatives.[42] **a)** Natural biosynthesis; **b)** Biosynthesis governed by precursor; **c)** Mutasynthesis

Combinatorial biosynthesis omits chemical synthesis because it is based only on gene manipulation. It combines genes from at least two biosynthetic clusters of

usually related organisms. The unnatural combination of genes codes for enzymes that enable production of unnatural, hybrid antibiotics.[44]

2.4 Secondary Metabolites Produced by Actinomycetes

Microorganisms in general and actinomycetes in particular produce a vast number of secondary metabolites. Their chemical diversity is enormous and it is hard to imagine a more heterogeneous group of chemical structures. The most important actinomycete secondary metabolites and other deliberately selected antibiotics (either structurally remarkable or clinically important) are introduced in the following overview. This section describes chemical structures of the compounds and outlines general liquid chromatography techniques for their analysis, focusing on UV absorption. Chemical structures and UV spectra of selected compounds are given in supplementary data. Besides, additional information is provided on lincosamides because these compounds were particularly researched in this work.

2.4.1 Aminoglycosides and Aminocyclitols

The first member of the aminoglycoside class, streptomycin, was discovered in 1944 by S. Waksman.[45] Since then over one hundred aminoglycoside antibiotics have been found, among them gentamycin (**1**), kanamycin (**2**), tobramycin (**3**), neomycin (**4**), paromomycin (**5**), spectinomycin (**6**) and others.[46] All the compounds are closely related water-soluble basic pseudosaccharides usually composed of two aminosaccharide units attached to an aminocyclitol, typically streptidine or 2-deoxystreptamine.[46, 47]

Analytical methods including chromatography techniques for aminoglycosides have been reviewed *e.g.* by D. A. Stead.[48] Since aminosaccharides form polybasic cations under low pH conditions, they can by separated by ion-exchange liquid chromatography[49] or ion-pair liquid chromatography employing reverse-phase chromatographic columns.[50] Reverse-phase liquid chromatography without ion-pair reagents in the mobile phase is not suitable due to high polarity of the analytes.[50] The aminoglycoside structure is lacking in free π electrons and any other chromophores or fluorophores.[51] Hence, they cannot be detected by UV or fluorescence unless a derivatization step is included. The derivatization not only enables the analyte

detection, but also allows separation of the less polar analyte derivatives in reverse--phase mode without ion-pair reagents.[52, 53] Alternatively, electrochemistry[54] or mass spectrometry[54, 55] can be applied for aminoglycoside detection.

2.4.2 Macrolides

Pikromycin (**7**) was the first macrolide antibiotic discovered by H. Brockman and W. Henkel in 1950.[56] Nowadays, more than 2000 macrolides belong to this heterogenous class of secondary metabolites. Macrolides can be defined as macrocyclic lactones. Their core is composed of a lactone ring, whose size ranges from 8- to 42-membered rings, as well as 44-, 48-, and 62-membered rings. Monolactones contain one lactone group in one macrocyclic ring, whereas macropolylides contain two or more lactone groups. Polyene monolactones are characterized by non-saturated bonds – typical polyenes are trienes, tetraenes, pentatenes, hexaenes, or heptaenes. Macrolide lactones with nitrogen in their skeleton (azamacrolides and macrolide lactams) or with oxazole or thiazole occur as well. The macrocyclic ring is generally ornamented by one or two mono- or disaccharide units attached by α- or β-glycosidic linkages. One of the sugar groups usually bears a tertiary amino group or is substituted in a different way. Apart from the sugar groups, glutarimide, long chains (epoxy olefins), phenyl- and cycloalkyl- derivatives or a ring fused to the macrocyclic skeleton are the most prevalent ornaments.[57] Among actinomycete products, the following are due to mention: octalactin A and juglorubin (both consisting of an 8-membered lactone ring); a macrodiolide actimycin A (9-membered); methymycin (12-membered); pikromycin, erythromycin (**8**), clarithromycin (**9**) and oleandomycin (**10**) (14-membered); avermectin, tylosin (**11**), angolamycin (**12**), carbomycin (**13**), kitasamycin (**14**), and spiramycin (**15**) (16-membered); tacrolimus (21-membered); and rapamycin (**16**) (29-membered). The 14- and 16-membered lactone ring compounds are those that are most important clinically, whereas tacrolimus and rapamycin are potent immunosuppressants.

The majority of macrolides are less polar lipophilic compounds. They exhibit basic properties provided that the tertiary amino group is present in the molecule. Methods for macrolide analysis have been reviewed by I. Kanfer *et al.*[58] or M.J.G. de la Huebra *et al.*[59] The employment of reverse-phase liquid chromatography fully complies with the relatively low polarity of the analytes. Macrolides are generally UV

detectable, but the response in UV and the absorption maxima of macrolides vary.[59] Basically, conjugated systems of double bonds in the macrocyclic ring cause the UV absorption. Maxima of the UV spectra depend on the number and the arrangement of the conjugated system. Erythromycin, clarithromycin, oleandomycin, troleandomycin, and roxithromycin lack any double bonds in the ring resulting in a poor absorption lower than 230 nm. By contrast, rapamycin, tylosin, angolamycin, carbomycin, kitasamycin, spiramycin, and avermectin,[60] all of them containing two double bonds in conjugation, exhibit a UV spectrum of one maximum (except rapamycin) between 230 and 290 nm. More complex conjugated systems can be found in polyenes such as natamycin (**17**) (four double bonds in conjugation), nystatin (**18)** (two and four), levorin (**19**) (seven) or trichomycin (**20**) (seven), which are characterized by very distinctive UV spectra with a strong absorption between 300 and 400 nm in three to five maxima. More special chromophores confer diverse UV maxima, such as 227 and 317 nm for actimycin A due to an aromatic chromophor[61] or 282 and 475-500 nm for a comlex chromophore of juglorubin.[62] Macrolides lacking in any strong chromophore may be derivatized and subsequently detected by fluorescence.[63, 64] They may also be detected by broadly applied electrochemical (amperometric[65, 66] or coulometric[67, 68]) or mass spectrometry[69, 70] detection.

2.4.3 β-Lactams

β-Lactam compounds undoubtedly started the entirely era of antibiotics when A. Fleming announced the discovery of penicillin as early as 1929.[71] As for the chemical structure, β-lactams may be divided into several groups, three of which are important in particular: (1) penicillins (penams), constituted of a sulphur-containing five-membered thiazolidine ring fused with a β-lactam ring ornamented with a variety of side-chains; (2) carbapenems, differing from the penicillins by the substitution of CH_2 for sulphur in the five-membered ring and by one unsaturated bond in the same ring; (3) cephalosporins derived from cephalosporin C, constituted of a sulphur--containing six-membered dihydrothiazine ring fused to a β-lactam substituted in various ways. Penicillin and cephalosporin C are produced by fungal microorganisms, *e.g. Penicillium chrysogenum* and *Cephalosporium acremonium*, respectively. However, actinomycetes produce secondary metabolites with β-lactam motif as well.

These include thienamycin (**21**), a hydrophillic carbapenem; cephamycin B (**22**), an analogue of cephalosporin C; nocardicin (**23**), a monocyclic β-lactam; and clavulanic acid (**24**), an oxopenam.[72]

The earliest separations of β-lactams obtained by ion-exchange chromatographic methods have been replaced by reverse-phase liquid chromatography techniques.[73] The detection of β-lactams can be performed by a UV detector.[74, 75] Penicillins including nocardicin reasonably absorb due to their phenyl ring(s) at low wavelengths in the range 200–240 nm (a tailing peak with its maximum around 202 nm) plus weakly at around 269 nm. Cephalosporins including cephamycin B show UV spectra with maxima around 200 and 260 nm conferred by a bicyclic β-lactam–dihydrothiazine structure. Alternatively, fluorescence after derivatization with a formaldehyd solution[76] or electrochemical (pulsed amperometric) detection of sulphur-containing β-lactams[77] have been applied. Moreover, various mass spectrometry detection techniques have been employed for analysis of β-lactams extensively.[78, 79]

2.4.4 Lincosamides

Lincosamides comprise a small group of compounds based on a unique structure unlike that of any other antibiotics. Lincosamides emerged in 1962 when D. J. Mason et al.[80] isolated lincomycin A (**25**) (hereinafter lincomycin). The number of lincosamides known today does not exceed a couple of substances including semi-synthetic derivatives. Lincomycin is composed of an amino acid trans-*N*-methyl--4-*n*-propyl-L-proline (propylhygric acid), linked *via* an amide bond to an amino-saccharide moiety, 6-amino-6,8-dideoxy-1-thio-D-erythro-α-D-galactooctopyranoside (methylthiolincosamide, MTL). By chloration of lincomycin, a clinically important semi-synthetic lincosamide clindamycin (**26**) is prepared. Unlike lincomycin, another natural lincosamide, celesticetin (**27**), lacks the propyl chain in the amino acid part, but bears a salicylate group attached to the aminosaccharide moiety.

Lincosamides are suitable analytes for reverse-phase chromatography, but ion--pair liquid chromatography has been applied as well. Lincosamides may be detected by all common detectors including mass spectrometry, electrochemical and UV detectors.[115] UV spectra of lincosamides are not distinctive since the UV absorption is

limited to low wavelengths (lower than 230 nm). The salicylate group confers to celesticetin maximal UV absorption at 240 and 305 nm.

2.4.4.1 Biosynthesis of Lincomycin

Lincomycin is biosynthesized by the actinomycete *Streptomyces lincolnensis* spp. as well as by *Micromonospora halophytica*, *S. espinosus*, *S. vellosus*, and *S. variabilis*. Biosynthetic pathway of lincomycin has not been described in details yet. The proposed biosynthetic pathway depicted in Fig. 2.5 proceeds *via* two separate branches from tyrosine and D-glucose (or D-octulose) to the amino acid unit, the aglycone 4-propyl-L-proline (PPL) and the aminosaccharide, methylthiolincosamide (MTL), respectively. Condensation of these two precursors *via* an amide bond yields *N*-demethyllincomycin (NDL), which is subsequently methylated to form lincomycin.[81-83]

Every step in the biosynthesis is determined by a gene(s) which codes for an enzyme that enables the conversion. The genes are arranged in the lincomycin biosynthetic cluster depicted in Fig. 2.6 and their proposed function is given in Table 2.1.[81, 84] The hypothetical biosynthesis of the lincomycin precursor PPL including putative functions of the participating biosynthetic genes (enzymes) responsible for PPL biosynthesis is suggested in Fig. 2.7.[81] In order to confirm the function of a specific gene (enzyme), a mutant strain with this gene inactivated is constructed. Then, exploring the production of lincomycin, its derivatives, and/or its precursors facilitates to reveal the function of the gene. Therefore, determination of lincomycin and related metabolites in the cultivation broth of *S. lincolnensis* represents a powerful tool for the biosynthetic pathway investigation. In addition to that, it should be emphasized that the precise knowledge of the lincomycin biosynthesis is a prerequisite for generation of improved derivatives by mutasynthesis or combinatorial biosythesis.

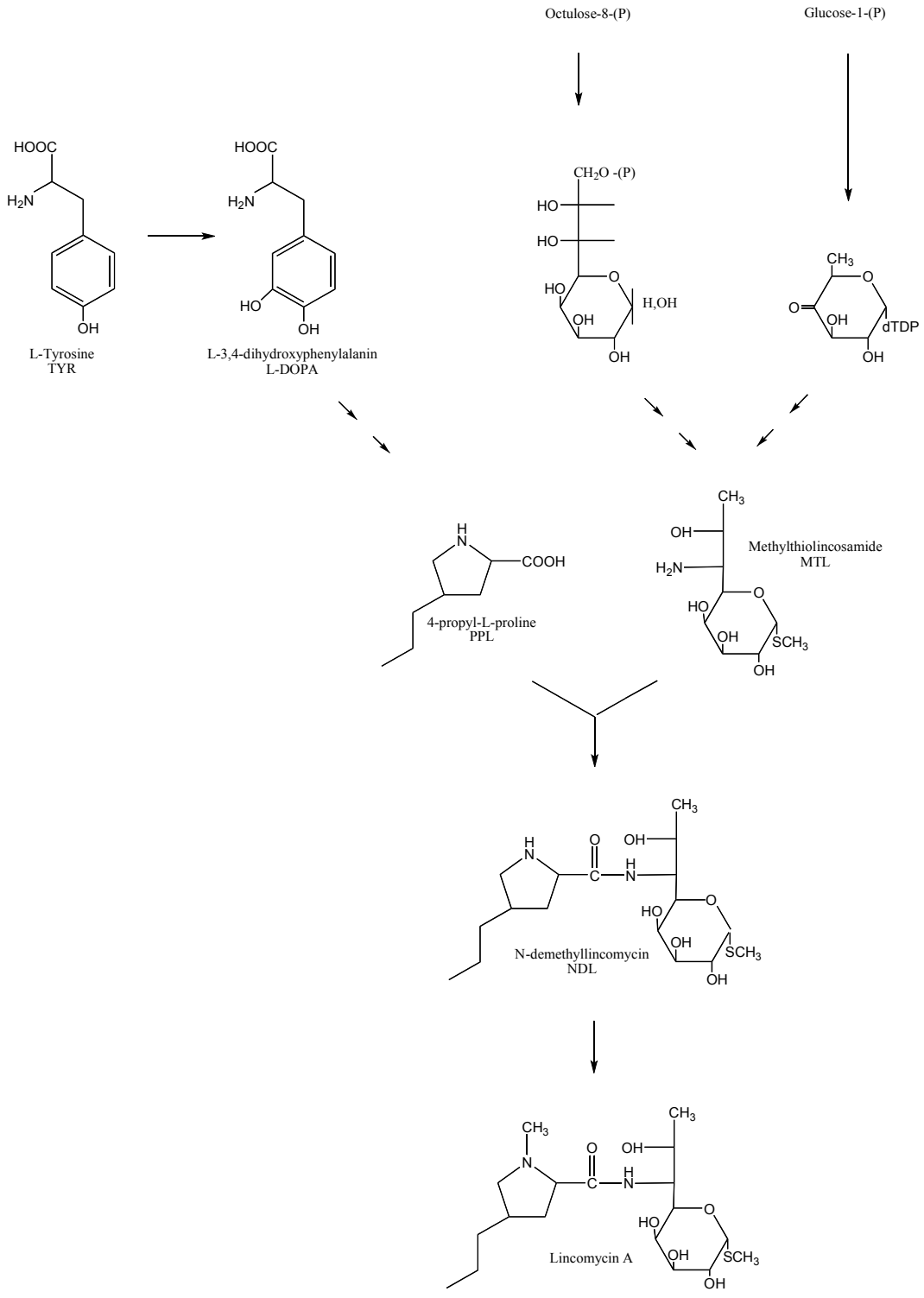

Fig. 2.5 Proposed scheme of the biosynthetic pathway of lincomycin.[81-83]

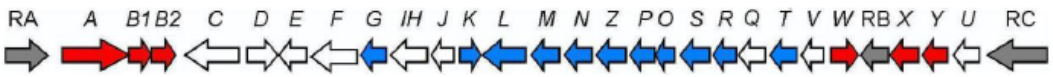

Fig. 2.6 Arrangement of genes in the lincomycin biosynthetic cluster.[84] Participation of the genes in the biosynthesis is marked: PPL synthesis (red), MTL synthesis (blue), other biosynthetic function and unknown function (white), resistance (grey).

Table 2.1 Proposed function of proteins (enzymes) coded by the genes of lincomycin biosynthetic cluster.[84] Colour coded as in Fig 2.6.

Gene	Participation in	Proposed enzyme function	Gene	Participation in	Proposed enzyme function
lmrA	resistance	transmembrane efflux protein	***lmbN***	MTL synthesis	isomerase
lmbA	PPL synthesis	unknown	***lmbZ***	MTL synthesis	oxidoreductase
lmbB1	PPL synthesis	L-DOPA converting	***lmbP***	MTL synthesis	kinase
lmbB2	PPL synthesis	L-DOPA converting	***lmbO***	MTL synthesis	nucleotidyl transferase
lmbC	condensation	adenylation of PPL	***lmbS***	MTL synthesis	aminotransferase
lmbD	condensation	unknown	***lmbR***	MTL synthesis	transaldolase
lmbE	condensation	N-acetyl-D-glucosamine acetylase	***lmbQ***	unknown	unknown
			lmbT	MTL synthesis	glycosyltransferase
lmbF	condensation	aminotransferase	***lmbV***	unknown	unknown
lmbG	MTL synthesis	methyltransferase	***lmbW***	PPL synthesis	methyltransferase
lmbIH	unknown	unknown	*lmrB*	resistance	rRNA methyltransferase
lmbJ	methylation of NDL	NDL-methyltransferase	***lmbX***	PPL synthesis	unknown
lmbK	MTL synthesis	phosphatase	***lmbY***	PPL synthesis	oxidoreductase
lmbL	MTL synthesis	dehydrogenase	***lmbU***	unknown	unknown
lmbM	MTL synthesis	dehydrogenase	*lmrC*	resistance	ABC transporter

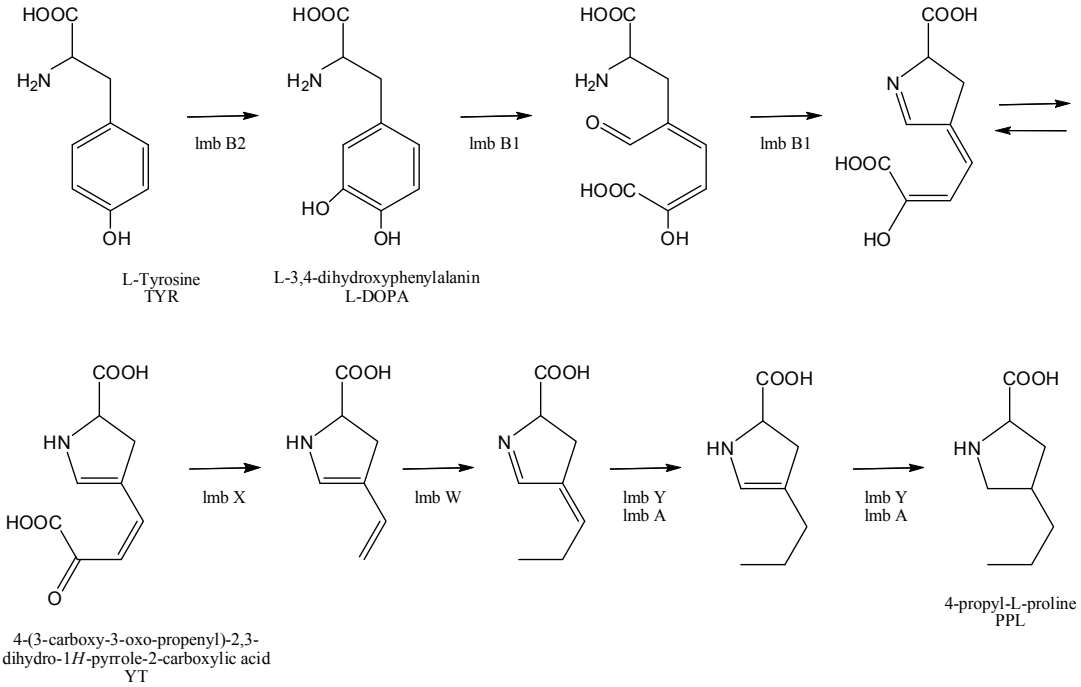

Fig. 2.7 Proposed scheme of the biosynthetic pathway of 4-propyl-L-proline, the lincomycin precursor.[81]

2.4.5 Tetracyclines

B. M. Duggar discovered the first member of the tetracycline family – chlortetracycline (**28**) (aureomycin) – in 1945.[85] Many semi-synthetic derivatives have been introduced since then. However, naturally occurring tetracyclines still represent a rather small pool of compounds, including tetracycline (**29**), chlortetracycline, oxytetracycline (**30**), and demeclocycline. Their structures are based on a hydronaphthacene nucleus containing four fused rings ornamented with hydroxyl groups, a tertiary amino group, an amide group or even chlorine.

Tetracyclines have been typically separated by reverse-phase liquid chromatography techniques as reviewed by C. R. Anderson *et al*.[86] The detection can be performed by UV detectors[87] because tetracyclines strongly absorb in UV, exhibiting maxima of their UV spectra at around 220, 270, and 355 nm. Since tetracyclines fluoresce naturally, fluorescence detectors are suitable for their detection as well.[88] Alternatively, electrochemical (pulsed amperometry)[89] and mass spectrometry[90] techniques have been utilized.

2.4.6 Glycopeptides

Glycopeptide antibiotics were discovered in 1955 when McCormick *et al.* isolated vancomycin (**31**) in Borneo.[91] Prior to 1984, the glycopeptide class included few members beyond vancomycin, teicoplanin (**32**), ristocetin A (**33**), and avoparcin. Since then, the class swelled to include thousands of natural and semi-synthetic compounds.[92] Most glycopeptides contain a core heptapeptide with a high degree of homogy in aromatic amino acids 4 to 7 and variation of amino acids 1 to 3, which can be either aromatic or aliphatic. Individual compounds are further characterized by sugar moieties attached to the amino acid residues. In addittion, vancomycin and teicoplanin contain chlorine.

In the view of analytical chemistry, it is worth mentioning that glycopeptides have been used for chiral stationary phases applicable in enantioseparations.[93] As for separation of glycopeptides, reverse-phase liquid chromatography has been applied most frequently, usually coupled with a UV detector.[94, 95] UV absorption is caused by the presence of aromatic amino acids in the molecule, which results in a strong absorption under 230 nm forming a broad maximum around 206 nm and a weaker,

but distinctive maximum around 282 nm. The same UV spectra, however, also apply for other peptide antibiotics containing aromatic amino acids. As alternative means of detection, fluorescence [96], electrochemical[97] and mass spectrometry[98] techniques have been described.

2.4.7 Peptides

Apart from glycopeptides, various antimicrobial peptides are biosynthesized by actinomycetes. Streptogramins (pristamycin IA and IB) discovered in the early 1950s[99] are a large group of peptides containing lactam and lactone linkages and typically incorporate a heterocyclus (a pyridine or an oxazole ring). Actinomycins are composed of two peptide rings and a phenoxazine derivative causing a UV absorption peak at 410 nm. Thiostrepton (**34**) discovered in 1955[100] forms a broad UV peak from 200 to 310 nm with its maximum at 206 nm. A semi-synthetic lipopeptide daptomycin containing a decanoyl side chain was discovered in late 1980s. In general, UV spectra of peptide antibiotics are either as described for glycopeptides or diverse if they depend on additional structures, usually heterocycles, attached to the peptide core. All other aspects of chemical analysis are in accordance with those for glycopeptides.

2.4.8 Aminocoumarins and Coumermycins

Aminocoumarins represent a small group of antibiotics based on a substituted coumarin nucleus. The best known antimicrobial aminocoumarin is novobiocin (**35**), which was discovered in 1955.[101, 102] Novobiocin is composed of a coumarin residue, a benzoic acid derivative, and a sugar novobiose. Coumermycins (**36**) discovered in the early 1960s[103] are symmetry-like structures composed of two coumarin residues, two saccharide moieties, and typically three 2-pyrrole carboxylic ester moieties.

Aminocoumarins as well as courmermycins have been analyzed by reverse--phase liquid chromatography with mass spectrometry[104] or UV[105, 106] detection. The UV spectrum of novobiocin exhibits two strong maxima at 210 and 330 nm. As for coumermycins, the maxima are shifted to 280 and 340 nm[106].

2.4.9 Miscellaneous Actinomycete Products

The production of actinomycete secondary metabolites is extremely diverse as follows from the previous subsections. However, the list of the compounds is not complete at all and contains only the most important classes of secondary metabolites. A few more miscellaneous actinomycete products are briefly mentioned in the following text.

- Chloramphenicol (**37**) with a nitro group and two chlorine atoms exhibits the maximum in its UV spectrum at 278 nm.
- Aminonucleosides such as blasticidin (**38**) (UV maxima at 206 and 275 nm) and puromycin (**39**) (203 and 269 nm) are analogues of nucleosides, cytidine and adenosine, respectively.
- Piperidines composed of a glutarimide residue and a substituted alicyclic chain absorb strongly at 200-205 nm; cycloheximide (**40**) and streptovitacin A (**41**) are the most known members.
- Anthracyclines with their tetracycline ring structure resemble tetracyline antibiotics. Unlike tetracyclines, anthracyclines including daunorubicin (**42**) and doxorubicin (**43**) contain a saccharide moiety, daunosamine.
- Polyether compounds, *e.g.* monensin (**44**) and salinomycin (**45**), with ionophore properties lack a strong chromophore. However, lasalocid (**46**) absorbs strongly at 215, 249 and 318 nm[107] due to its methylsalicylate moiety.
- Rifamycins (*e.g.* rifampicin **47**) are polyketide compounds with an incorporated naphtalene ring. The absorption maxima of rifampicin are at 233, 259, 339, and 472 nm.
- Benzoquinones produced by actinomycetes include for instance mitomycins, which contain aziridine moiety (*e.g.* mitomycin C exhibiting the maximal absorption around 360 nm[108]).
- Pyrrolo[1,4]benzodiazepines such as sibiromycin (**48**), anthramycin and porothramycin (**49**) are not effective antimicrobials, but represent potent antitumor agents. Two UV maxima are observed, around 230 and between 310 and 340 nm.
- Fosfomycin is an example of a very simple structure – a polar phosphoric acid derivative undetectable by UV.

- Manumycins (*e.g.* **manumycin A**, **asukamycin**) are a small class of antibiotics composed of two unsaturated alifatic chains (one of them is a triene) linked together with an oxiram-containing residue.
- **Mithramycin** (aureloic acid) is an antineoplastic antibiotic consisting of tetrahydroanthracene derivative core ornamented by saccharide moieties and a substituted five-carbon aliphatic chain. The UV spectrum maxima are at 230, 278, and 413 nm.[109]

2.4.10 Antibiotic Precursors

Not only the final secondary metabolites, but also their precursors may be detected in the culture broths of actinomycetes. This is especially true for mutants defected in some biosynthetic step so that an enzyme is not produced and its substrate (metabolite precursor) accumulates. With respect to the antibiotic structures, the precursors may be represented by anything from various amino acids, saccharides and specific structures usually originating from the primary metabolism up to anything reminding of the final secondary metabolite. Especially the precursors corresponding to the beginning of the biosynthetic pathway are small polar compounds lacking any chromophores. Hence, both reversed-phase chromatography and UV detection may fail to analyze them unless a derivatization step is involved.

3 LIQUID CHROMATOGRAPHY AIMED AT GAINING NOVEL ANTIBIOTICS

This chapter introduces liquid chromatography from a general point of view. It includes sample treatment prior to analysis, defines liquid chromatography techniques, emphasizing current trends in this field, and finally gives an overview of detectors compatible with liquid chromatography and suitable for detection of secondary metabolites.

3.1 Sample Pre-Treatment

Biological samples including cultivation broths represent a complex matrix often containing low amounts of analytes. Therefore, it cannot be applied directly to instrumentation for chemical analysis without preceding extraction and/or derivatization.

3.1.1 Extraction

The main purposes of sample extraction are clean-up and pre-concentration of the analyte. Liquid-liquid extraction, solid-phase extraction (SPE), and solid-phase microextraction are the most common techniques applied for extraction of semi--volatile compounds from liquids.[110]

Liquid-liquid extraction has been widely applied for extraction of secondary metabolites.[111, 112] However, there are a great number of disadvantages of this technique including low recovery and reproducibility, exposure to large volumes of organic solvents, and time-consuming procedures. What is more, many solvents commonly applied for extraction are toxic.[110]

Even though, some advances in microfluidics amenable to automation of the liquid-liquid extraction process have been achieved,[113, 114] the alternative techniques are preferred. Among them, SPE is particularly remarkable because it proved to be a very efficient tool for extraction of secondary metabolites.[112, 115] Undoubtedly, it follows from better characteristics of SPE compared to liquid-liquid extraction. The most cited benefits include improved recovery and reproducibility, reduced analysis

time, and reduced cost.[110, 116] Besides that, the variety of sorbents available nowadays provides greater opportunity for either more selective or more universal extractions. Several examples of SPE sorbents are depicted in Fig. 3.1. Some of them are particularly suitable for extraction of secondary metabolites, considering the interactions performed by the sorbent and physico-chemical properties exhibited by secondary metabolites.[115, 117-120] Firstly, sillica-based sorbents with the C_8 or C_{18} ligands are based on reverse-phase interactions. Secondly, polymeric resins such as styrene/vinyl benzene (Amberlite XAD) contain aromatic structures for hydrophobic interactions. Thirdly, hydrophilic-lipophilic balanced copolymer (HLB) involves polar interactions (performed by the 2-pyrrolidone functionality) as well as hydrophobic interactions. Given the combination of these two different interactions, HLB sorbent is universal rather than selective. Therefore, it appears to be especially suitable for non-target extractions aimed at compounds of diverse structures. Moreover, HLB requires a low elution volume to release the analyte and is not susceptible to drying effects.[115] Lastly, ion-exchange sorbents including mixed-mode sorbents (combination of ion-exchange and other interactions) represent an option for more specific applications (ionic and ionizable compounds).[110, 121]

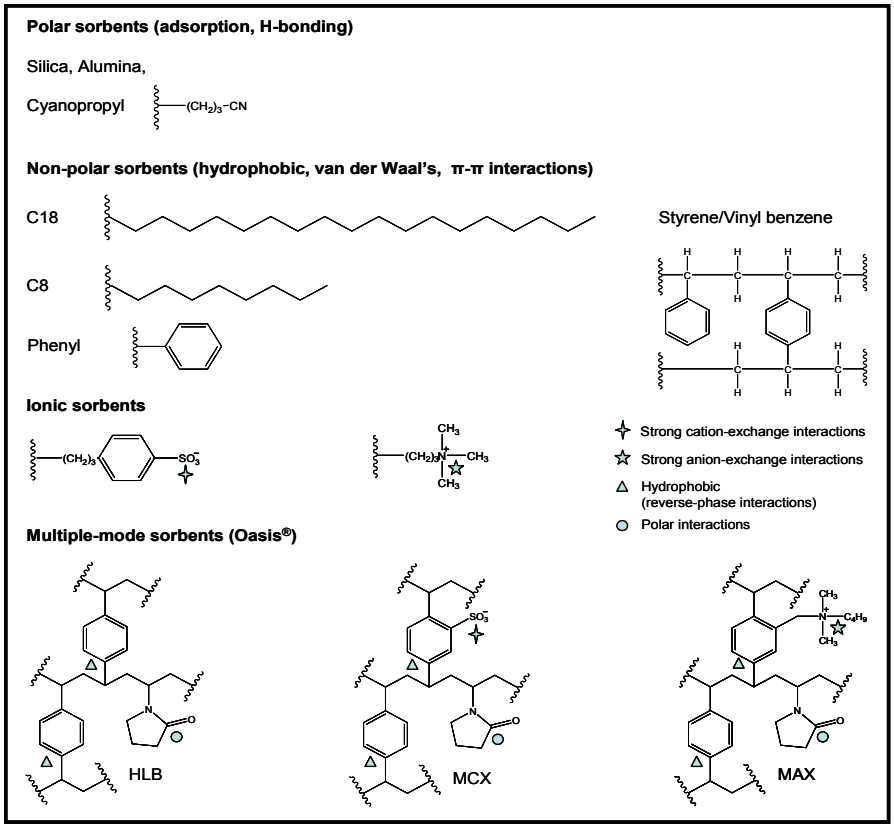

Fig. 3.1 Examples of solid-phase extraction sorbents.[121, 122]

Another extraction technique, solid-phase microextraction, is derived from SPE. By contrast, the sorbents are not filled in a cartridge as in SPE, but configured as a fiber, a stirrer, suspended particles, or a disc immersed into the liquid containing the analyte. Solid-phase microextraction can be considered for extraction of secondary metabolites as well. Nevertheless, one should be aware of the principal difference between SPE, which is an exhaustive extraction technique, and solid-phase microextraction, which is an equilibrium-based extraction technique.[110]

3.1.2 Derivatization

Derivatization of the analyte is carried out to enable or improve its detection by carrying a specific group into the analyte molecule, which enhances the detector response. Derivatization has been applied for UV detection, but it is much more common with fluorescence detection.[123] Incorporating a chromophor or fluorophor into a molecule is usually a difficult task dependent on the type of the derivatization agent. Common problems include insufficient stability of the reaction products, interfering side products, and necessity of removal of the agent.[124] Due to these difficulties, the derivatization generally represents a time-consuming procedure and suffers from problems with reproducibility. On the other hand, analysis of fluorescent derivatives does not require extraction because fluorescence detection is more specific and sensitive than *e.g.* UV detection[124], and likewise, fluorescence detection does not demand as cleaned-up samples as *e.g.* mass spectrometry.

In general terms, derivatization is performed either before the sample separation (pre-column derivatization) or after its separation (post-column derivatization).[121] The former technique not only enables the detection, but it also improves chromatographic properties of the analyte (typically, less polar derivatives suitable for reverse-phase chromatography are created). Meanwhile, the latter technique is limited to derivatizations based on fast reactions and requires an additional pump in order to introduce the derivatization agent to the chromatographic column effluent before it enters the detector.

3.2 Liquid Chromatography

Regardless of the sample pre-treatment procedure, samples of complex matrices contain a great number of compounds. The compounds have to be separated from each other in order to detect them effectively. Without a doubt, the most widespread separation technique nowadays is liquid chromatography, which is commonly applied in pharmacology, toxicology, clinical analysis as well as in various research fields.[125-127] Liquid chromatography enables separation of majority of the actinomycete secondary metabolites with respect to their physico-chemical properties.

3.2.1 Techniques of Liquid Chromatography

Liquid chromatography techniques include paper, thin-layer, column atmosphere- and middle-pressure, high-performance (HPLC) and ultra high--performance (UHPLC) liquid chromatography. Paper and thin-layer chromatography are very simple techniques, often considered to be outdated. However, the low costs and demands on operators are their great advantages. Moreover, these techniques are compatible with detection by chemical agents. A great variety of agents differing in their specificity can be used in order to detect the analytes and also reveal important information on their chemical structures, usually the presence of specific functionalities in the analyte molecule.[128] Column atmosphere- and middle-pressure liquid chromatography are limited to preparative purposes aimed at isolation of a relatively high (milligrams and more) amounts of analytes. HPLC represents the most commonly applied liquid chromatography technique today. The HPLC separation occurs on a solid stationary phase inserted into a chromatographic column, through which the analytes are carried away with a liquid mobile phase. The separation is determined by analyte/stationary phase and analyte/mobile phase interactions. Polar stationary phase and non-polar mobile phase are used in normal--phase liquid chromatography, whereas non-polar stationary phase and polar mobile phase are used in reversed-phase liquid chromatography. Also, stationary phases for ion-exchange or size exclusion chromatography are available. UHPLC was developed from HPLC by improvements of the separation process efficiency and adjustments of the instrumentation as described in section 3.2.2.

HPLC and UHPLC, but also paper and thin-layer liquid chromatography can be employed in three different modes: target analysis, profiling, and fingerprinting. Target analysis aims to identify and quantify specific analytes of interest (*e.g.* lincomycin). Profiling requires that the signals in the profile (peaks in a chromatogram) can be assigned to a specific analyte whether it is of known structure or not (*e.g.* an unknown analyte eluted in a specific retention time or exhibiting a specific UV spectrum). Finally, fingerprinting aims to get a "chemical picture" or "chemical bar code" of the sample where the signals cannot necessarily be used to detect specific analytes.[129]

3.2.2 Ultra-High-Performance Liquid Chromatography

UHPLC represents current state-of-the-art liquid chromatographic technique. It was launched in 2003 by Waters company. Since then, other companies (Agilent, ThermoElectron, Shimadzu, Dionex, and Perkin Elmer) have adopted the concept and the offer of UHPLC instrumentations and columns has extended a lot.

The efficiency of chromatographic separation can be described by van Deemter equation. The comparison of van Deemter curves for chromatographic column particles of different size reveals that the smaller particles are used the more effective separation is obtained. This reflects the fact that the use of smaller particle size ensures better mass transfer and minimizes band broadening. This statement led to the development of columns with sub-2 μm particles giving birth to UHPLC. The sub-2 μm particles employed in UHPLC columns enable approximately six times faster analyses than HPLC adding to its suitability for application in the screening of a large number of samples. Also, selectivity and sensitivity is considerably higher than that with standard HPLC columns. On the other hand, particles of this size are responsible for significantly higher back pressure. Only hardware adjustments allow UHPLC systems to work at pressures up to 100 MPa as required for sub-2 μm particle columns.[115, 130-132]

The particles in UHPLC columns capable of dealing with high pressure are based on the bridged ethylene hybrid (BEH) technology. This sorbent provides not only high mechanical robustness, but also chemical stability in wide pH range.[132] UHPLC columns with various ligands bonded to the BEH sorbent have been introduced by Waters. These include linear alkyl chains C_8 and C_{18} for common

reverse-phase applications, Shield RP 18 ligand (alkyl chain with inserted polar carbamate group suitable for *e.g.* polyphenolic compounds), and phenyl ligand on a C_6 alkyl chain. Further, two UHPLC columns based on hydrophilic interaction chromatography (HILIC) have been developed: one with an amidic group as a ligand and the other without any ligand. With increasing popularity of the UHPLC technique, chromatographic columns compatible with this techniques are available from several other companies: Restec (Pinnacle DB columns), Grace (Vision HT columns), Thermo Scientific (Hypersil GOLD), Agilent (Eclipse Plus C_{18} and StableBond SB-C_{18}), and Perkin Elmer (Brownlee Analytical DB C_{18}).

3.2.3 Current Trends in Liquid Chromatography

A great effort has been made in order to develop chromatographic columns with similar efficiency and short analysis time compared to UHPLC, but compatible with HPLC systems working only up to 40 MPa. These requirements are accomplished in recently introduced technologies of fused-core particles and analogous core-shell particles. As depicted in Fig. 3.2[133], core-shell particles are composed of a solid core (1.70 µm) surrounded by a thin porous silica layer (0.50 µm). This technology enables that the analyte can diffuse only into the pores of the thin porous layer. The particle size together with porous silica layer provides separation efficiency and analysis time similar to UHPLC, but with lower column backpressure, making these columns compatible to conventional HPLC systems.[134, 135]

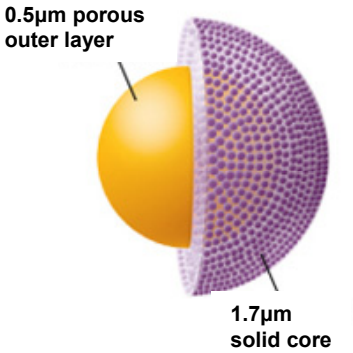

Fig. 3.2
Core-shell particle.

Another alternative is represented by monolithic columns, which overcome the pressure limitation due to their higher permeabilities. Comparative studies proved that compared to conventional reversed-phase silica-based columns, the separation efficiency on monolithic columns was higher.[136, 137] On the other hand, compared to sub-2 µm particle columns, it performed considerably worse[138]. However, it is still believed that monolith may play an important role in column chromatography in the future and research on this issue continues.[139]

3.3 Detectors for Liquid Chromatography

The following detectors compatible with liquid chromatography are considered in this section: ultraviolete (UV) including diode-array (DAD) detectors, fluorescence, evaporative light-scattering (ELSD) and condensation nucleation light--scattering (CNLSD), and finally mass spectrometry (MS) and nuclear magnetic resonance (NMR) detectors.

3.3.1 Ultraviolet and Diode-Array Detectors

UV detectors, based on UV absorption of compounds, are undoubtedly the most widespread in liquid chromatography due to their initial and operating low costs, simplicity, and high universality. However, the utilization of UV detectors is limited only to analytes with a chromophore.[140] In other words, compounds lacking π- or unshared electrons can be easily overlooked. Among actinomycete secondary metabolites, this applies for aminoglycosides, which are practically undetectable by UV detectors. Furthermore, compounds having weak chromophores can be detected only at short wavelengths (around 200 nm). In this case, the mobile phase solvents exhibiting high UV cut-offs should be avoided because they would blind the detection of these compounds.[141] This is true *e.g.* for lincomycin or some macrolides (erythromycin, oleandomycin).

The diode-array UV technology additionally provides information on the analyte structure by measuring UV spectra during the analysis. UV spectrum cannot be used for structure elucidation of unknowns because it lacks in specificity.[142] However, UV spectrum increases the reliability of the analyte identification if it is based on comparison of retention times of the standard and the analyte. Moreover, distinct UV spectra of complex chromophore systems can classify the analyte[143] to a specific group of secondary metabolites such as polyene macrolides, glycopeptides, or tetracylines. In this respect, the information in UV spectra lacking somewhat in specificity is more valuable than the information from MS because classification of compounds according to their masses is not possible.

It is worth mentioning that UV/DAD detectors are non-destructive; therefore, they are often accompanied by another detector in the series (usually ELSD or MS) in order to enhance the method universality.[144]

3.3.2 Fluorescence Detectors

Fluorescence detection is based on the process where the molecular absorption of a photon triggers the emission of another photon with a longer wavelength. Fluorescence is more sensitive and selective than UV.[144] Unfortunately, relatively few secondary metabolites fluoresce in a practical range of wavelengths. However, many compounds can be made to fluoresce by forming appropriate derivatives (see subsection 3.1.1).[140] Further, fluorescence lacks in universality and does not bring any structural information.

3.3.3 Evaporative and Condensation Nucleation Light-Scattering Detectors

ELSD detectors work by measuring the light scattered from the solid solute particles remaining after nebulization and evaporation of the mobile phase. ELSD detectors represent a universal detector because it is capable of detecting any analyte less volatile than the mobile phase regardless other physico-chemical properties including the presence or absence of chromophores.[145] Unlike refractometric detectors, ELSD can work also under gradient chromatoghraphic conditions. However, the mobile phase applied should be considered in a similar way as in MS because only evaporative solvents are suitable.[141]

CNLSD (*e.g.* the Nano Quantity Analyte Detector, NQAD^{TM}) is a recently launched, also aerosol-based detector. In principle, CNLSD represents an innovated version of ELSD detectors, but it uses the condensation nucleation technology. This technology is based on nebulization and evaporation of the mobile phase at elevated temperature and consequent analyte condensation with super-saturated auxiliary water vapour. This leads to creation of relatively large droplets that are later detected using scattered light with a laser photodetector system. The increase in particle size increases the light-scattering signal and thus the sensitivity in comparison to ELSD.[146]

Additionally, neither of these detectors provides any structural information on the analytes. Therefore, these non-destructive detectors have been used mostly as additional detectors rather than substitutes.[141]

3.3.4 Mass Spectrometry Detectors

MS detection is considered to be the most universal detection technique by far. However, ionization of the analyte represents here the main prerequisite for obtaining a signal.[143] The most popular ionization techniques employed in connection with liquid chromatography are electro spray (ESI) and atmosphere pressure chemical ionization (APCI). Both techniques remove the mobile phase solvents prior to ionization and produce ions of the analytes by combination of high voltage and heat. The ions are directed toward the mass analyzer to be separated and assayed by measuring their mass-to-charge ratio (*m/z*).[141]

The main breakthrough brought by MS is the structural information it gives. This information is strongly dependent on the ionization technique and the mass analyzer employed in the MS detector. Both ESI and APCI provide a soft ionization predominantly producing molecular ion species in the form of either protonated molecules $[M + H]^+$ in the positive mode or deprotonated molecules $[M - H]^-$ in the negative mode. Also, different adducts are produced, *e.g.* $[M + Na]^+$, $[M + K]^+$, or $[M + HCOO]^-$, depending on the analyte and mobile phase composition.[141] Mass spectrum containing these ion species can be easily used to deduce molecular mass of the analyte. More complex structural information may be provided by fragmentation of the ion species. It can be induced in one of the high-pressure regions of the ion beam from the ESI or APCI source to the mass analyzer by collision-induced dissociation (CID) either at the source (in-source CID) or in conjunction with tandem MS.[147] Further structural information is dependent on the type of the mass analyzer. For instance, current time-of-flight analyzers provide accurate *m/z* of ions (<5 ppm) and high resolution (>10 000) sufficient to resolve peaks of isotopic ions. Based on the accurate mass and the isotopic ion pattern, elemental composition of the analyte ions is predicted.

The acquired molecular mass or elemental composition of the analytes can be entered to a chemical database (*e.g.* ChemIDplus[148] or Reaxys[149]) in order to tentatively identify the analyte. The analyte structure can be then confirmed using the fragmentation data from the in-source CID MS and MS^n spectra.

3.3.5 Nuclear Magnetic Resonance Detectors

NMR detectors are rarely connected to liquid chromatography mainly due to high initial and operating costs. Furthermore, NMR traditionally requires high amounts of analytes because it lacks in sensitivity. However, with recent advances in NMR, the hyphenated liquid chromatography techniques employing NMR have been successfully developed and applied.[150] The main advantage of NMR is obtaining of superior structural information which can be directly used for database search as a powerful tool for dereplication of known compounds and identification of novel compounds.[144, 150] NMR is also often used off-line to elucidate the analyte structure.

Other detectors compatible with liquid chromatography include chemiluminescence, electrochemical, and refractometric detectors. None of the detectors discussed in this section is fully universal; in fact the difference between universality, selectivity, sensitivity and provided structural information is vast. It is mandatory to consider all characteristics of the detectors in order to select the most suitable detection system.

4 DISSERTATION OBJECTIVES

This dissertation thesis aimed at developing liquid chromatography methods for analysis of actinomycete secondary metabolites and antibiotics. The specific objectives were as follows.

1. **To develop and apply liquid chromatography methods for analysis of lincomycin precursors, the precursor analogues, and lincomycin derivatives (Paper 1 and 2).**

2. **To compare sub-2 µm-particle UHPLC columns with core-shell particle chromatographic columns applied for antibiotic analysis (Paper 3).**

3. **To test the compatibility of UHPLC with CNLSD (NQAD^{TM}) detector and to compare CNLSD and UV detection techniques using a set of antibiotics (Paper 4).**

4. **To develop and apply a universal liquid chromatography method for fingerprinting of actinomycete secondary metabolites in cultivation broths (Paper 5).**

5 EXPERIMENTAL

The experimental sections specify the instrumentation used for development and application of the liquid chromatography methods and describe experimental procedures for the unpublished results. For experimental data concerning the published results, see the respective paper attached in the appendices.

5.1 Instrumentation

HPLC analyses were performed on Waters system equipped with flow controller 600, autosampler 717, UV detector 486, and fluorescence detector 474. Data were processed with Millenium 32 software (Waters, USA).

UHPLC analyses were carried on the Acquity UPLC system equipped with the 2996 photodiode array detection system detector and since 2010 coupled with the LCT Premier XE orthogonal accelerated time-of-flight mass spectrometer with an electrospray interface (Waters, USA). Data were processed with Empower 2 and MassLynx V4.1 software (Waters, USA). Alternatively, the Quant Nano Quatity Analyte Detector (NQAD^{TM}, Quant Technologies, USA) was applied and the data were processed with the integrator LCI-100 (PerkinElmer, USA).

5.2 Experimental Procedures for Unpublished Results

5.2.1 Experimental –

– 4-(3-carboxy-3-oxo-propenyl)-2,3-dihydro-1*H*-pyrrole-2-carboxylic acid

The experimental procedures described in this subsection refer to the results in subsection 6.1.1.2. The standard of 4-(3-carboxy-3-oxo-propenyl)-2,3-dihydro--1*H*-pyrrole-2-carboxylic acid (YT) was obtained as described by Novotna *et.al.*[151] The cultivation broth, into which YT was spiked, and the cultivation conditions were described in the same publication. The chemicals are listed and specified in Paper 5.

Three Oasis SPE sorbents: HLB, MAX and MCX (Waters, USA) were tested for extraction of YT using protocols given in Table 5.1. pH of the samples was prior to the extraction adjusted by either formic acid or ammonium hydroxide. The

extraction with the HLB sorbent was tested with samples of its original pH and with pH adjusted to 3.0. The extraction with the MAX sorbent was tested with samples adjusted to pH 8.5. The extraction with the MCX sorbent was tested with the samples adjusted to pH 2.0, 2.5, 3.0, 3.5, and 4.0.

The eluents were evaporated to dryness and reconstituted in 300 µL 5% methanol for the UHPLC-DAD analysis using the chromatographic column Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm i.d., particle size 1.7 µm, Waters), hereinafter referred to as BEH C_{18} column. The chromatographic conditions were as follows: mobile phase, 0.1% formic acid and acetonitrile; linear gradient program, min/% acetonitrile: 0/5, 1.5/5, 4.5/25 with subsequent equilibration (2 min); flow rate, 0.4 mL min^{-1}; colum temperature, 30 °C; injection volume: 1 µL; DAD detection: 194–600 nm (chromatograms extracted at 410 nm); data sample rate, 20 pts s^{-1}; filter constant, 0.5.

Table 5.1 Tested SPE protocols for extraction of YT.

Sorbent	Oasis HLB 3cc	Oasis MAX 3cc	Oasis MCX 3cc
Conditioning	3 mL CH_3OH	3 mL CH_3OH	3 mL CH_3OH
Equilibration	3 mL H_2O	3 mL H_2O	3 mL H_2O
Sample	3 mL 0.5 mmol L^{-1} YT in cultivation broth*		
Wash	3 mL H_2O	3 mL 5% NH_4OH	3 mL 2% HCOOH
Elution 1	1.5 mL CH_3OH	1.5 mL CH_3OH	1.5 mL CH_3OH
Elution 2	---	1.5 mL 2% HCOOH in CH_3OH	1.5 mL 5%NH_4OH in CH_3OH

** pH of the sample adjusted as specified earlier in the text.*

Two chromatographic columns and a variety of other UHPLC conditions were investigated. Firstly, the Acquity UPLC BEH Amide column (50 mm × 2.1 mm inner diameter, particle size 1.7 µm, Waters), hereinafter referred to as BEH Amide column was investigated with the following UHPLC conditions as follows: mobile phase, 1 mM or 10 mM ammonium acetate (pH 5) and acetonitrile; linear gradient programs, min/% acetonitrile: 0/90, 8/50 or 0/90, 8/40 with subsequent column equilibration (5 min); flow rate, 0.4 mL min^{-1}; colum temperature, 35 °C; injection volume: 1 µL. Secondly, the BEH C_{18} column was investigated under conditions as described in the previous paragraph dealing with YT extraction. This column was tested with the following mobile phases: 0.05%, 0.1% formic acid, or 0.1% trifluoroacetic acid, and

acetonitrile or methanol as the organic modifier. For the parameters of mass spectrometry detection, see Figs. 6.4 and 6.5.

5.2.2 Experimental – Other Lincomycin Derivatives

The experimental procedures described in this subsection refer to the results in subsection 6.1.2.2. The cultivation conditions for the *S. lincolnensis* mutant strains are described in Paper 2; the chemicals are listed and specified in Paper 5.

The cultivation broth was centrifuged (5000×g, 10 min, 4 °C) and the supernatant was extracted using the Oasis HLB 3cc cartridges (Waters) as follows. Supernatants (3 mL) were loaded onto the cartridge pre-conditioned and equilibrated with methanol (3 mL) and water (3 mL). The cartridge was washed with water (3 mL) and the retained compounds were eluted with methanol (1.5 mL).

The eluent was evaporated to dryness, reconstituted in 150 µL 50% methanol, and analyzed by UHPLC-DAD. For MS detection, the samples were 100× diluted with 50% methanol. The following UHPLC conditions were applied for the analysis: chromatographic column, BEH C_{18} column (specified in section 5.2.1); mobile phase, 1 mM ammonium formate (pH 9) and acetonitrile; linear gradient program, min/% acetonitrile: 0/5.0, 1.5/5.0, 15/90 with subsequent column clean-up (2min, 100% acetonitrile) and equilibration step (2 min); flow rate, 0.4 mL min^{-1}; colum temperature, 30 °C; injection volume: 5 µL (DAD detection) or 1 µL (MS detection); DAD detection: 194–600 nm (chromatograms extracted at 194 nm); data sample rate, 20 pts s^{-1}; filter constant, 0.5; MS detection: cone voltage, 40 V; capillary voltage, +2500 V, −2500 V; ion source block temperature, 120°C; nitrogen desolvation gas temperature, 350°C; desolvation gas flow rate, 800 L h^{-1}, cone gas flow, 50 L h^{-1}. Full scan spectra from m/z 100 to 1500 were acquired in the W dynamic range enhancement mode with the scan time of 0.1 s and the interscan delay time of 0.01 s (0.3 s for polarity switch, 0.1 s for lock spray). Mass accuracy was maintained by the lock spray using Leucine Enkephalin as the reference compounds. The fragmentation using in-source collision induced dissociation was achieved by the Aperture I value set to 50 V.

5.2.3 Experimental – Application of the Fingerprinting Method

The experimental procedures described in this subsection refer to the results in subsection 6.3.2. The cultivation, extraction and UHPLC analyses are described in Paper 5. MS conditions used for the tentative identification of the secondary metabolite were as follows: cone voltage, 40 V; capillary voltage, +2500 V, −2500 V; ion source block temperature, 120°C; nitrogen desolvation gas temperature, 350°C; desolvation gas flow rate, 800 L h^{-1}, cone gas flow, 50 L h^{-1}. Full scan spectra from m/z 100 to 1500 were acquired in the W dynamic range enhancement mode with the scan time of 0.1 s and the interscan delay time of 0.01 s (0.3 s for polarity switch, 0.1 s for lock spray). Mass accuracy was maintained by the lock spray using Leucine Enkephalin as the reference compounds.

6 RESULTS AND DISCUSSION

This chapter briefly summarizes and comments on the published results (subsections 6.1.1.1, 6.1.2.1, section 6.2, and subsection 6.3.1). For more detailed information, see the respective paper in the appendices. Further, the chapter includes yet unpublished achievements (subsections 6.1.1.2, 6.1.2.2, and 6.3.2).

6.1 Analysis of Lincomycin Precursors, Lincomycin Precursor Analogues, and Lincomycin Derivatives

Development of chromatographic methods for analysis of lincomycin precursors, lincomycin precursor analogues, and lincomycin derivatives was required for investigation of the lincomycin biosynthesis and preparation of improved derivatives of lincomycin by mutasynthesis. The chemical structures of all the analytes of interest are shown in Fig. 6.1.

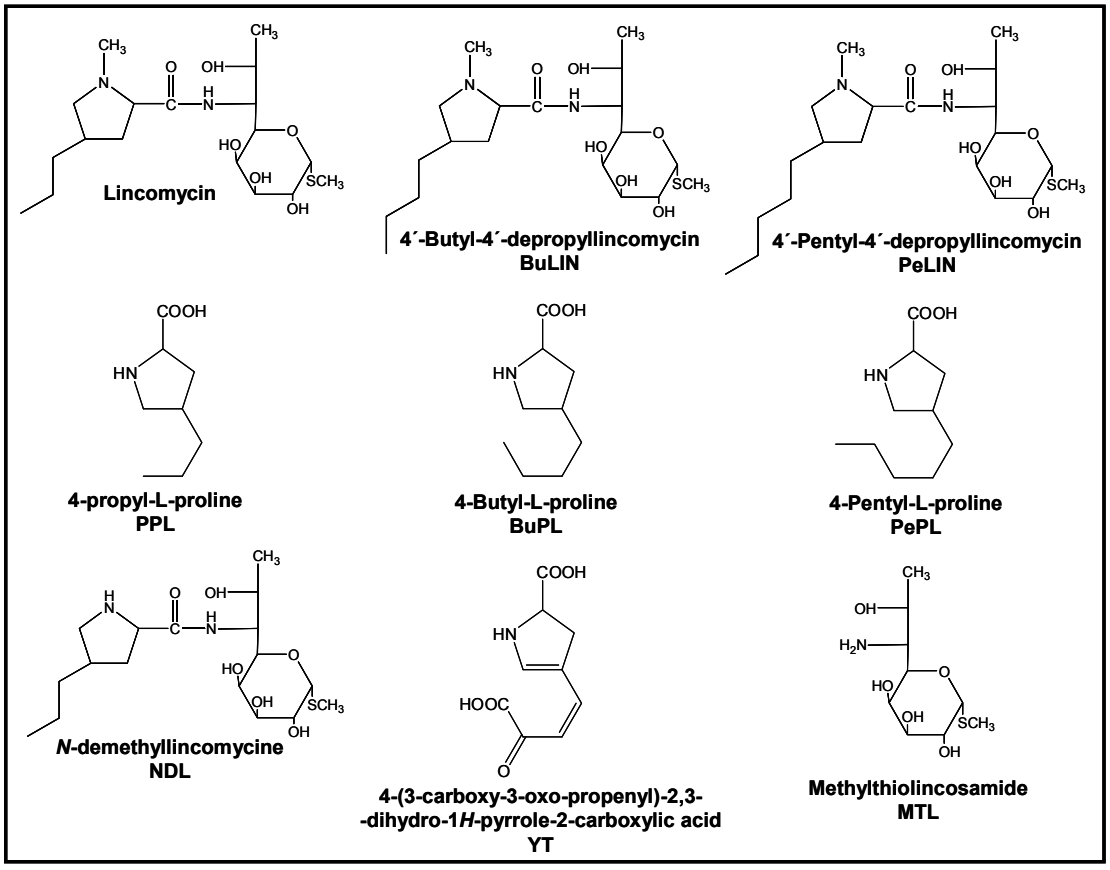

Fig. 6.1 Lincomycin, lincomycin derivatives, lincomycin precursors and their analogues.

The function of a lincomycin biosynthetic gene is investigated by inactivation of this gene. It means that a mutant of the natural lincomycin producer *S. lincolnensis* is constructed. While the natural producer is named the wild type (WT) strain, *i.e.* WT *S. lincolnensis*, the mutant is named according to the inactivated gene, *e.g. ΔlmbW S. lincolnensis* for the mutant with the *lmbW* gene inactivated. The absence of the inactivated gene results in production and accumulation of different compounds compared to the WT *S. lincolnensis*. Analyzing these compounds, particularly lincomycin precursors and derivatives, in the cultivation broth of the mutant strains facilitates to assign the function to the inactivated gene.

The preparation of improved lincomycin derivatives by mutasynthesis (see section 2.3.5) involved chemical analysis of the lincomycin precursor analogues (4-butyl-L-proline, BuPL and 4-pentyl-L-proline, PePL), which were added to the cultivation broth of the *ΔlmbX S. lincolnensis* mutant defective in biosynthesis of 4-propyl-L-proline. Further, it was necessary to detect potential production of the desired products, 4´-butyl-4´-depropyllincomycin (BuLIN) and 4´-pentyl--4´-depropyllincomycin (PeLIN).

6.1.1 Analysis of Lincomycin Precursors and Lincomycin Precursor Analogues

6.1.1.1 4-Butyl-L-proline, 4-Pentyl-L-proline, and Methylthiolincosamide (Paper 1)

Two HPLC methods with fluorescence detection for determination of PPL and NDL in cultivation broth of *S. lincolnensis* have been reported in the past.[152]

The validated method for PPL analysis based on the pre-column derivatization with *o*-phthaldialdehyde was optimized in order to analyze also PPL analogues, BuPL and PePL. The calibration curves were prepared on five concentration levels: 5, 10, 20, 50, and 100 μg mL^{-1}. The equations of the calibration curves were $A = 1.63 \cdot 10^5 c - 1.82 \cdot 10^5$ ($R^2 = 0.9996$) for BuPL and $A = 1.63 \cdot 10^5 c - 1.82 \cdot 10^5$ ($R^2 = 0.9998$) for PePL. Selectivity of the method is demonstrated in Fig. 6.2, which depicts analysis of PePL in cultivation broth. The method was applied for semi--quantitative analysis of BuPL and PePL in cultivation broths where they were added at the beginning of the cultivation during mutasynthetic experiments. The measurements enabled to observe the consumption of BuPL and PePL during mutasynthesis of BuLIN and PeLIN, respectively.

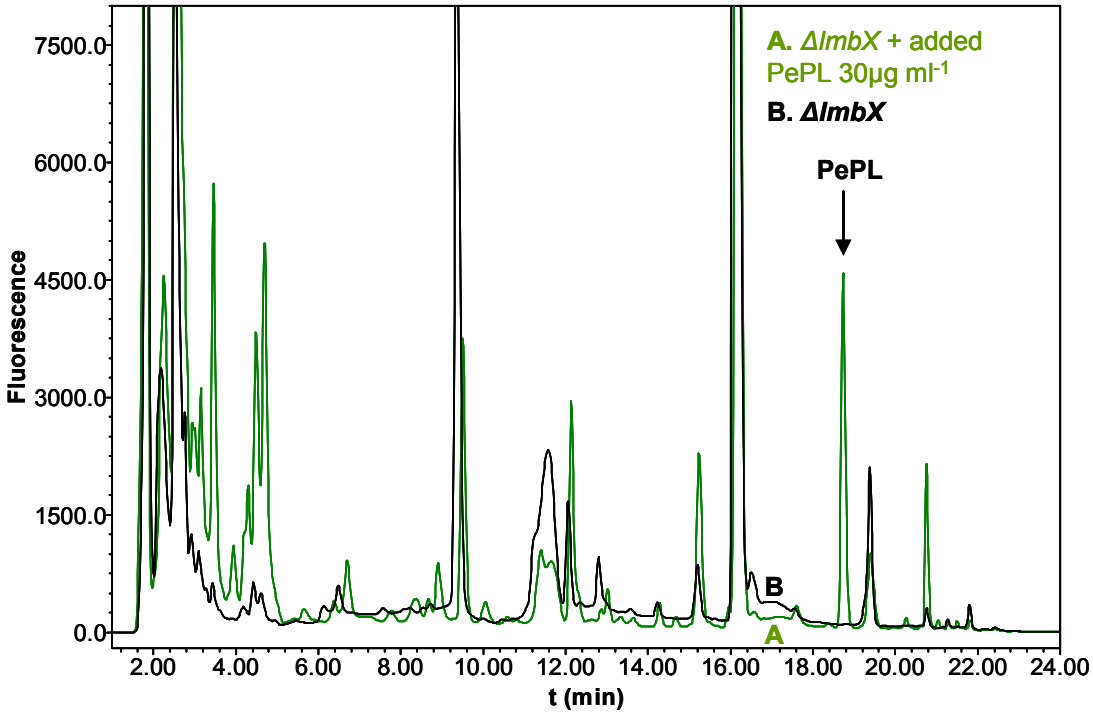

Fig 6.2 Chromatogram overlay – analysis of PePL. Analyte (30 µg mL^{-1}) added into cultivation broth of *ΔlmbX* mutant strain at the beginning of cultivation. HPLC: Luna C_{18} column (250 × 4.6 mm i.d.; particle size, 5 µm; Phenomenex); mobile phase: (A) 20 mM ammonium formate, pH 4.7 and acetonitrile (10:1 *v/v*) and (B) acetonitrile; flow rate, 1.25 mL min^{-1}; injection volume, 25 µL; linear gradient elution (min/%B): 0/30; 1/30; 13/65; 15/100, column clean-up (4 min, 100% B), equilibration (7 min, 30% B). Fluorescence detection: λ_{ex} = 240 nm; λ_{em} = 417 nm, gain switch from 10 to 100 at 6th min of analysis.

The validated method for NDL analysis based on the pre-column derivatization with 4-chloro-7-nitrobenzofurazan was extended to another lincomycin precursor, MTL. The calibration curve was prepared at six concentration levels: 2.5, 5.0, 7.5, 15, 20, and 40 µg mL^{-1}. The equation of the calibration curve was $A = 2.38 \cdot 10^6 c - 1.55 \cdot 10^6$ ($R^2 = 0.9998$). The method was validated with the lower limit of quantification of 2.50 µg mL^{-1} and the inter- and intra-day accuracies and precisions within 12%. The method has been applied for analysis of MTL in several *S. lincolnensis* mutants. However, MTL has not been detected even in samples where it presumably should be present. It can be explained by the fact that MTL – an amino saccharide – is metabolized in an alternative pathway; hence, it does not accumulate during the cultivation. The chromatogram of simultaneous analysis of MTL and NDL standards spiked into the cultivation broth is presented in Fig. 6.3.

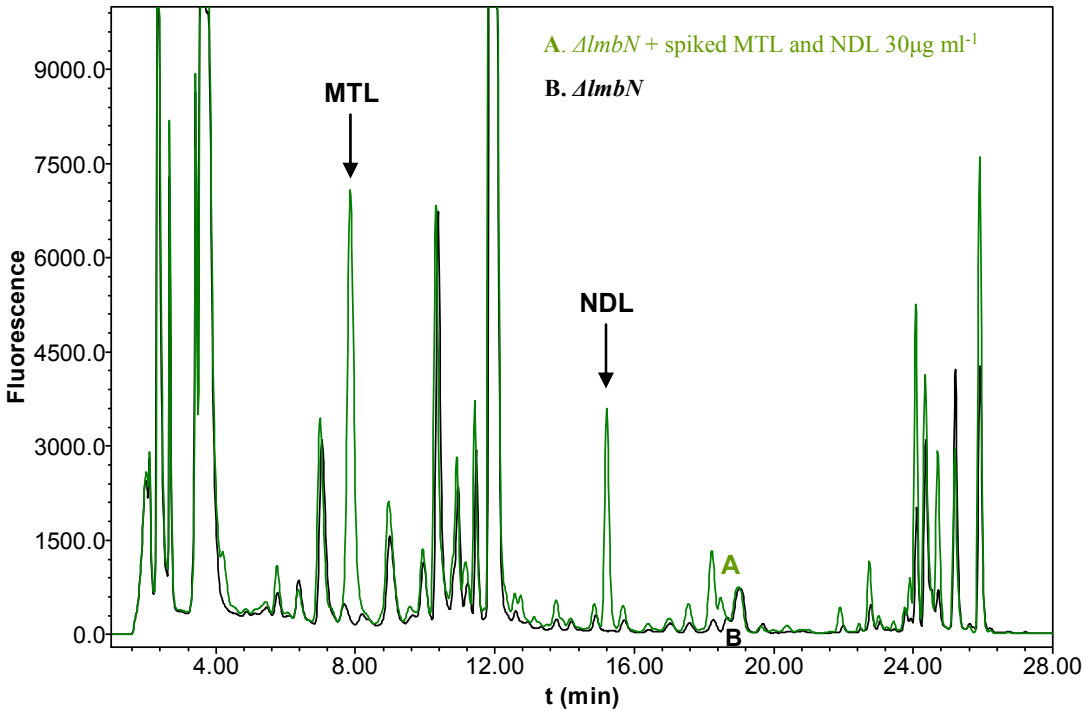

Fig 6.3 Chromatogram overlay – analysis of MTL and NDL. Analytes (30 µg mL^{-1}) spiked into spent cultivation broth of MTL and NDL non-producing *ΔlmbN* mutant strain. HPLC: Luna C_{18} column (250 × 4.6 mm i.d.; particle size, 5 µm; Phenomenex); mobile phase: (A) 20 mM ammonium formate, pH 4.7 and acetonitrile (10:1 *v/v*) and (B) acetonitrile; flow rate, 1 mL min^{-1}; injection volume, 10 µL; linear gradient elution (min/%B): 0/15; 5/33; 15/44; 16/100, column clean-up (4 min, 100% B), equilibration (7 min, 15% B). Fluorescence detection: λ_{ex} = 420 nm; λ_{em} = 525 nm, gain 100.

6.1.1.2 4-(3-carboxy-3-oxo-propenyl)-2,3-dihydro-1*H*-pyrrole-2-carboxylic acid (unpublished)

The lincomycin precursor 4-(3-carboxy-3-oxo-propenyl)-2,3-dihydro-1*H*--pyrrole-2-carboxylic acid (YT) is composed of a 5-membered nitrogen heterocyclus, to which a carboxylic group and an unsaturated chain with an oxocarboxylic moiety are attached (see Fig. 6.1). Consequently, YT is a very polar, water-soluble molecule containing two acidic carboxylic groups and a basic imino group. YT was extracted from cultivation broth and analyzed by a newly developed method as described in the following paragraphs.

For extraction of YT, three SPE sorbents were tested: HLB sorbent (hydrophilic and lipophilic interactions), MAX sorbent (combination of hydrophilic and lipophilic interactions with an anion-exchange group) and MCX sorbent (combination of hydrophilic and lipophilic interactions with a cation-exchange group). The functionalities of the sorbents are depicted in Fig. 3.1. The extraction with the HLB sorbent was not successful indicating that YT is too polar even for the 2-pyrrolidone group, which is responsible for the hydrophilic interactions of the HLB sorbent. The

MAX sorbent, having a strong anion-exchange group, was expected to extract YT because the analyte contains two carboxylic groups. But surprisingly, the MAX sorbent also failed to retain YT. Conversely, sulfonic acid group in the MCX sorbent, representing the strong cation-exchange group, managed to retain YT apparently because of its interaction with the ionized imino group present in YT. In addition to that, the best retention of YT was achieved when the sample applied on the cartridge was acidified to pH 2.5 (the pH range 2 to 4 was tested). Interestingly, YT was released from the MCX sorbent by pure methanol. Hence, 5% NH_4OH in methanol, usually required to interrupt the ion-exchange interactions, was not necessary. The recovery of 0.5 mM YT extracted by MCX sorbent and eluted with pure methanol was 75.2%. In conclusion, the observations that YT was not retained by the HLB sorbent and was eluted with pure methanol from the MCX sorbent suggested that both the 2-pyrrolidone and the sulfonic acid groups were required to retain YT under the tested conditions.

For analysis of YT by ultra-high performance liquid chromatography with diode-array and mass spectrometry detections, BEH Amide and BEH C_{18} UHPLC columns were tested. The BEH Amide column was chosen with respect to high polarity of YT and the presence of nitrogen in its molecule. Indeed, the retention time (1.56 min) and the peak shape of YT were satisfactory when 10 mM ammonium acetate (pH 5) and acetonitrile as components of mobile phase were used. Nevertheless, it was observed that the BEH Amide column caused degradation of YT. This is evident from Fig. 6.4, which shows the analysis of YT using this column and MS spectra of the eluted compound in positive and negative modes. According to MS spectra, a degradation product instead of YT was eluted from the column. The structure of the degradation product (M) deduced from the MS spectra suggests that YT was decarboxylated and the remaining carbonyl group was oxidized to a carboxyl group. By contrast, BEH C_{18} column not only preserved YT, but also separated it with a symmetrical peak shape and a reasonable retention time of 3.47 min (see Fig. 6.5). These results were achieved with 0.1% formic acid and acetonitrile as mobile phase components and with a gradient elution beginning at the ratio of 5:95 acetonitrile:0.1% formic acid (*v/v*). The calibration curve was prepared at six concentration levels: 78.13, 156.3, 312.5, 625.0, 1250 and 2500 μmol L^{-1}, and was measured with DAD detection (410 nm). The equation of the calibration curve was $A = 221.5\ c - 7.646$ ($R^2 = 0.9998$). More validation data were not acquired because the

method has been used only for detection and semi-quantitative analysis of YT and related compounds in reaction mixtures and cultivation broths.

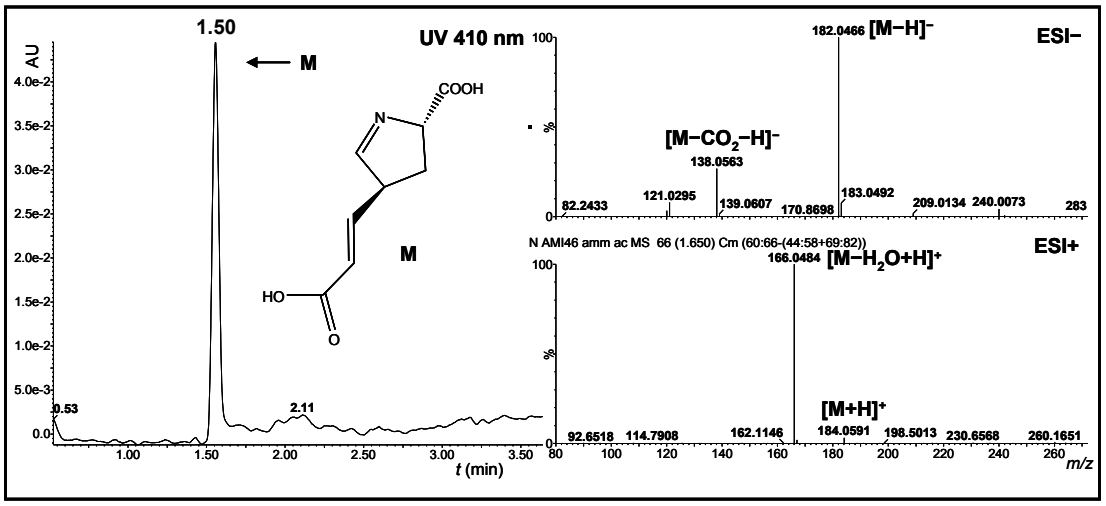

Fig. 6.4 Analysis of YT with BEH Amide column – chromatogram and MS spectra of YT degradation product (M). UHPLC: Acquity UPLC BEH Amide column (50 mm × 2.1 mm i.d., particle size 1.7 µm, Waters); mobile phase, (A) 10 mM ammonium acetate, pH 5, (B) acetonitrile; flow rate, 0.4 mL min^{-1}; linear gradient elution (min/%B), 0/90, 8/50, equilibration (5 min, 90%B); colum temperature, 35 °C; injection volume, 1 µL. DAD detection: 194–600 nm (chromatograms extracted at 410 nm); data sample rate, 20 pts s^{-1}; filter constant 0.5. MS detection: cone voltage, 40 V; capillary voltage, +2800 V, −2500 V; ion source block temperature, 120°C; nitrogen desolvation gas temperature, 350°C; desolvation gas flow rate, 800 L h^{-1}; cone gas flow, 50 L h^{-1}; W dynamic range enhancement mode; scan time, 0.1 s; interscan delay time, 0.01 s (for polarity switch, 0.3 s, for lock spray, 0.1 s).

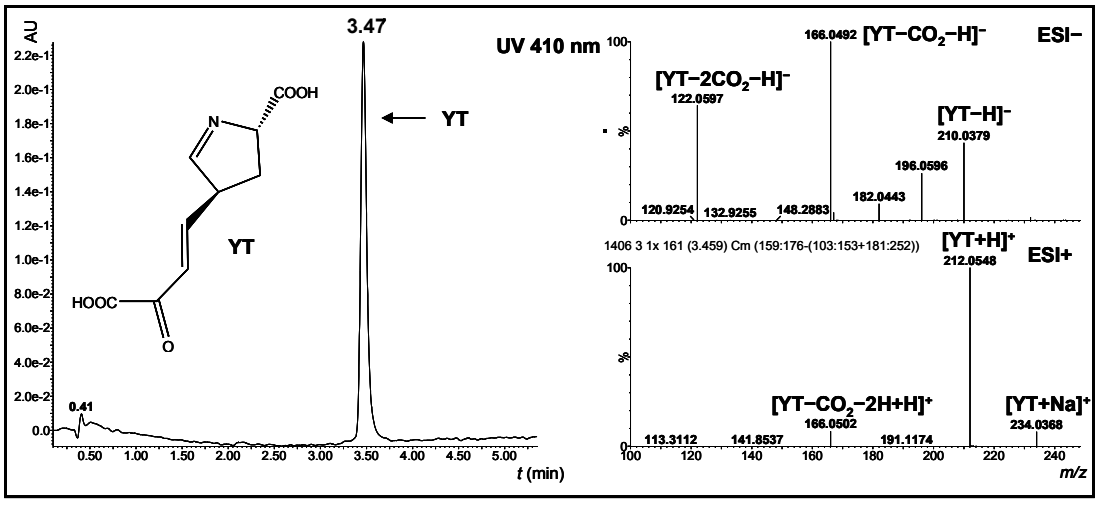

Fig. 6.5 Analysis of YT with BEH C_{18} column – chromatogram and MS spectra of YT. UHPLC: Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm – i.d., particle size 1.7 µm, Waters); mobile phase, (A) 0.1% formic acid, (B) acetonitrile; flow rate, 0.4 mL min^{-1}; linear gradient elution (min/%B), 0/5, 1.5/5, 4.5/25, column clean-up (1 min, 100% B), equilibration (2 min, 5%B); column temperature, 30 °C; injection volume, 1 µL. DAD detection: 194–600 nm (chromatograms extracted at 410 nm); data sample rate, 20 pts s^{-1}; filter constant 0.5. MS detection: cone voltage, 40 V; capillary voltage, +2800 V, −2500 V; ion source block temperature, 120°C; nitrogen desolvation gas temperature, 350°C; desolvation gas flow rate, 800 L h^{-1}; cone gas flow, 50 L h^{-1}; W dynamic range enhancement mode; scan time, 0.1 s; interscan delay time, 0.01 s (for polarity switch, 0.3 s, for lock spray, 0.1 s).

6.1.2 Analysis of Lincomycin Derivatives

6.1.2.1 4´-Butyl-4´-depropyllincomycin and 4´-Pentyl-4´-depropyllincomycin (Paper 2)

The *ΔlmbX S.lincolnensis* mutant defective in PPL biosynthesis was fed with PPL synthetic analogues BuPL and PePL. As a result of this mutasynthetic experiment, the lincomycin derivatives BuLIN and PeLIN should be biosynthesized (see Fig. 6.1 for the chemical structures). However, the derivatives presumably present in the cultivation broth were not detected when the published method for lincomycin analysis was used.[115] It can be explained by the longer alkyl chains of the derivatives, which significantly reduced their polarity compared to lincomycin. Less polar lincomycin derivatives were retained on the BEH C_{18} column under isocratic conditions longer. Only significantly prolonged time of analysis revealed both derivatives separated in very broad peaks. Hence, a gradient elution was developed, so that lincomycin and its both derivatives were analyzed without these difficulties. The comparison of the lincomycin, BuLIN and PeLIN analyses under the original isocratic and the developed gradient conditions is shown in Figs. 6.6 and 6.7, respectively.

Further, the optimal conditions for semi-preparative HPLC analysis were found in order to isolate the putative derivatives for structure elucidation and bioassays. In total, 2.5 mg of isolated BuLIN and PeLIN were used for MS and NMR assays, which proved their structure depicted in Fig. 6.1. Moreover, the bioassays revealed that PeLIN was more active than lincomycin against clinical *Staphylococcus* isolates with genes determining low-level resistance. Additionally, it was proven that the mutasynthetic approach enabled preparation of the lincomycin derivatives without lincomycin contamination.

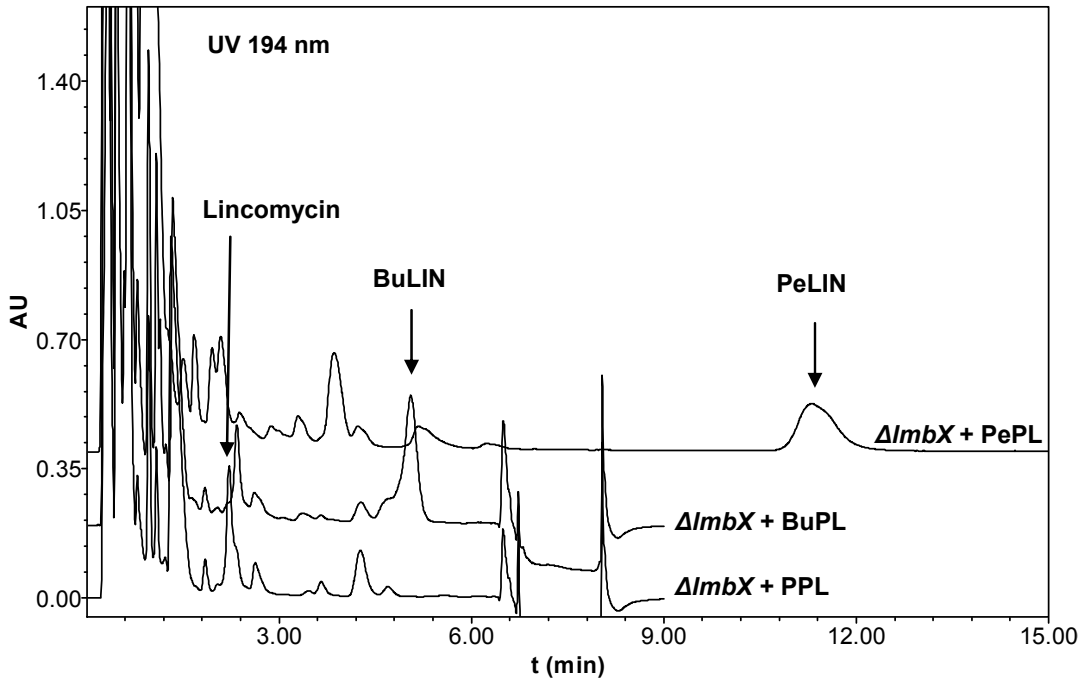

Fig. 6.6 Analysis of lincomycin and its derivatives under isocratic conditions.

UHPLC: Acquity BEH C_{18} column (50 × 2.1 mm – i.d., particle size 1.7 µm, Waters); mobile phase, (A) 1 mM ammonium formate, pH 9.0, (B) acetonitrile; flow rate, 0.4 mL min^{-1}; linear isocratic elution 24% B, column clean-up (1 min, 100%B), equilibration (1 min, 24%B); column temperature, 35 ^{o}C; injection volume, 5 µL. DAD detection: 194–600 nm (chromatograms extracted at 194 nm), data sample rate, 20 pts s^{-1}; filter constant 0.5.

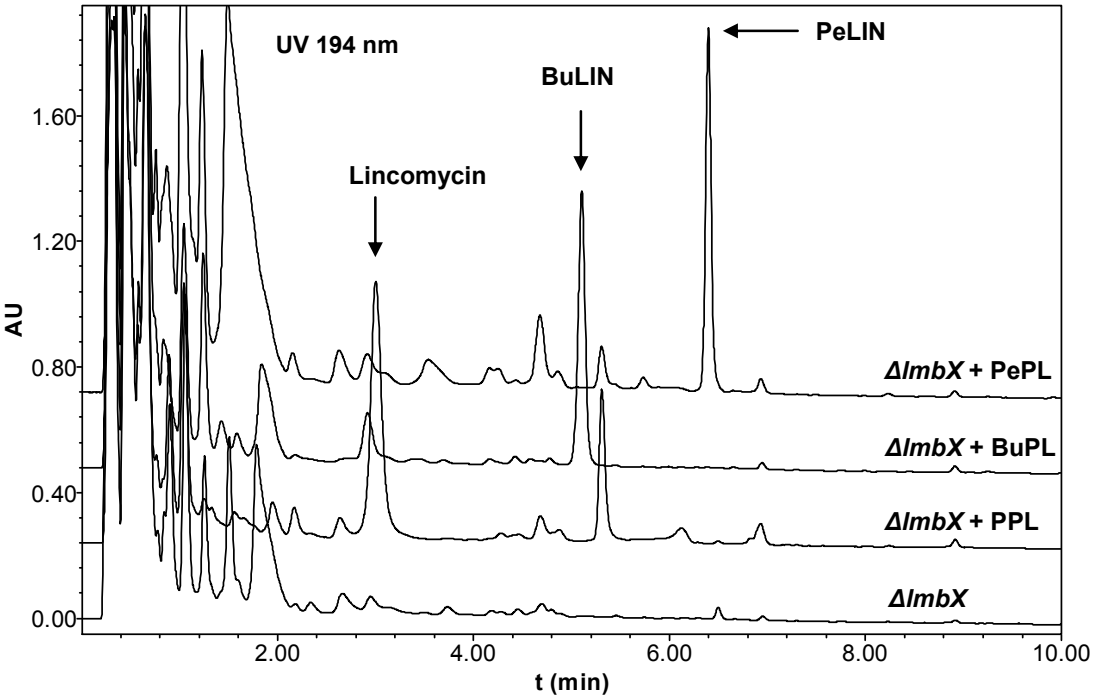

Fig. 6.7 Analysis of lincomycin and its derivatives under gradient conditions.

UHPLC: Acquity BEH C_{18} column (50 × 2.1 mm – i.d., particle size 1.7 µm, Waters); mobile phase, (A) 1 mM ammonium formate, pH 9.0, (B) acetonitrile; flow rate, 0.4 mL min^{-1}; linear gradient elution (min/%B), 0/22, 3/22, 10/52.5, column clean-up (1 min, 100%B), equilibration (1.5 min, 22%B); column temperature, 35 ^{o}C; injection volume, 5 µL. DAD detection: 194–600 nm (chromatograms extracted at 194 nm); data sample rate, 20 pts s^{-1}; filter constant 0.5.

6.1.2.2 Other Lincomycin Derivatives (unpublished)

The chromatographic method described in the previous subsection 6.1.2.1 was modified (see experimental procedures in subsection 5.2.2) and applied on cultivation broths of several mutants of *S. lincolnensis* with specific genes for the lincomycin biosythesis inactivated. Unexpectedly, compounds corresponding to unknown lincomycin derivatives (based on UV spectrum and comparison with analysis of culture broth of WT *S. lincolnensis*) were detected in *ΔlmbX* and *ΔlmbA S. lincolnensis* mutants. MS and in-source CID MS spectra and their comparison with the respective data for lincomycin (Fig. 6.8a) tentatively identified the metabolites produced by these mutants.

The mutant *ΔlmbX S. lincolnensis* produced a dehydrogenated derivative of lincomycin. To be more specific, according to the CID MS spectrum, the double bond was present in the amino acid part of the dehydrogenated lincomycin. Fig. 6.8b depicts the proposed structure; however, more variants of the double bond position are apparently possible.

The mutant *ΔlmbA S. lincolnensis* produced a lincomycin derivative bearing a carbonyl group probably attached to the propyl chain of lincomycin as depicted in Fig. 6.8c. These results were surprising and encouraged us to completely revise the originally proposed biosynthetic pathway of PPL moiety of lincomycin (see Fig. 2.7), particularly the function of *lmbX* and *lmbA* genes. In addition to that, the metabolite of *ΔlmbA* mutant strain is potentially utilizable in biotechnology.

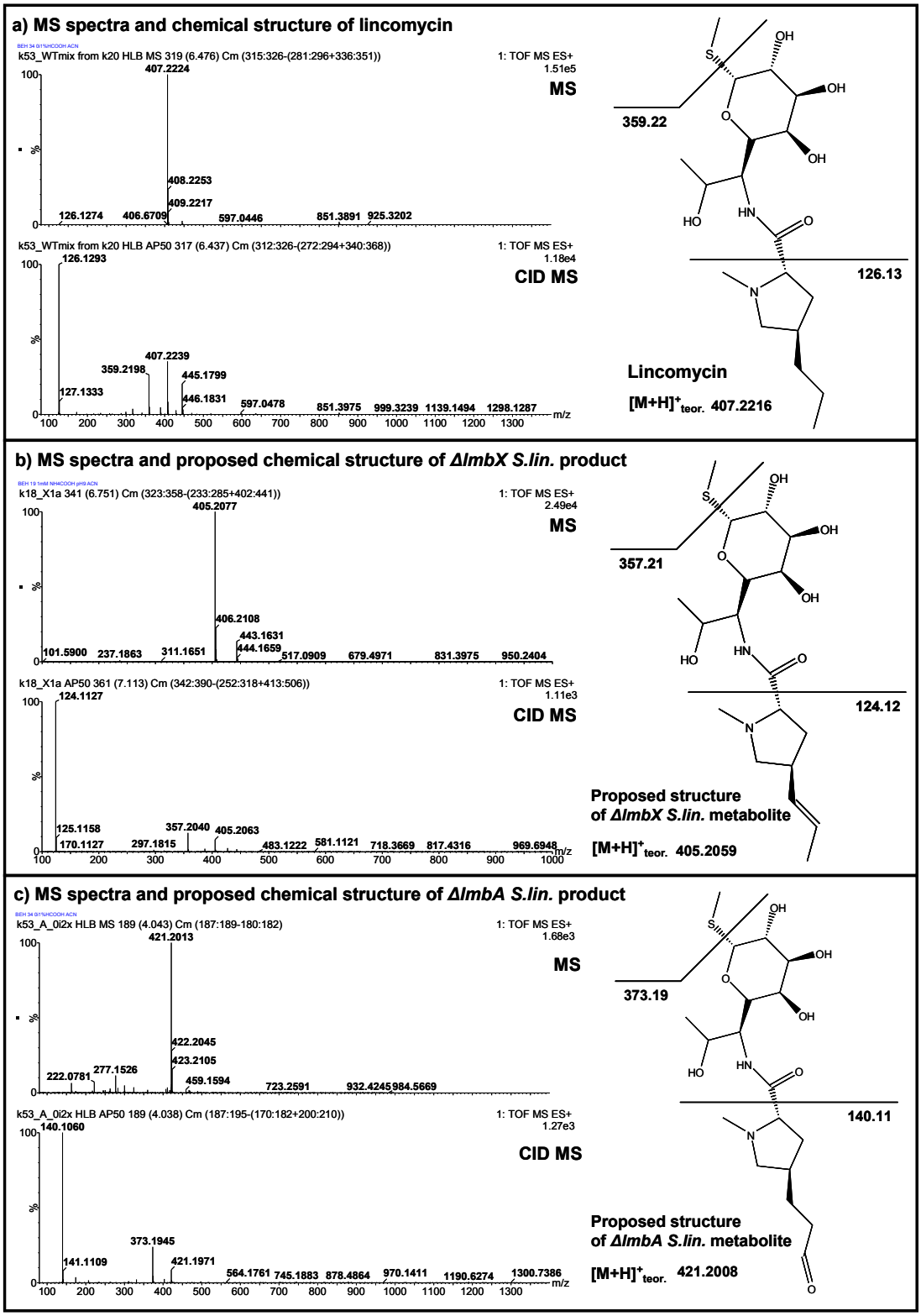

Fig. 6.8 MS spectra and structures of metabolites of *S. lincolnensis* mutant strains.

a) lincomycin; b) metabolite of *ΔlmbX S.lincolnensis* c) metabolite of *ΔlmbA S.lincolnensis*.

MS detection: cone voltage, 40 V; capillary voltage, +2500 V; ion source block temperature, 120 °C; nitrogen desolvation gas temperature, 350 °C; desolvation gas flow rate, 800 L h^{-1}; cone gas flow, 50 L h^{-1}; W dynamic range enhancement mode; scan time, 0.1 s; interscan delay time 0.01 s. In-source CID mass spectra: aperture I, 50 V.

6.2 Alternatives to Common Techniques for Antibiotic Analysis

6.2.1 Core-Shell Particle columns for Antibiotic Separation (Paper 3)

The recently introduced Kinetex C_{18} chromatographic column packed with core--shell 2.6 μm particles is declared to provide similar efficiency and short analysis as Acquity BEH C_{18} column with 1.7 μm porous particles. Unlike Acquity BEH C_{18} column, Kinetex C_{18} column exhibited lower column backpressure making this column compatible to conventional HPLC systems. The performance of Kinetex C_{18} column (2.1 × 50 mm) and Acquity BEH C_{18} column (2.1 ×50 mm) for gradient separation of tetracyclines under acidic conditions (oxytetracycline, tetracycline, chlortetracycline, and doxycycline) and macrolides under alkaline conditions (tylosin, clarithromycin, roxithromycin, and carbomycin) were studied.

The columns were compared by evaluation of their experimental peak capacity and its dependence on linear velocity and gradient slope. Maximal experimental peak capacities for analysis of tetracyclines were 51.8 (Acquity BEH C_{18} column) and 48.4 (Kinetex C_{18} column). This indicated that Kinetex C_{18} was a suitable alternative to Acquity BEH C_{18} column for analysis of tetracyclines under acidic conditions. On the contrary, maximal experimental peak capacities for analysis of macrolides on Acquity BEH C_{18} column was higher (46.7) than that on Kinetex C_{18} column (36.9). Moreover, application of Kinetex C_{18} column for analysis of two macrolides (clarithromycin and roxithromycin) under alkaline conditions was limited with respect to its decreasing performance with growing number of injections on the column. This phenomenon was connected to alkaline conditions (pH 9), but was not observed for the other macrolides (tylosin and carbomycin), nor tetracyclines analyzed under acidic conditions. To conclude, Kinetex C_{18} column represents a convenient alternative to Acquity BEH C_{18} column for analysis of tetracyclines under acidic conditions, but exhibited substantial limitations for analysis of macrolides under alkaline conditions.

6.2.2 Condensation Nucleation Light-Scattering Detector for Detection of Antibiotics (Paper 4)

The connection of the condensation nucleation light-scattering detector NQAD^{TM} (Nano Quantity Analyte Detector) with the UHPLC system was investigated. The detector was employed for detection of selected antibiotic compounds – macrolides (oleandomycin, erythromycin, troleandomycin, clarithromycin, and roxithromycin) that are difficult to detect by classical UV detectors due to the lack of strong chromophores. The comparison of NQAD^{TM} and UV detection of these compounds analyzed under isocratic conditions is shown in Fig. 6.9. The determined lowest detection limits for these compounds detected by the NQAD^{TM} detector ranged from 3.0 to 5.4 μg mL^{-1}, which was on average three times lower than when a UV detector was employed. Furthermore, it was revealed that the mobile phase composition (buffer type, concentration, additives) represents the limiting parameters in the NQAD^{TM} universal detection.

The suitability of the detector connected with ultra-high performance liquid chromatography in the gradient mode was tested on a more complex mixture containing twelve antibiotics. The detector exhibited full compatibility under both, isocratic and gradient, elution modes when the separations were achieved in relatively short run times. However, the sensitivity of structurally different analytes on UV versus NQAD^{TM} varied: lincomycin, clindamycin, clarithromycin and roxithromycin, achieved a better signal-to-noise ratio with NQAD^{TM}, whereas vancomycin, streptovitacin A and metronizadole exhibited lower sensitivity by NQAD^{TM} compared with the UV detector. Comparable sensitivity on both detectors was obtained for chloramphenicol, cycloheximide, griseofulvin, rapamycin, and carbomycin. To conclude, the NQAD^{TM} detector is suitable for detection of analytes in the UHPLC effluents and represents a promising alternative to UV detectors for compounds with weak or no chromophores.

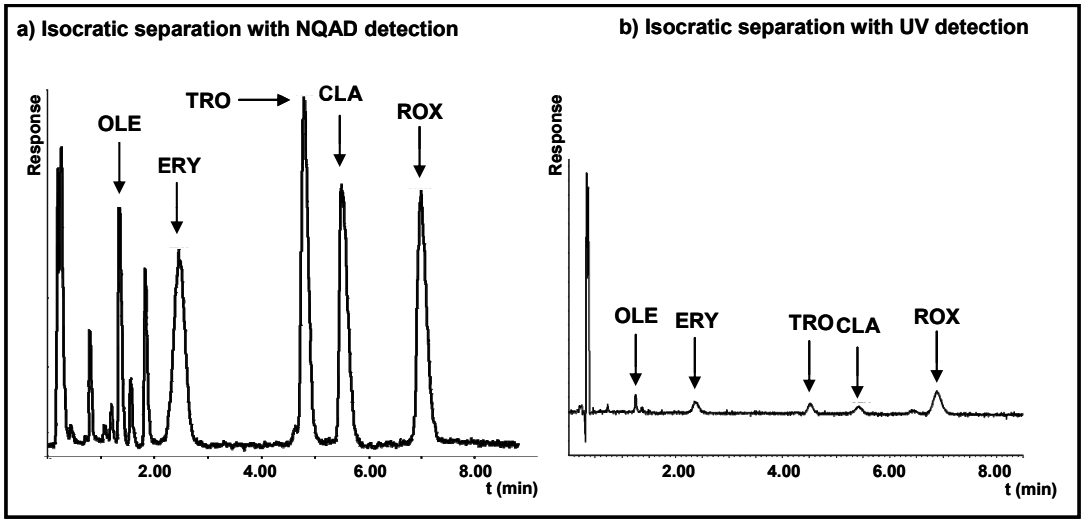

Fig. 6.9 Isocratic separation of macrolides. a) CNLSD (NQAD^{TM}); b) UV detection.

OLE – oleandomycin, ERY – erythromycin, TRO – troleandomycin, CLA – clarithromycin, ROX – roxithromycin, 50 µg mL^{-1}. UHPLC: Acquity BEH C_{18} column (50 × 2.1 mm – i.d., particle size 1.7 µm, Waters); mobile phase, (A) water (B) acetonitrile, both containing 0.01% NH_4OH; flow rate, 0.4 mL min^{-1}; isocratic elution, 45% B; injection volume, 5 µL. NQAD^{TM} detection: 35 °C, gain 10×. UV detection: 194 nm; data sample rate, 20 pts s^{-1}; filter constant 0.5.

6.3 Fingerprinting of Secondary Metabolites in Cultivation Broth

6.3.1 Development of the Fingerprinting Method (Paper 5)

A fingerprinting method for chemical screening of microbial secondary metabolites, potential antibiotics, in spent cultivation broths has been developed. The method is based on high-throughput UHPLC separation with photodiode array UV detection. Thirteen antibiotic standards and four cultivation broths were used for the method optimization. The comparison of ten liquid-liquid and solid phase extraction protocols for sample clean-up and pre-concentration revealed that HLB sorbent gives the best recoveries. The Acquity BEH C_{18} chromatographic column was chosen for the samples separation with respect to its universality, selectivity, efficiency and robustness. The sample extraction and method validation were assessed with relative standard deviations of 0.5, 5.0 and 20.0% for retention times, peak areas and minor compound peak areas, respectively. The method is presented by two 3D fingerprints for every sample, which were obtained under different, acidic and alkaline, UHPLC conditions (see Fig 6.10). The acidic mobile phase consisted of 0.5% phosphoric acid with methanol and the alkaline mobile phase of 1 mM ammonium formate, pH 9 with acetonitrile.

Each pair of the 3D fingerprints includes the following information on the physico-chemical properties: polarity (retention time), presence and characterization of chromophores (UV spectra), compound concentration (detector response), and acid-base properties (influence of different pH of the aqueous parts of mobile phase on retention times). Provision of the latter physico-chemical information is illustrated by different retention order of the compounds C1 to C3 in Figs. 6.10a and 6.10b.

The fingerprints can be further used for statistical comparison in order to dereplicate already known compounds, or to seek correlation between physico--chemical information of the fingerprint and genetic or ecological markers (*e.g.* presence of selected genes, locality of strain origin or their taxonomic identification).

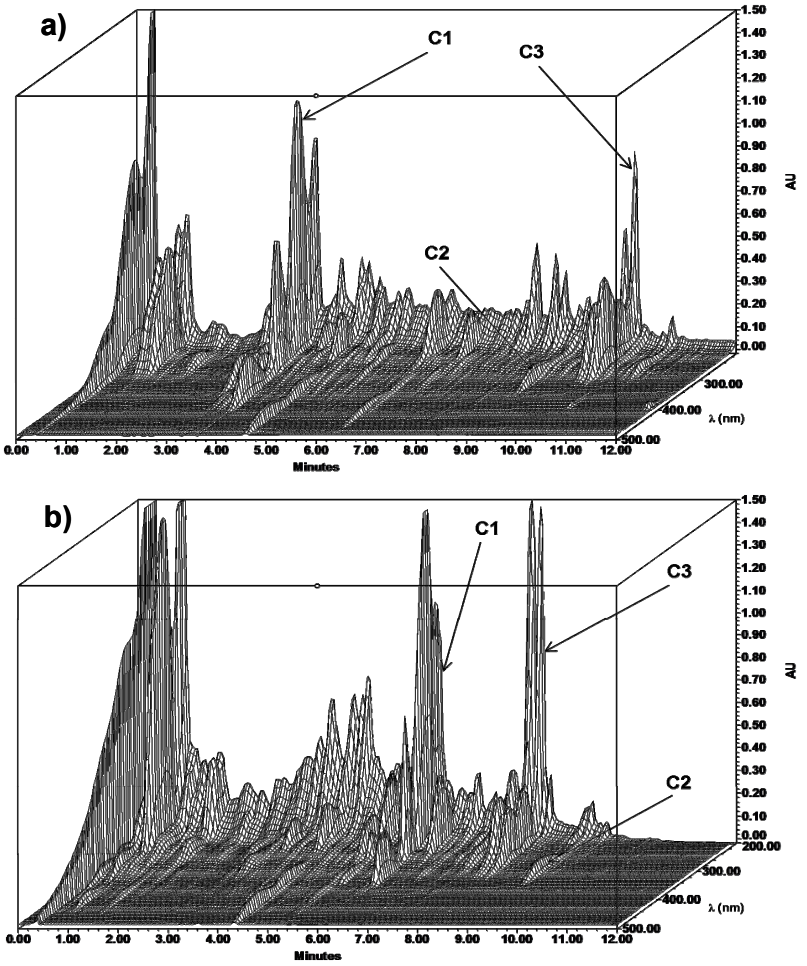

Fig. 6.10 UHPLC 3D fingerprint pair of secondary metabolites in an actinomycete cultivation broth; a) acidic UHPLC conditions; b) alkaline UHPLC conditions.

C1, C2, and C3 – unknown compounds. UHPLC: Acquity BEH C_{18} column (50 × 2.1 mm i.d., particle size 1.7 µm), Waters; flow rate, 0.4 mL min^{-1}; injection volume, 5 µL; DAD detection: data sample rate, 20 pts s^{-1}; filter constant 0.5. *a) acidic conditions:* mobile phase, (A) 0.5% H_3PO_4 in water, (B) methanol; linear gradient elution (min/%B), 0/5, 1.5/5, 16.5/100, 18.0/100, equilibration 1 min; column temperature, 55 °C; wavelength range, 210–600 nm. *b) alkaline conditions:* mobile phase, (A) 1 mM ammonium formate, pH 9 (B) acetonitrile; linear gradient elution (min/%B), 0/5; 2.0/5.0, 18.0/65.8, 18.0/100, 19.0/100, equilibration 1 min; column temperature, 30 °C; wavelength range, 194–600 nm.

6.3.2 Application of the fingerprinting method (unpublished)

The extraction protocol of the fingerprinting method (subsection 6.3.1) was applied on a complex set of compounds in order to confirm universality of the method. The set consisted not only of actinomycete secondary metabolites, but also of antibiotics produced by other microorganisms as well as their semi-synthetic analogues. It included β-lactames (penicillin G, penicillin V, ampicillin, amoxicillin, oxacyllin, celaphalosporin C), macrolides (erythromycin, clarithromycin, troleandomycin, oleandomycin, roxithromycin, tylosin, spiramycin, carbomycin, turimycin), tetracyclines (tetracycline, oxytetracyline, doxycycline, chlortetracycline, granaticin), peptides and glycopeptides (polymyxin, thiostrepton, vancomycin, ristocetin A), lincosamides (lincomycin, clindamycin), polyene macrolides (natamycin, nystatin), fluoroquinolones (ciprofloxacin, ofloxacin), griseofulvin, novobiocin, chloramphenicol, metronidazole, purimycin, cycloheximide and streptovitacin A. The recovery rates of these antibiotics extracted by the HLB sorbent and eluted with methanol-water solvents of different ratios are given in Fig. 6.11.

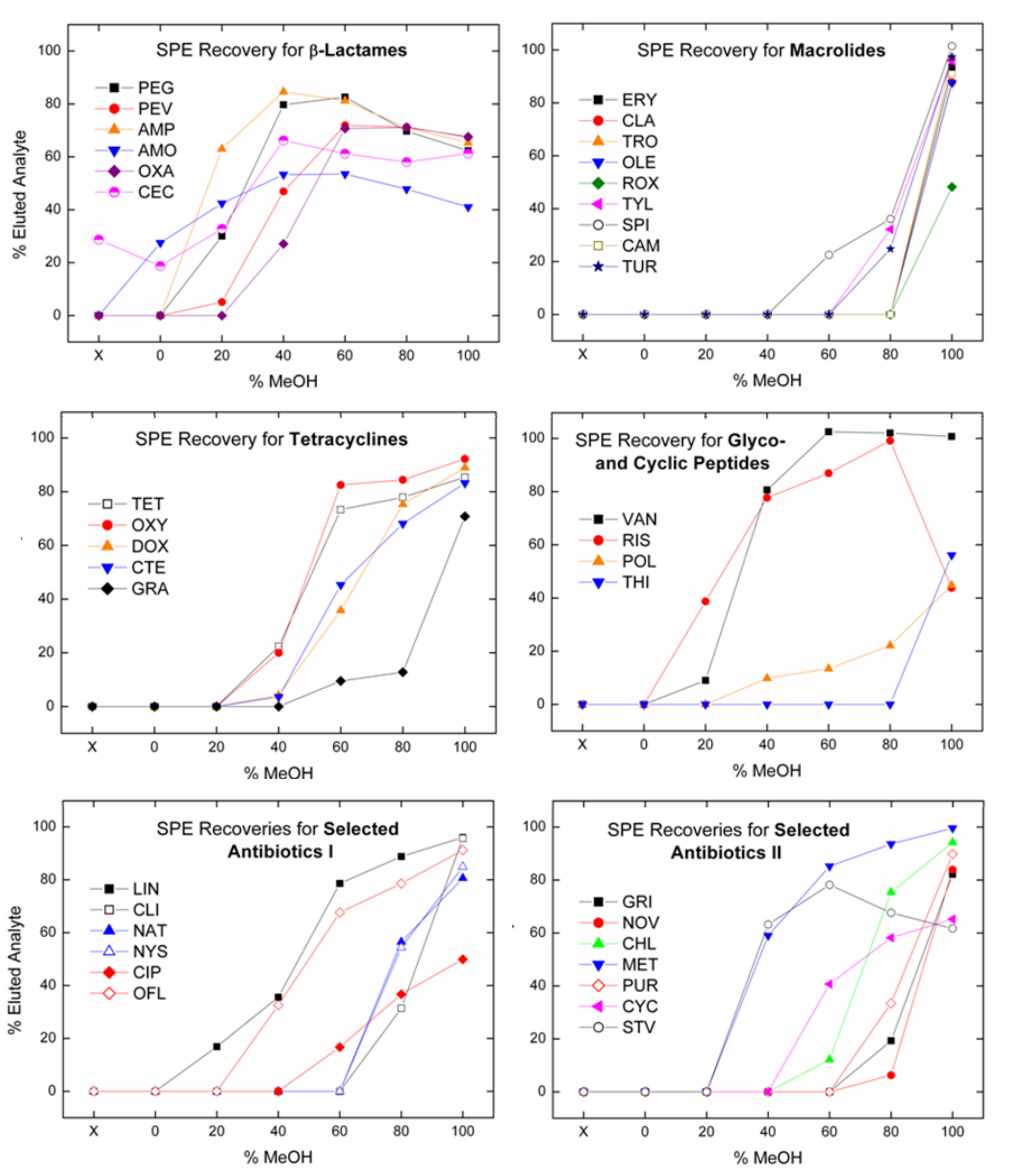

Fig. 6.11 Recovery rates of antibiotics extracted by HLB sorbent. PEG – penicillin G, PEV – penicillin V, AMP – ampicillin, AMO – amoxicillin, OXA – oxacillin, CEC – cephalosporin C, ERY – erythromycin, CLA – clarithromycin, TRO – troleandomycin, OLE – oleandomycin, ROX – roxithromycin, TYL – tylosin, SPI – spiramycin, CAM – carbomycin, TUR – turimycin, TET – tetracycline, OXY – oxytetracycline, DOX – doxycycline, CTE – chlortetracycline, GRA – granaticin, VAN – vancomycin, RIS – ristocetin A, POL – polymyxin, THI – thiostrepton, LIN – lincomycin, CLI – clindamycin, NAT – natamycin, NYS – nystatin, CIP – ciprofloxacin, OFL – ofloxacin, GRI – griseofulvin, NOV – novobiocin, CHL – chloramphenicol, MET – metronizadole, PUR – purimycin, CYC – cycloheximide, STV – streptovitacin A. Analyte concentration 10 μg mL^{-1}. Fraction X represents the eluent eluted when loading the sample.

Apparently, only cephalosporin C failed to retain on the HLB sorbent (fraction X) at all and amoxicillin was eluted already with water. All the other compounds were well retained and eluted with pure methanol with the recovery rates greater than 40%, which is a very satisfactory result for a non-target extraction. In addition to that, the elution profiles tended to be class-specific; particularly the

elution profiles of β-lactames, macrolides and tetracyclines are distinctive within these classes.

Furthermore, the method has been applied for screening of 448 unknown actinomycete strains isolated from nine habitats contrasting in pH, soil bedrock, and vegetation type. The fingerprints were transported into the ASCII code for further statistical evaluations. So far, several actinomycete secondary metabolites in the fingerprints were selected according to their UV spectra. Based on MS spectra of these compounds (accurate mass, isotopic peak ratio, Reaxys database[149]) and positive bioassay tests with *Kocuria rhizophilla* (described in Paper 2), these compounds were tentatively identified as known secondary metabolites. The compounds include: a 16-membered ring macrolide, angolamycin; a macrocyclic lactone, desertomycin (Fig. 6.13a); an aminocoumarin, novobiocin (Fig. 6.13b); various macrolide polyenes including rimocidin (Fig. 6.13c); a polyketide, granaticin (Fig. 6.13d); a peptide, siamycin I (Fig. 6.13e); and an aminonucleoside futalosine (Fig. 6.13f). Based on UV spectra and retention times, the occurrence of these compounds among all 448 samples was explored and the frequency of these compounds among the habitats of isolation was established (see Table 6.1).

Table 6.1 Occurence of actinomycetes producing selected secondary metabolites in different habitats.

Habitat of isolation	Number of Isolates	ANG	DES	GRA	POL	NOV	SIA
DE	35			+	+		
KO	20				+	+	
ME	60	+	+		+		
NE	38	+			+	+	
OB	36		+		+		
SR	86	+			+	+	
ST	46						
TR	94				+	+	
VY	33					+	+

The codes for habitats of isolation represent different localities in Czech Republic, Germany, and Austria. ANG – angolamycin, DES – desertomycin, GRA – granaticin, POL – various polyenes, NOV – novobiocin, SIA – siamycin I; "+" indicates that at least one strain isolated from the respective habitat produced the given antibiotic under laboratory conditions.

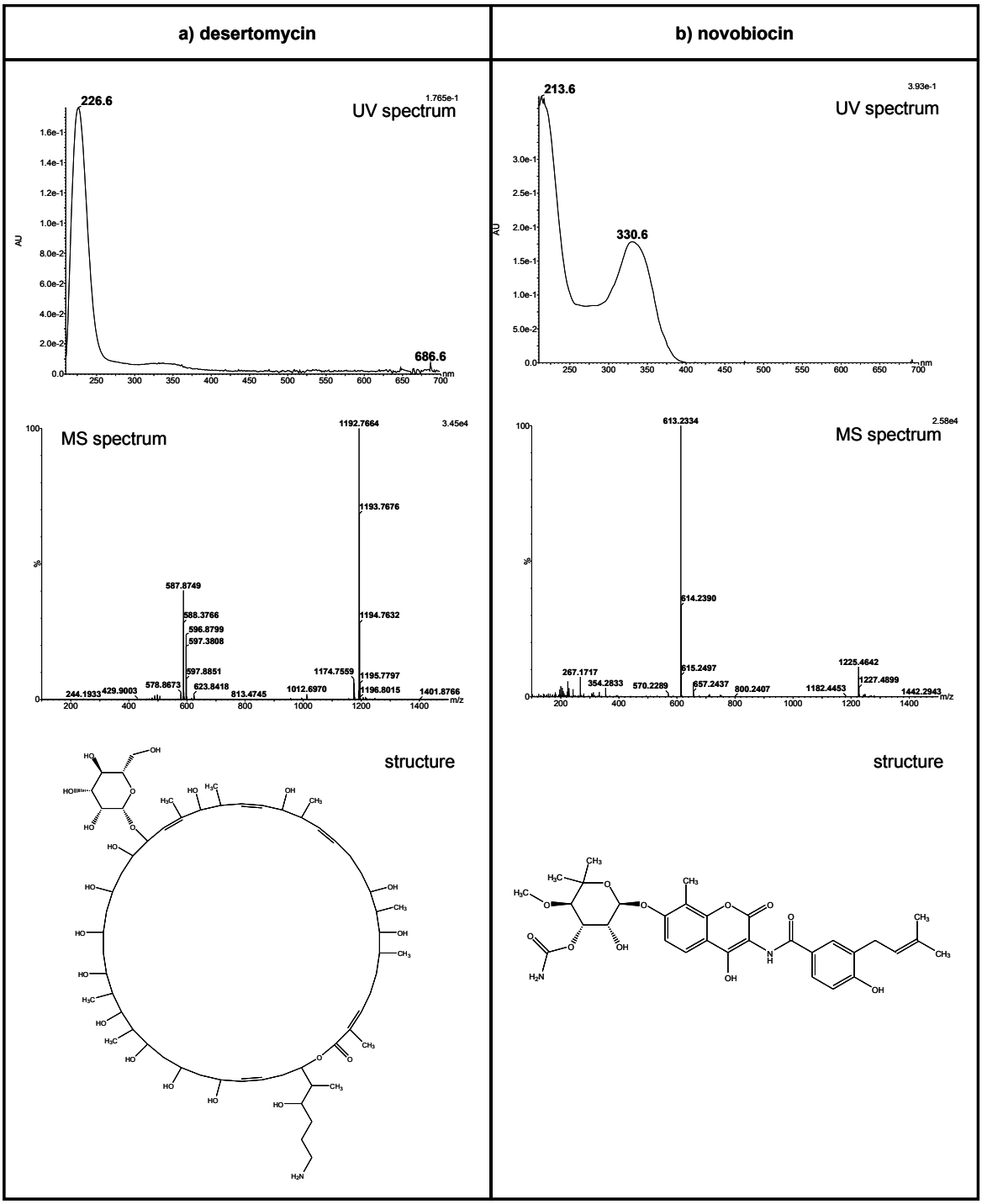

Fig. 6.12a,b UV and MS spectra of secondary metabolites found in actinomycete cultivation broths and their proposed structures; a) desertomycin, b) novobiocin.

MS conditions: cone voltage, 40 V; capillary voltage, +2500 V, −2500 V; ion source block temperature, 120°C; nitrogen desolvation gas temperature, 350°C; desolvation gas flow rate, 800 L h^{-1}, cone gas flow, 50 L h^{-1}. W dynamic range enhancement mode; scan time, 0.1 s; interscan delay time, 0.01 s (0.3 s for polarity switch, 0.1 s for lock spray).

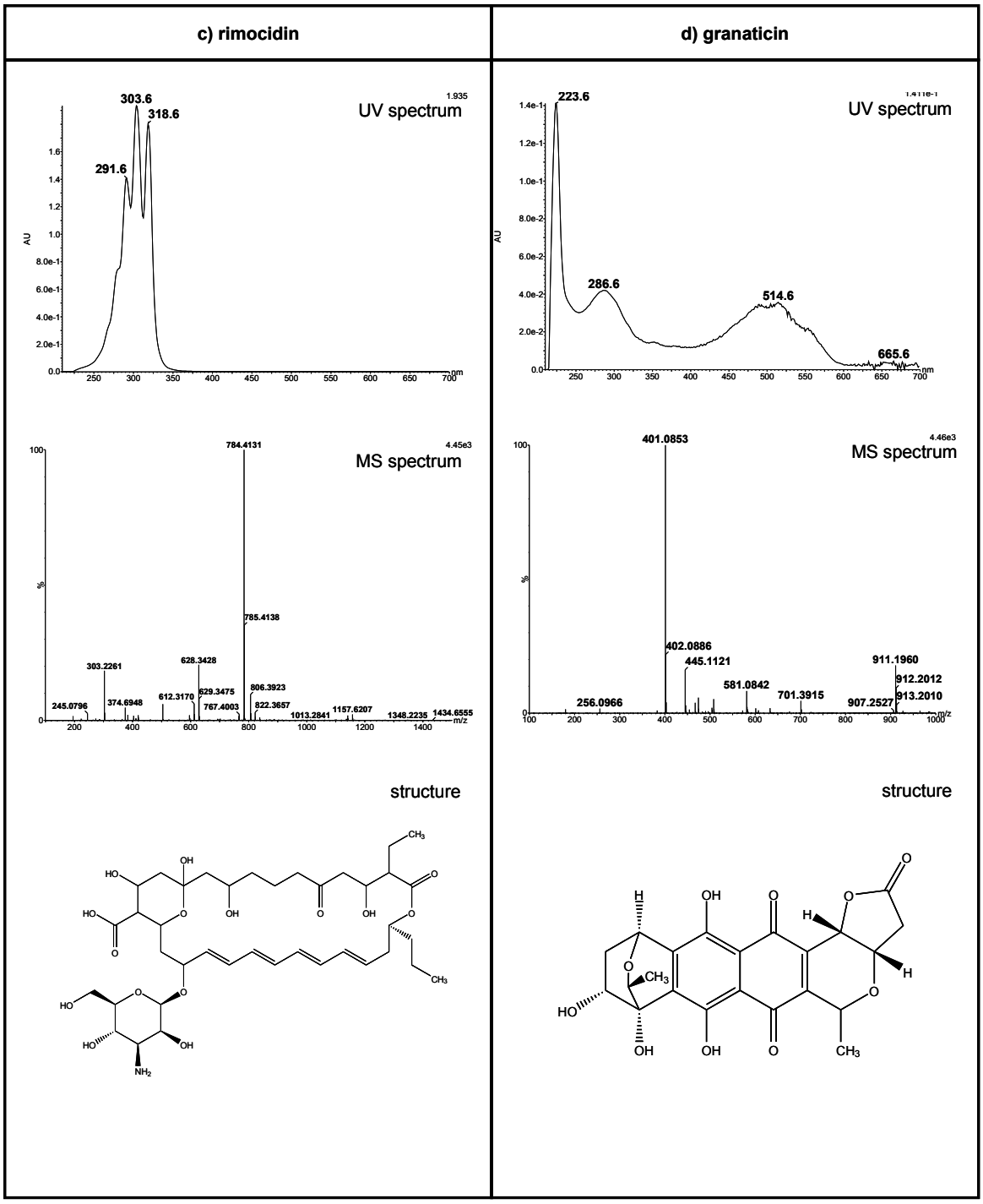

Fig. 6.12c,d UV and MS spectra of secondary metabolites found in actinomycete cultivation broths and their proposed structures; c) rimocidin, d) granaticin.

MS conditions: cone voltage, 40 V; capillary voltage, +2500 V, −2500 V; ion source block temperature, 120°C; nitrogen desolvation gas temperature, 350°C; desolvation gas flow rate, 800 L h^{-1}, cone gas flow, 50 L h^{-1}. W dynamic range enhancement mode; scan time, 0.1 s; interscan delay time, 0.01 s (0.3 s for polarity switch, 0.1 s for lock spray).

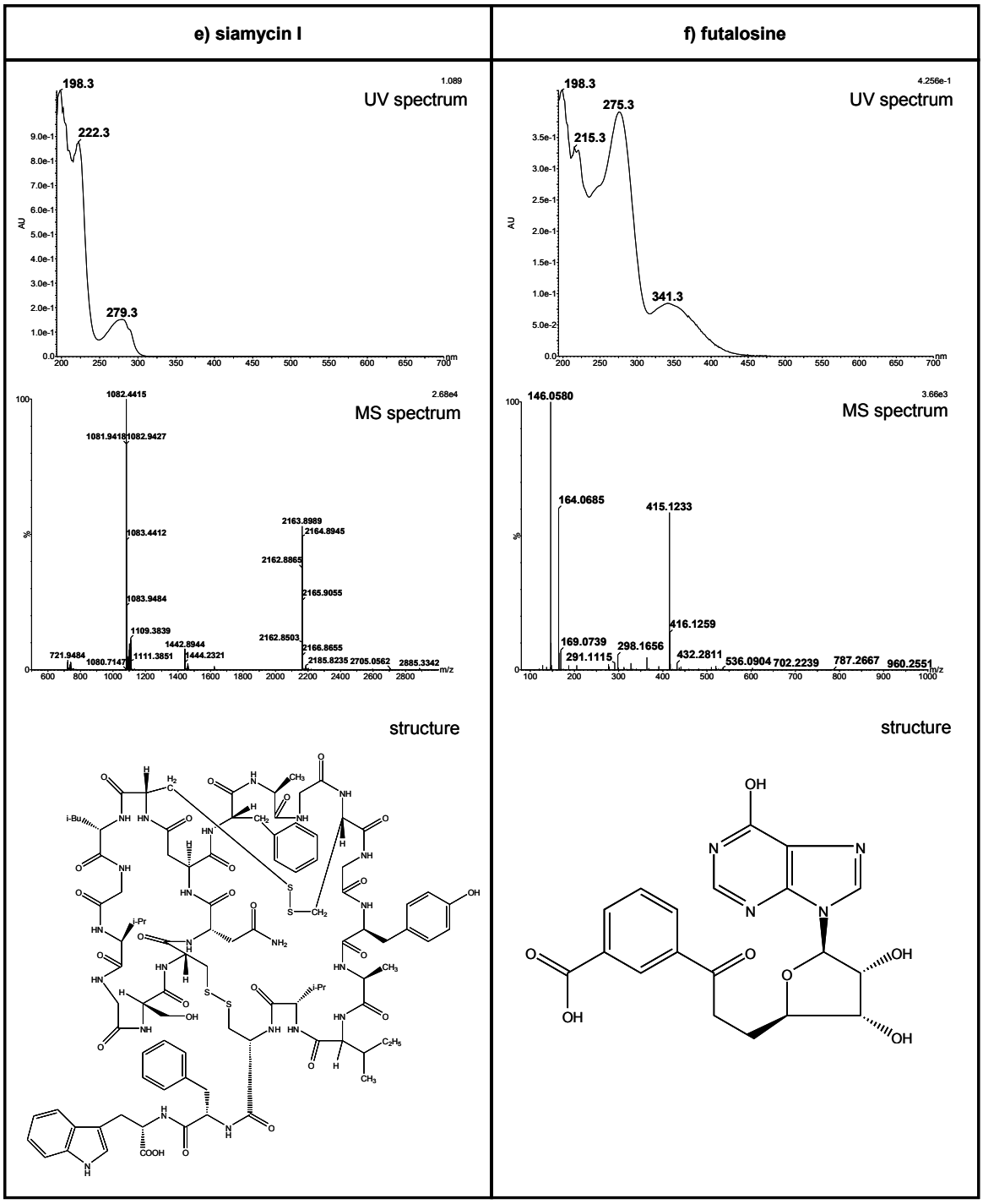

Fig. 6.12e,f UV and MS spectra of secondary metabolites found in actinomycete cultivation broths and their proposed structures; e) siamycin I, f) futalosine.

MS conditions: cone voltage, 40 V; capillary voltage, +2500 V, −2500 V; ion source block temperature, 120°C; nitrogen desolvation gas temperature, 350°C; desolvation gas flow rate, 800 L h^{-1}, cone gas flow, 50 L h^{-1}. W dynamic range enhancement mode; scan time, 0.1 s; interscan delay time, 0.01 s (0.3 s for polarity switch, 0.1 s for lock spray).

7 CONCLUSIONS

The work in the presented dissertation thesis contributed to the research aimed at developing and searching for novel antibiotics. For this purpose, liquid chromatography methods with diode-array UV, fluorescence, nucleation condensation light-scattering and mass spectrometry detection techniques have been investigated, developed, and applied.

The specific aims stated in chapter 4 were accomplished as follows.

1. Novel or optimized liquid chromatography methods for analysis of analytes crucial for the study of the lincomycin biosynthetic pathway and for mutasynthesis of lincomycin derivatives were developed. The analytes include methylthiolincosamide (validated method), 4-(3-carboxy-3-oxo-propenyl)--2,3-dihydro-1*H*-pyrrole-2-carboxylic acid, butyl-L-proline, pentyl-L-proline (semi-quantitative method), 4´-butyl-4´-depropyllincomycin, and 4´-pentyl--4´-depropyllincomycin (separation, detection, and identification). Application of the methods has allowed the preparation of improved derivatives of lincomycin by mutasynthesis and revealed two unexpected lincomycin derivatives produced by *S. lincolnensis* mutant strains, which consequently resulted in revision of the biosynthetic pathway of 4-propyl-L-prolin, the lincomycin precursor.

2. The comparison of the UHPLC sub-2 μm particle (Acquity BEH C_{18}) and the core-shell particle (Kinetex C_{18}) columns revealed that Kinetex C_{18} represents a convenient alternative to Acquity BEH C_{18} column for analysis of tetracyclines under acidic conditions. However, Kinetex C_{18} exhibited substantial limitations for analysis of macrolides under alkaline conditions.

3. The condensation nucleation light-scattering NQAD^{TM} detector proved to be fully compatible with UHPLC under both isocratic and gradient chromatographic conditions. Further, several macrolides poorly detectable with UV detector were detected by the NQAD^{TM} detector with the limits of detection three times lower than those with UV detection.

4. A universal UHPLC method for screening of actinomycete secondary metabolites was developed. The method provides a pair of 3D fingerprints for one sample, and includes the following physico-chemical information on the secondary metabolites: polarity (retention times), structure (presence of chromophores), concentration (detector response), and acid-base properties (the influence of mobile phase pH on retention times). The method was applied to screen several hundreds of samples and provided data for subsequent statistical evaluation. Further, the presence of six known antibiotics of diverse structures in the actinomycete cultivation broths was confirmed.

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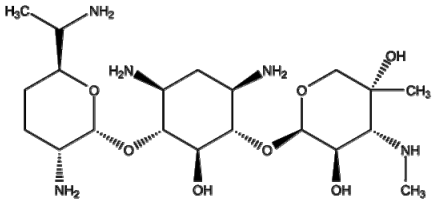
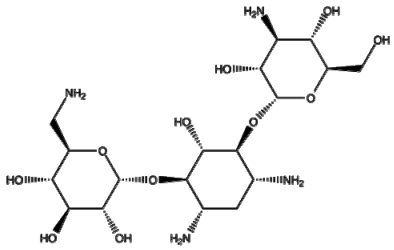
SUPPLEMENTARY DATA

Supplementary data contain chemical structures of selected compounds stated in section 2.4. Also, available UV spectra of the compounds are included.

Gentamycin **1**



Kanamycin **2**



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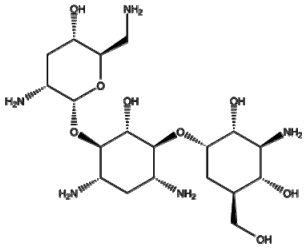
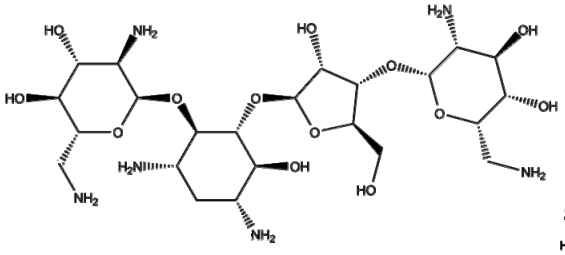


Neomycin **4**



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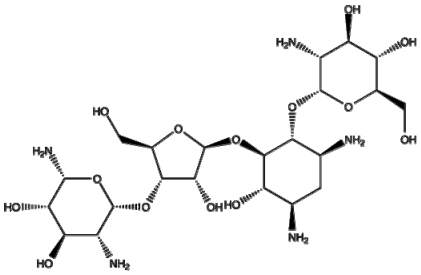

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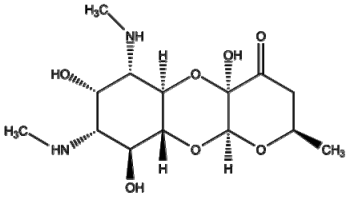
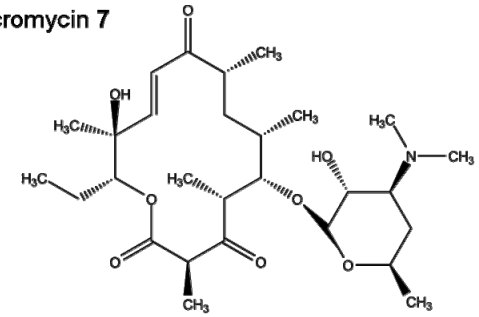
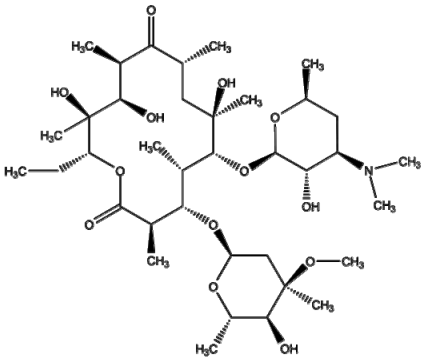


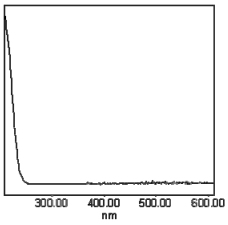
Aminoglycosides and aminocyclitols

Picromycin **7**



Erythromycin **8**





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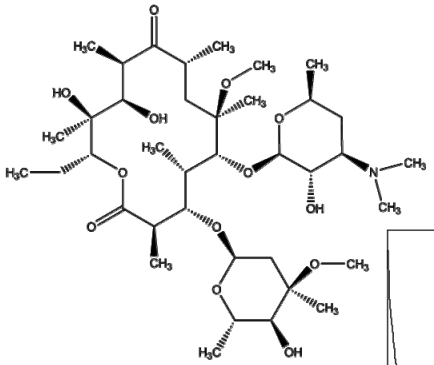
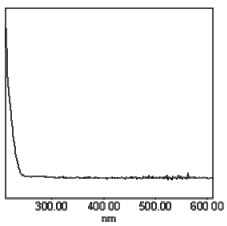
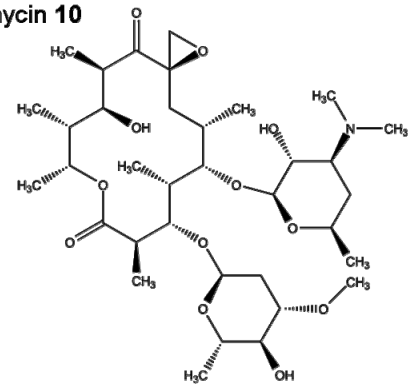
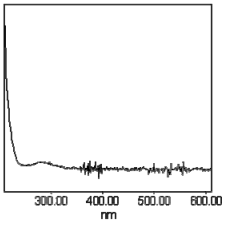




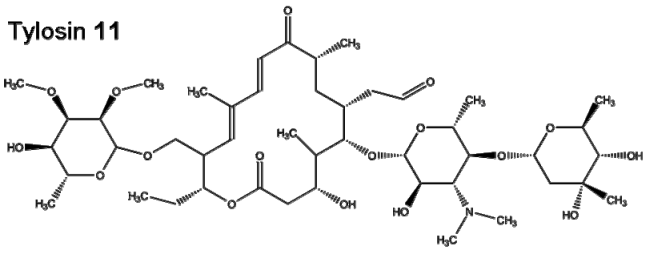
Oleandomycin **10**

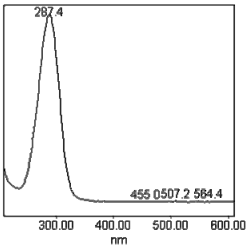




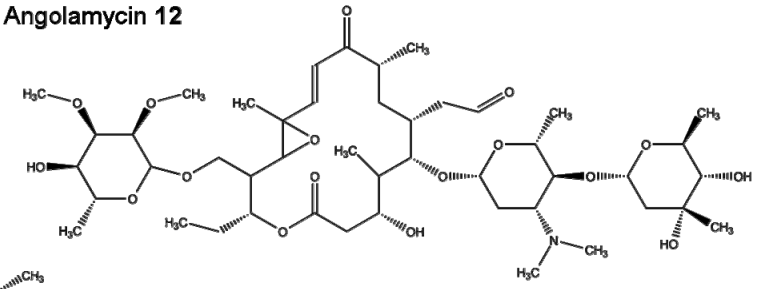
Macrolides I

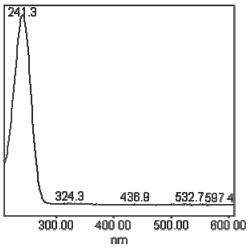
Tylosin **11**



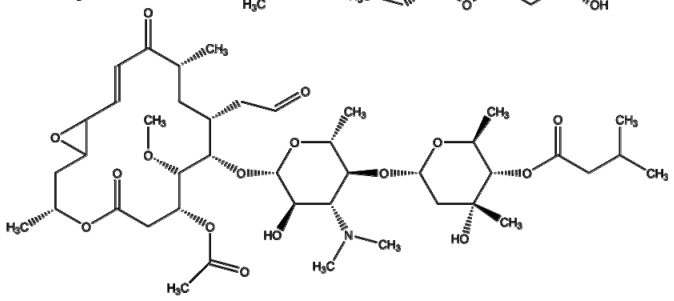


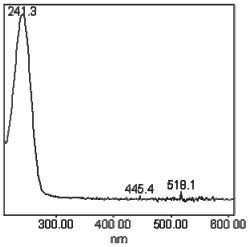
Angolamycin **12**





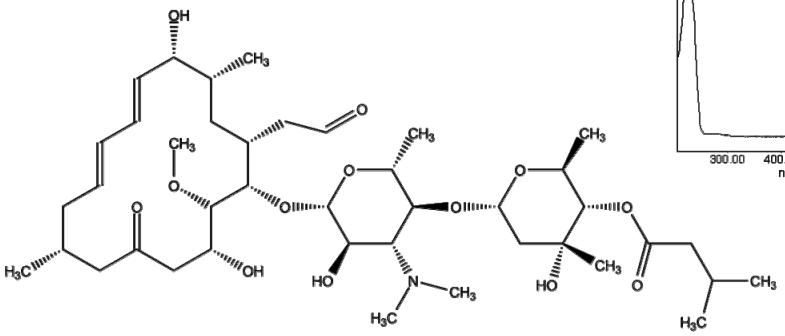
Carbomycin **13**

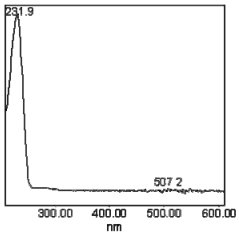




Macrolides II

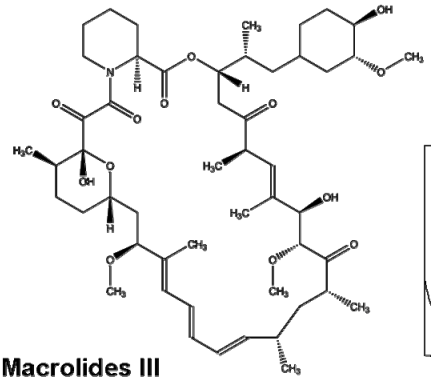
Kitasamycin **14**

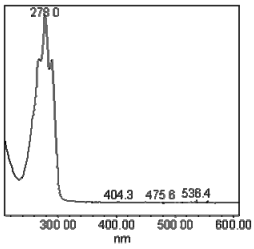


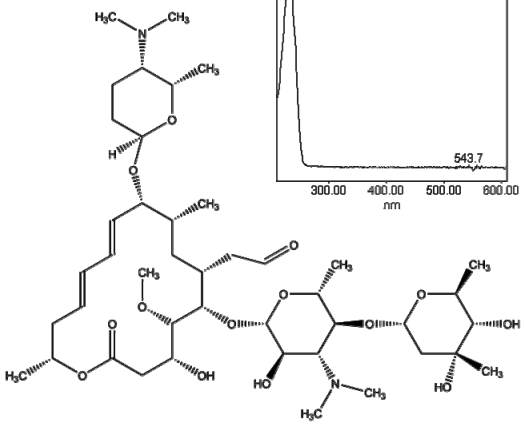


Spiramycin **15**

Rapamycin **16**

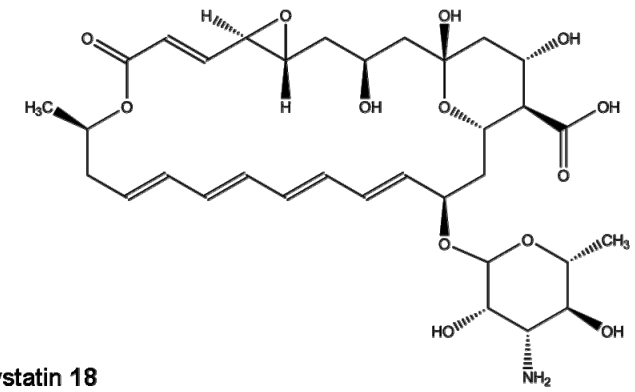


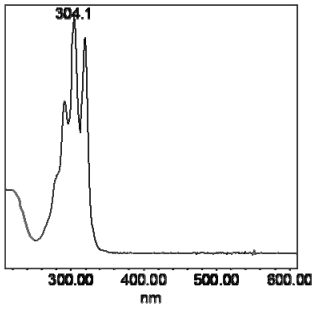




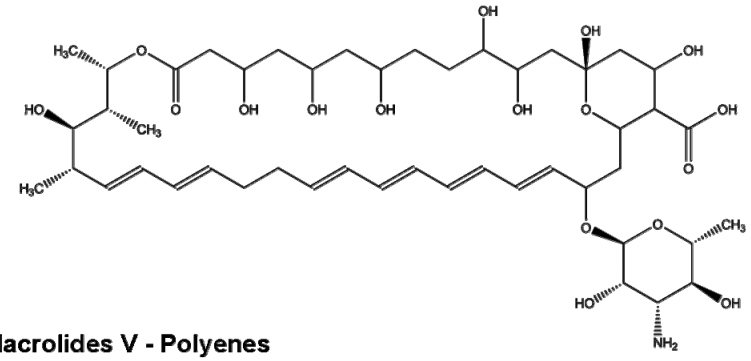
Macrolides III

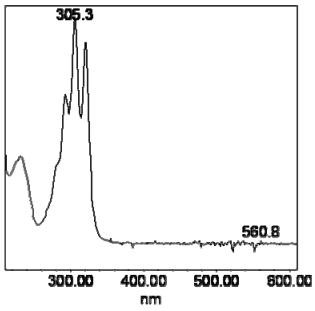
Natamycin **17**





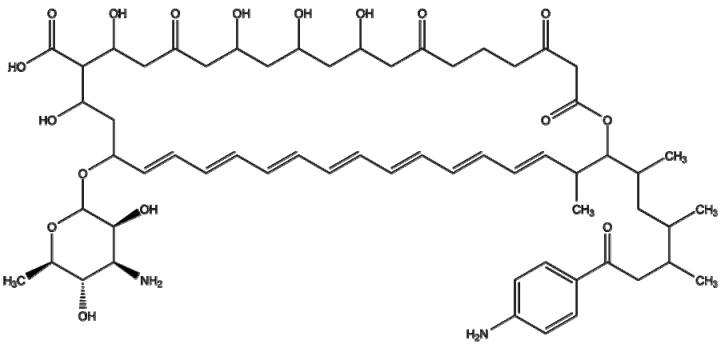
Nystatin **18**



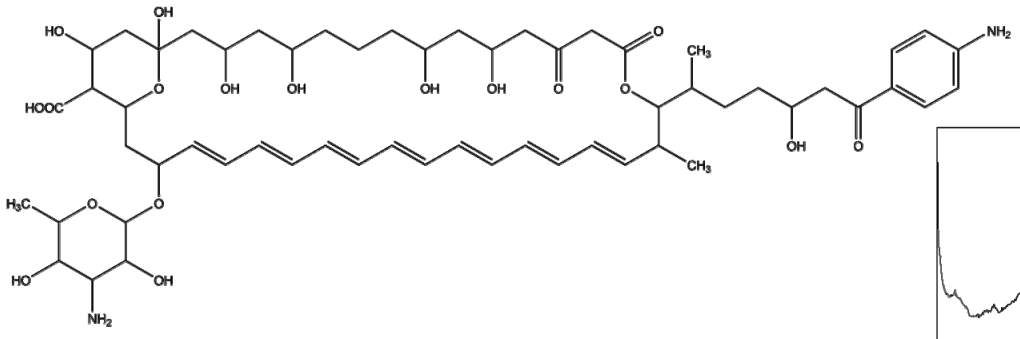


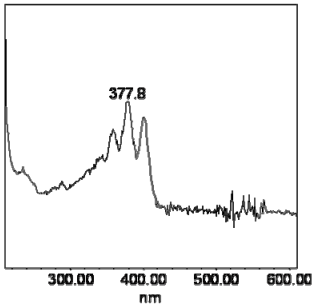
Macrolides V - Polyenes

Levorin **19**



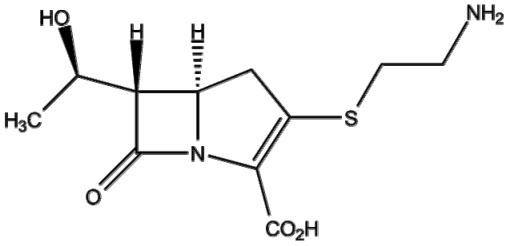
Trichomycin **20**



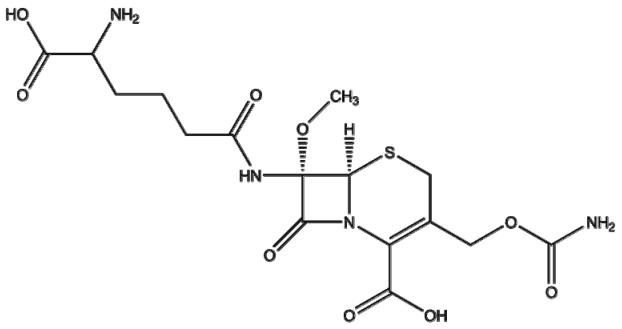


Macrolides VI - Polyenes

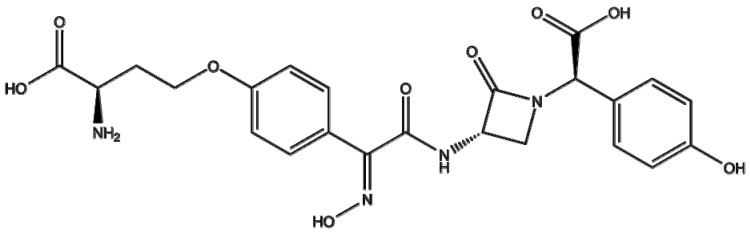
Thienamycin **21**



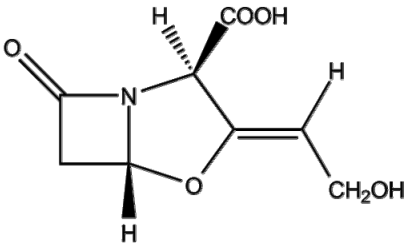
Cephamycin B **22**



Nocardicin **23**

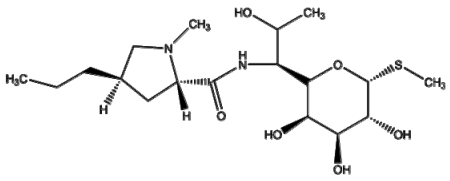


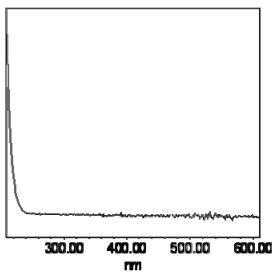
Clavulanic acid **24**



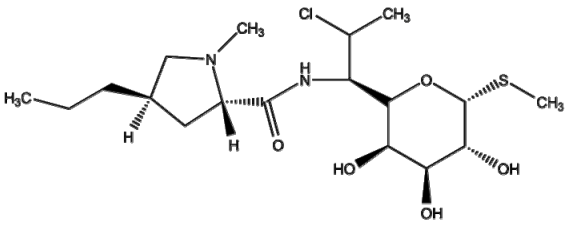
β-Lactames

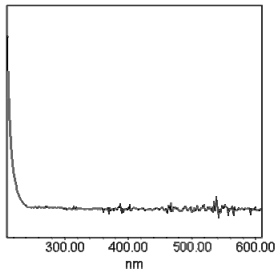
Lincomycin A **25**



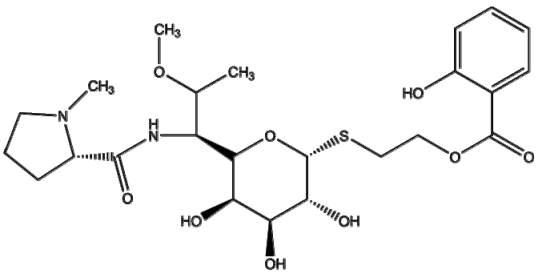


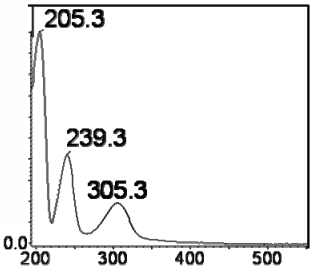
Clindamycin **26**





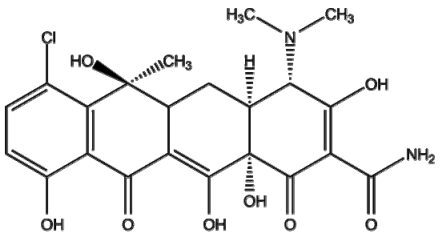
Celesticetin **27**

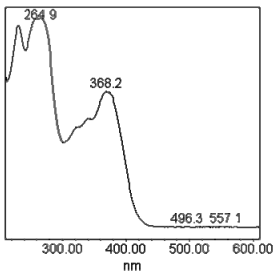




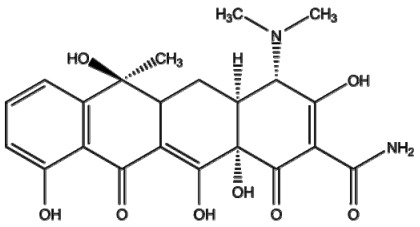
Lincosamides

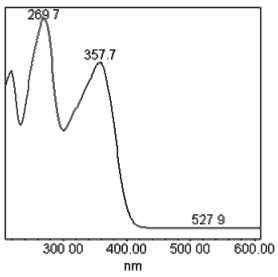
Chlortetracycline **28**



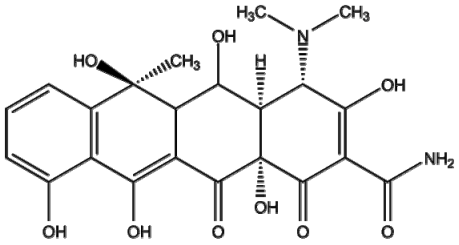


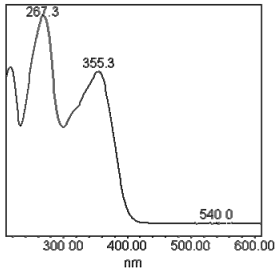
Tetracycline **29**





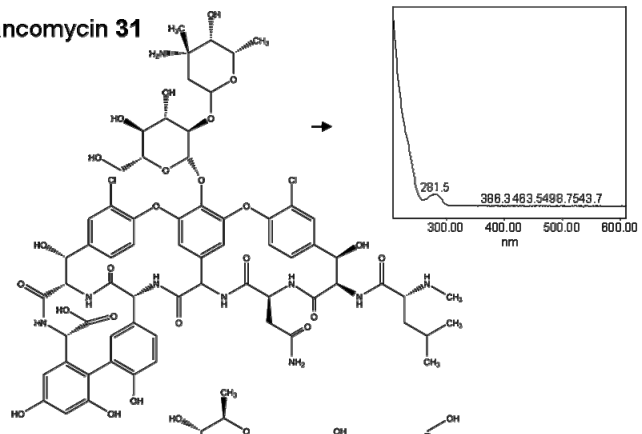
Oxytetracycline **30**



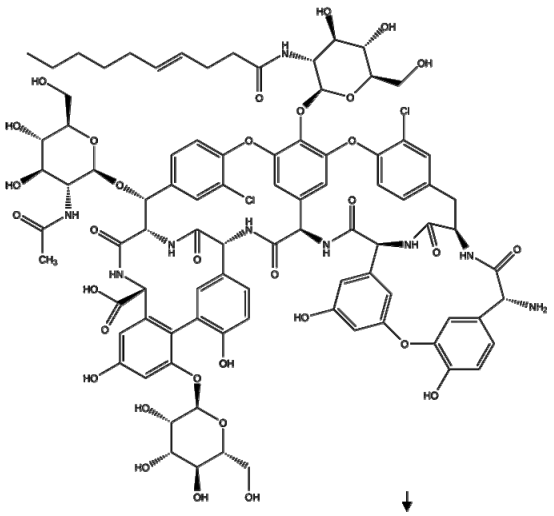


Tetracyclines

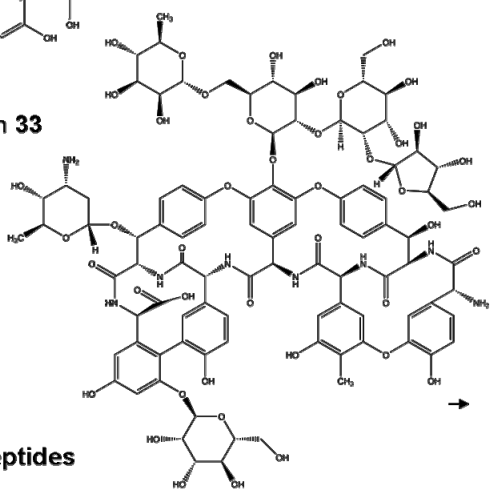
Vancomycin **31**

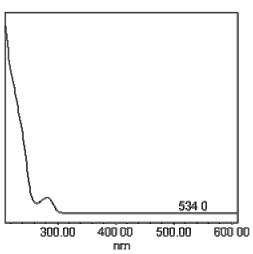


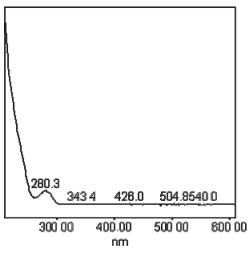
Teicoplanin **32**



Ristocetin **33**

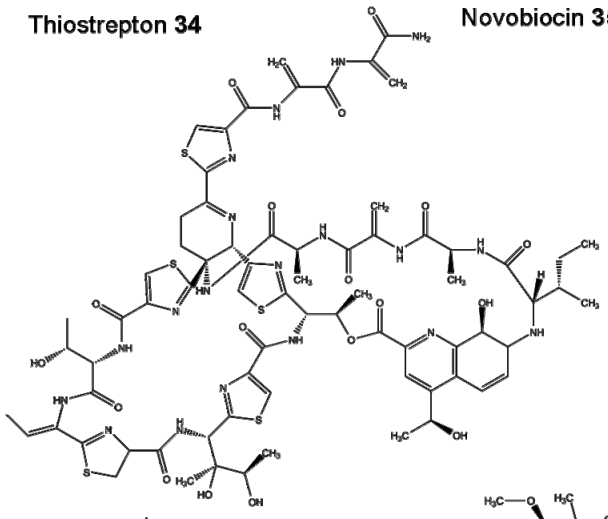


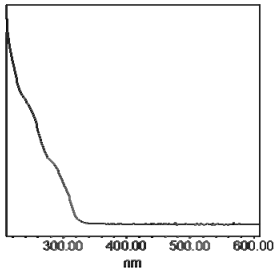




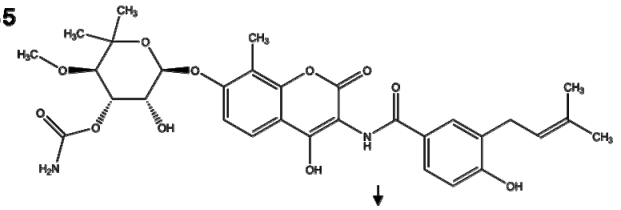
Glycopeptides

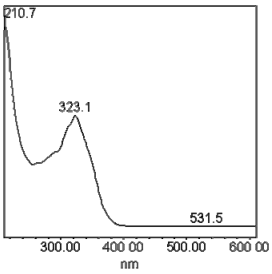
Thiostrepton **34**



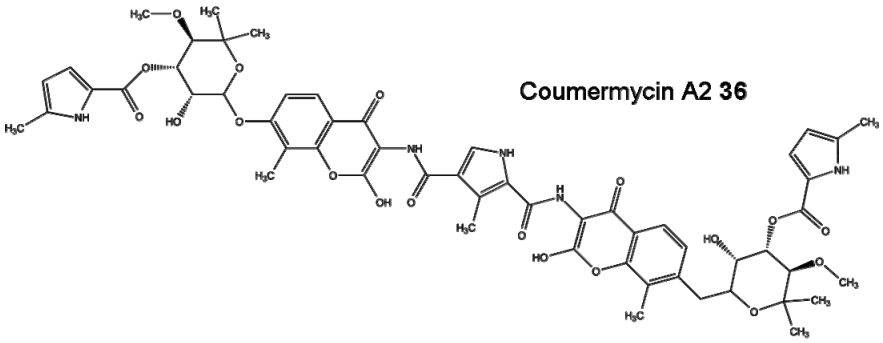


Novobiocin **35**

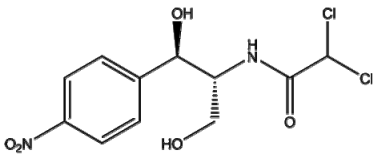


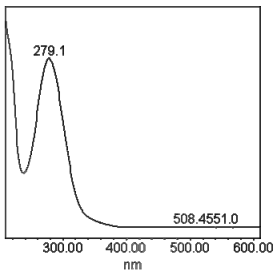


Coumermycin A2 **36**

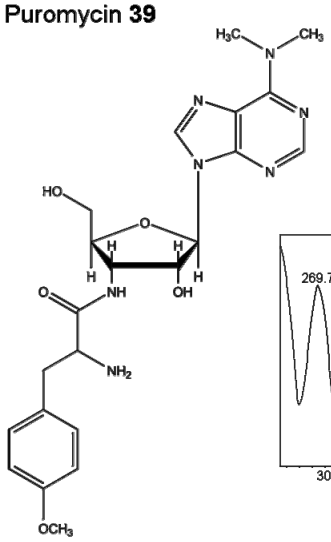


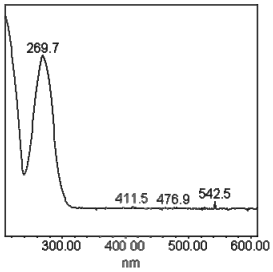
Chloramphenicol **37**



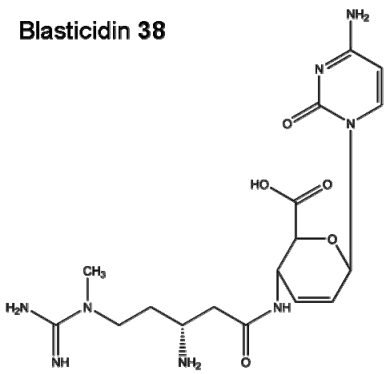


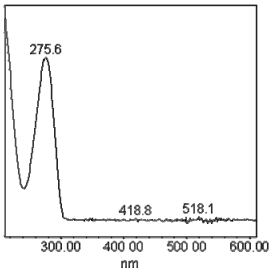
Puromycin **39**



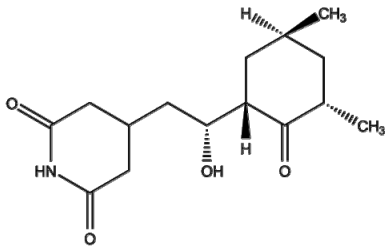


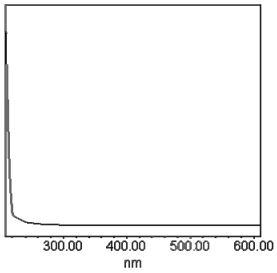
Blasticidin **38**



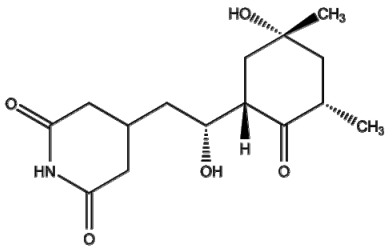


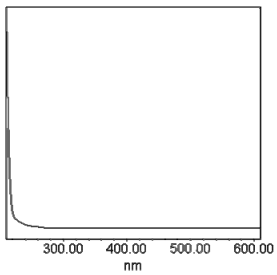
Cycloheximide **40**



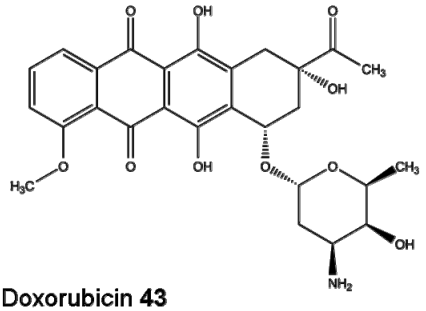


Streptovitacin A **41**

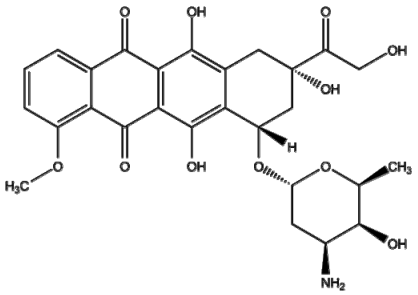




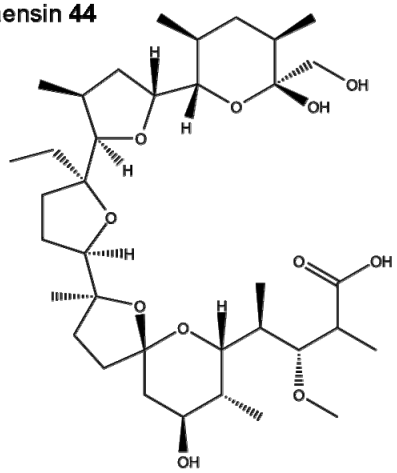
Daunorubicin **42**



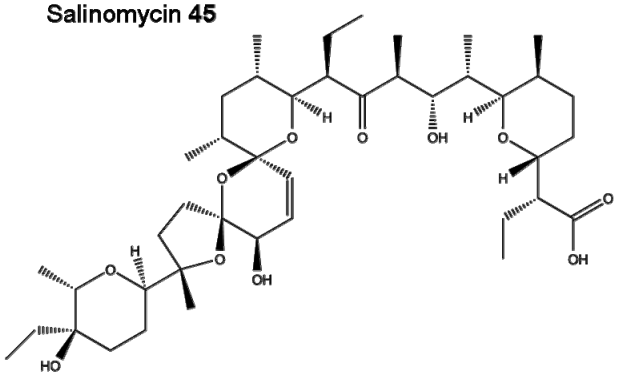
Doxorubicin **43**



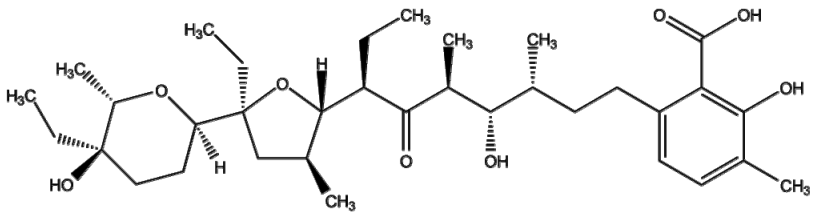
Monensin **44**

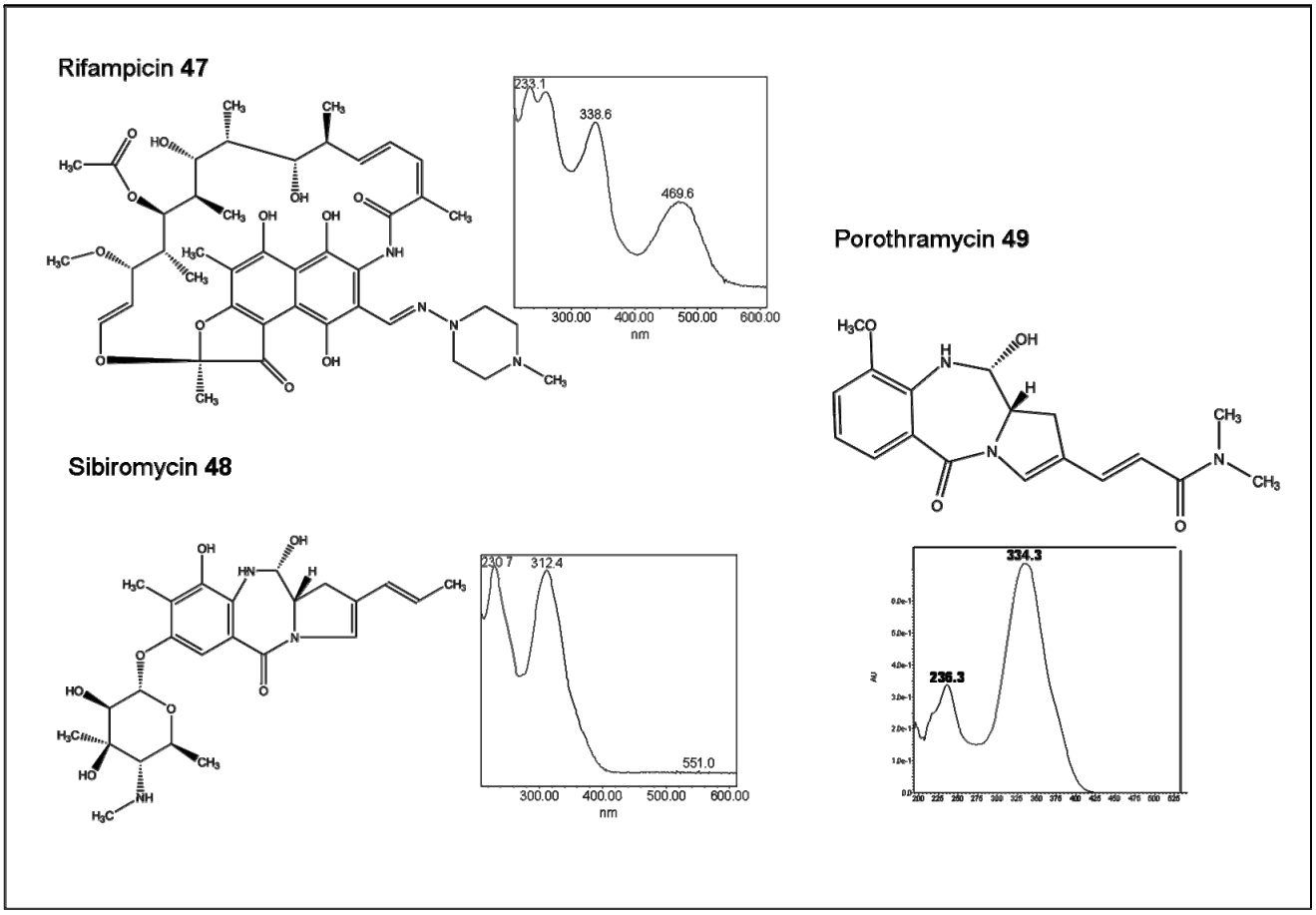


Salinomycin **45**



Lasalocid **46**





The UV spectra were acquired during UHPLC-DAD analysis under acidic chromatographic conditions. UHPLC: Acquity BEH C_{18} column (50 × 2.1 mm i.d., particle size 1.7 µm, Waters); mobile phase, (A) 0.5% H_3PO_4 in water, (B) methanol; linear gradient elution (min/%B), 0/5, 1.5/5, 16.5/100, 18.0/100, equilibration (1 min, 5%B); flow rate, 0.4 mL min^{-1}; column temperature, 55 °C; DAD detection: 210–600 nm; data sample rate, 20 pts s^{-1}; filter constant 0.5.

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Also, I thank Franz Hadacek and Vladimír Chobot for fruitful discussions and advice provided at the Department of chemical ecology and ecosystem research, University of Vienna.

APPENDICES (PAPERS 1 – 5)

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ORIGINAL PAPER

HPLC-fluorescence detection method for determination of key intermediates of the lincomycin biosynthesis in fermentation broth

Zdeněk Kameník · Jan Kopecký · Markéta Marečková · Dana Ulanová · Jitka Novotná · Stanislav Pospíšil · Jana Olšovská

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Abstract The biosynthetic pathway of the clinically important antibiotic lincomycin is not known in details. The precise knowledge of the lincomycin biosynthesis is a prerequisite for generation of improved derivatives by means of combinatorial genetics. Methods allowing determination of the key intermediates are very important tools of the pathway investigation. Two new high-performance liquid chromatography methods with fluorescence detection for determination of lincomycin precursors in fermentation broth of *Streptomyces lincolnensis* and its lincomycin nonproducing mutants were developed. The first one enables simultaneous analysis of methylthiolincosamide (MTL) and *N*-demethyllincomycin (NDL), whereas the second one is suitable for 4-propyl-L-proline (PPL) assay. Both methods are based on the pre-column derivatization: MTL and NDL with 4-chloro-7-nitrobenzofurazan; PPL with *o*-phthaldialdehyde. The methods were validated with lower limit of quantification values of 2.50, 3.75, and 3.75 μg ml^{-1} for MTL, NDL, and PPL, respectively. The inter- and intra-day accuracies and precisions were all within 12%. Stability of oxidized and derivatized analytes was investigated.

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Keywords Lincomycin precursors · *o*-Phthaldialdehyde · 4-Chloro-7-nitrobenzofurazan · HPLC · Fluorescence detection

Introduction

The rapidly growing number of antibiotic resistant bacterial pathogens represents one of the most serious public health problems [1]. Therefore, the need for new compounds with broader spectrum and improved pharmacological characteristics is still growing.

Lincosamides, some of which exhibit not only important and clinically used antibacterial but also antiprotozoal effects, have a great potential as a source of new more powerful compounds that has already been suggested by derivatization experiments [2].

Today, combinatorial genetics based on in vivo design of new combinations of genes coming from various gene clusters by methods of molecular biology which might lead to the production of hybrid compounds with new biological activities is considered to be one of the most promising ways for the extension of the spectrum of antibacterial compounds [3]. The strategy is applicable also in the case of lincosamides; however, a detailed knowledge of the respective biosynthetic pathway, which is essential for the successful targeted manipulations of the biosynthetic pathway [4], is still missing.

Information of the biosynthesis of the most important naturally produced lincosamide, lincomycin A (thereinafter lincomycin) is rather fragmentary. The complete lincomycin biosynthetic pathway has only been proposed based on determination of the biosynthetic origin of the carbon and nitrogen atoms [5, 6]. According to the proposal, two basic

precursors of the lincomycin molecule, the aglycone 4-propyl-L-proline (PPL) and the sugar moiety methylthiolincosamide (MTL), are synthesized via independent metabolic pathways and subsequently condensed to *N*-demethyllincomycin (NDL) and finally methylated into lincomycin (Fig. 1). Mutants disrupted in selected genes of the lincomycin cluster were prepared in order to confirm particular steps in lincomycin biosynthesis. Determination of the pathway key intermediates PPL, MTL, and NDL in the fermentation broth of *Streptomyces lincolnensis* DSM40355 and its above-mentioned mutants can lead to assignment of the particular genes to specific steps of the lincomycin biosynthesis.

To date, an assay for MTL was described by Yurek et al. [8], while analysis of PPL or NDL has not been reported at all. The already existing methods for proline analysis based on the pre-column derivatization with subsequent fluorescence detection [9–13] seem to match the selectivity and sensitivity requirements for PPL analysis. For the NDL and MTL assay, a similar approach can be employed, as both contain the amino group in their molecules. Another approach to NDL analysis can be derived from the previously described determination of lincomycin using UV detection and solid-phase extraction purification [14].

Such a complex matrix like fermentation broth represents the main factor limiting the selectivity and sensitivity

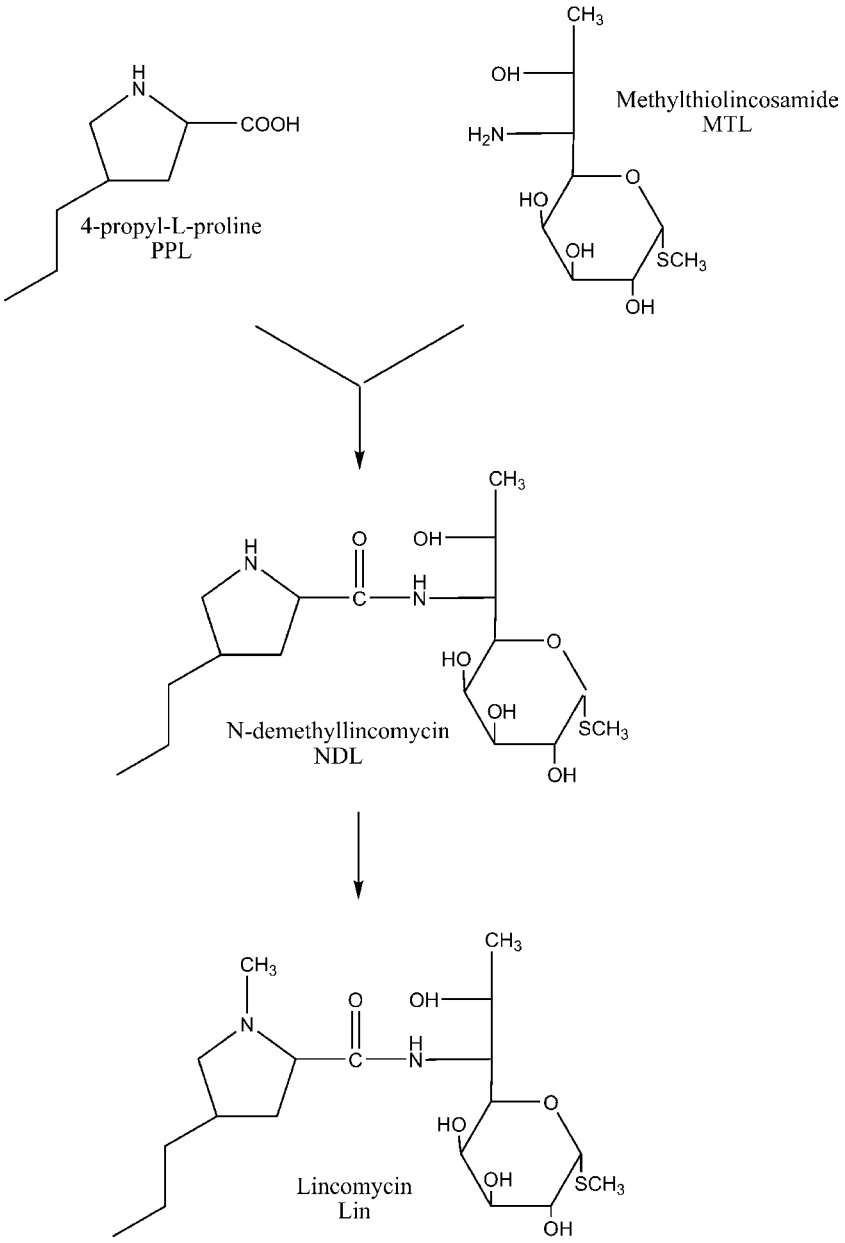

Fig. 1 The end of the lincomycin biosynthetic pathway [7]

(expected concentration of precursors is about 10 μg ml^{-1}) of the developed methods for MTL, NDL, and PPL analyses.

In this study, we describe two novel and validated methods for PPL analysis and for simultaneous analysis of MTL and NDL in fermentation broth of *S. lincolnensis* and its nonproducing mutants defective in PPL or MTL biosynthesis. The procedures were optimized with respect to sensitivity and selectivity of the methods and finally characterized by assessing the calibration and lower limit of quantitation (LLOQ), accuracy, precision, and stability in accordance with the generally accepted standards [15]. The methods represent a basic prerequisite for study of the lincomycin biosynthetic pathway especially because intermediates are not expected to be biologically active and, therefore, cannot be detected by help of biological assays.

Experimental

Strains and preparation of the mutant strains

Parental *S. lincolnensis* DSM40355, lincomycin A-producing strain, was obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany). Its nonproducing mutants B2, N, and Q, defective in PPL or MTL biosynthesis, were prepared by the Redirect PCR targeting system purchased from Plant Bioscience (Norwich) [16] (Novotná and Ulanová, manuscript in preparation).

Cultivation conditions

A seed *S. lincolnensis* culture was prepared by inoculating of 50 ml yeast extract malt extract medium (0.3% yeast extract, 0.5% peptone, 0.3% malt extract, pH 7.2; supplemented after sterilization with glucose and $MgCl_2$ to a final concentration of 2% and 0.048%, respectively) with spores from a glucosa yeast malt (GYM) agar plate (0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.2% $CaCO_3$, 1.2% agar, pH 7.2) and incubating for 30 h at 30°C with shaking. Twenty-five milliliters of avermectin medium (AVM) medium [14] was inoculated with the seed culture to a final concentration of 5%. The culture was then incubated for 120 h at 30°C with shaking. Then, the culture was sonicated (six pulses at 50 W, 30 s each, 30 s pause between pulses) with the Ultrasonic aparatus BBraun, Melsungen, Germany. The cell debris was removed by centrifugation at 5,000×*g* for 10 min at 4°C. The supernatant was stored at −20°C and used for preparation of the standard solutions or represented the sample for chemical analysis.

Chemicals, standard solutions

Solvents used in high-performance liquid chromatography (HPLC) were of gradient grade. Acetonitrile (ACN) was purchased from J.T. Baker (Holland), methanol from Merck (Germany). All other chemicals including derivatization agents were obtained from Sigma (Germany). All chemicals were stored according to the supplier instructions.

The standards were prepared at the Institute of Experimental Botany and Institute of Microbiology of the ASCR (Academy of Sciences of the Czech Republic), Prague. PPL and NDL standards were prepared by Libor Havlicek [17, 18], MTL standard was prepared by Radek Gazak and Stanislav Pospisil [19]. Structure and purity of the standards were approved by mass spectrometry (MS) and Nuclear magnetic resonance (NMR) (data not shown). The standard stock solutions were prepared with the HPLC grade water at a concentration level of 1 mg ml^{-1}. Standard solutions at required concentration were obtained by spiking the standard stock solutions to fermentation broth of the respective compound nonproducing mutants of *S. lincolnensis* DSM40355. Fermentation broth of B2 mutant strain was used as matrix for PPL standard solutions; similarly, broth of N mutant strain was used as matrix for MTL and NDL standard solutions.

Derivatization procedure

PPL oxidation and derivatization One hundred microliters of 10 mM solution of chloramin-T in 400 mM Na_3BO_3 (pH 9.5) and dimethyl sulphoxide, 4:1 (*v*/*v*) was preheated in a water bath (70°C) for 1 min. One hundred microliters of PPL standard solution or sample was added; the mixture was mixed and heated at 70°C for another minute. Then, 100 μl of 300 mM $NaBH_4$ in 600 mM LiOH was added, the mixture was mixed, heated at 70°C for 10 min, and centrifuged at 12,000×*g* for 5 min at room temperature (RT). Forty-five microliters of the mixture was added to 10 μl of derivatization agent (5 mg of *o*-phthaldialdehyde (OPA) dissolved in 50 μl of ACN, then diluted with 50 μl of 200 mM Na_3BO_3—pH 9.5, and finally, 4 μl of mercaptoethanol (MCE) was added).

MTL and NDL derivatization Two hundred fifty microliters of 30 mg ml^{-1} 4-chloro-7-nitrobenzofurazan in methanol was mixed with 50 μl of 1 M $NaHCO_3$ and 200 μl of MTL and NDL standard solution or sample. The mixture was vortexed for 20 s, heated in a water bath (70°C) for 4 h, and then frozen at −20°C for 20 min.

Chromatographic conditions

HPLC analyses were performed on the Waters system equipped with flow controller 600, autosampler 717, and fluorescence detector 474. Millenium 32 software was used for data processing.

Derivatized sample was loaded on the analytical Luna C_{18} column (250×4.6 mm i.d.; particle size, 5 μm;

Phenomenex) kept at RT, connected to a security guard C_{18} cartridge (30×20 mm i.d.; particle size, 5 μm; Phenomenex).

The mobile phase consisted of solvents: (A) 20 mM ammonium formate pH 4.7, ACN (10:1 *v*/*v*) and (B) ACN.

Gradient program for PPL determination Twenty-five microliters of derivatized sample was eluted by a flow rate of 1.25 ml min^{-1} with following linear gradient (min/%B): 0/30; 1/30; 13/65; 15/100. The column was washed with 100% ACN for 4 min and equilibrated for 7 min prior to following analysis. The column effluent was detected with a scanning fluorescence detector (λ_{ex}=240 nm; λ_{em}= 417 nm) with a gain switch from 10 to 100 at 6 min of analysis.

Gradient program for MTL and NDL determination Ten microliters of derivatized sample was eluted by a flow rate of 1 ml min^{-1} with following linear gradient (min/% B): 0/15; 5/33; 15/44; 16/100. The column was washed with 100% ACN for 4 min and equilibrated for 7 min prior to following analysis. The column effluent was detected with a scanning fluorescence detector (λ_{ex}=420 nm; λ_{em}= 525 nm) with gain set on the value of 100 for the whole analysis.

Method validation

Selectivity Chromatograms of the respective compound-free sample matrix and chromatograms of sample matrix spiked with PPL or mixture of MTL and NDL were compared in order to evaluate the method selectivity.

Calibration curve Calibration curves over linear ranges from 3.75 to 100 μg ml^{-1} for PPL, from 2.50 to 40.0 μg ml^{-1} for MTL, and from 3.75 to 40.0 μg ml^{-1} for NDL were determined. The applied range is sufficient with regards to the expected levels of analytes in real samples.

Lower limit of quantification LLOQ was determined as the lowest concentrations of PPL, MTL, and NDL quantified with precision (relative standard deviation (RSD)) and accuracy lower than 20%. Six replicates of samples were spiked with PPL (3.75 μg ml^{-1}), MTL (2.50 μg ml^{-1}), and NDL (3.75 μg ml^{-1}); concentrations at which the signal-to-noise ratio was found to be larger than 10 were measured.

Accuracy and precision To evaluate the intra-day and inter-day precision and accuracy, quality control samples were prepared at concentrations of 3.75, 40.0, 100; 2.50, 15.0, 40.0; and 3.75, 15.0, 40.0 μg ml^{-1} for PPL, MTL, and NDL, respectively, and were done in six replicates. RSD was taken as a measure of precision, and the percentage difference between determined and spiked amounts was considered as a measure of accuracy.

Stability Stability of the oxidized PPL, oxidized PPL derivative, MTL, and NDL derivatives was assessed for samples stored at −20°C and at RT (conditions usually encountered during the actual sample handling and analysis).

Stability of the oxidized PPL was investigated at two concentration levels (3.75 and 100 μg ml^{-1}) at −20°C (derivatized and analyzed after 1, 2, 3, 4, 7, 14, 21, and 28 days) and at RT (derivatized and analyzed after 0, 2, 4, 6, and 8 h). All samples were measured in triplicates.

Stability of the oxidized PPL derivative was investigated at two concentration levels (3.75 and 100 μg ml^{-1}) at RT after 0, 1, 2.5, 5, 10, 25, 60, and 120 min. All samples were measured in triplicates.

Stability of MTL and NDL derivatives was investigated at two concentration levels (2.50, 40.0 μg ml^{-1} for MTL and 3.75, 40.0 μg ml^{-1} for NDL) at −20°C (analyzed after 1, 2, 3, 4, 7, 14, 21, and 28 days) and at RT (analyzed after 0, 2, 4, 6, 8, 12, 18, and 24 h). All samples were measured in triplicates.

Results and discussion

Method development–PPL determination

Several derivatization agents (9-fluorenyl methylchloroformate (FMOC), dansyl chloride (DNS-Cl), 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), and OPA) were evaluated for PPL derivatization. FMOC agent has to be extracted after the derivatization, making it a complex procedure [13]. DNS-Cl agent needs more than 30 min for the reaction and yields side-products [13], and pre-experiments showed a very low sensitivity for PPL. AQC [12] is expensive for routine analyses. OPA is highly sensitive, the derivatization has a very good reproducibility, is simple and fast and, therefore, it was chosen for derivatization. However, OPA can react with primary amine only. Therefore, similar to the method for derivatization of proline standard [9], chloramin-T was used for PPL oxidation. Then, parameters of derivatization and chromatographic conditions were optimized in order to achieve required sensitivity (LLOQ) and selectivity in such a complex matrix like *S. lincolnensis* fermentation broth. Also, conditions were optimized with respect to stability of the OPA derivative.

Concentration of OPA was tested in order to obtain the highest possible concentration of PPL derivative in analyzed sample. OPA solutions of 5.0, 25, and 50 mg ml^{-1} of the volume of 100, 20, and 10 μl, respectively, were tested.

The volume of MCE added was always 4 μl in order to keep the substance ratio between OPA and MCE unaltered. OPA solution of 50 mg ml^{-1} showed the highest response (data not shown) and, therefore, the concentration was selected for further derivatization reactions.

Temperature of oxidative reaction The temperature influence on the oxidative reaction was tested in the range from 50°C to 80°C with 5°C steps. The efficiency of oxidation increased from 50°C to 70°C, and then it stagnated or even slightly decreased (data not shown). The reaction temperature of 70°C was chosen as optimal.

pH of Na_3BO_3 solution pH condition of the oxidative and derivatization reactions was tested simultaneously within the range from pH 7.0 to 10 resulting in pH of 9.5 (data not shown).

Sample volume added to the reaction The excess of oxidative and derivatization agents was tested simultaneously. The experiment was conducted by monitoring the dependence of the derivatized product concentration on the amount of the sample added to the reaction. Sample volumes of 25, 50, 100, 125, and 150 μl were examined resulting in the optimal volume of 100 μl (data not shown).

Chromatographic procedure In order to develop a mobile phase allowing an efficient separation of the PPL derivate from the matrix interferents, several buffers were examined: potassium phosphate [9], sodium borate, and ammonium formate. The usage of ammonium formate brought the best separation efficiency expressed by the resolution. It was ascertained that the separation is pH independent in the range of the buffer stability (from 2.75 to 4.75). The sample was eluted by gradient elution, which was modified in order to separate the analyte from the matrix (see "Experimental").

Method development—MTL and NDL determination

The method for lincomycin determination [14] seems to be suitable also for NDL determination with regards to their related chemical structures. However, the missing methyl group in NDL molecule has a significant influence on its separation. NDL is eluted at the very beginning of the analysis resulting in a considerable interference with the matrix. It was found out that the interference cannot be avoided even after optimation of the gradient elution or pH of the mobile phase. However, NDL can be derivatized with a fluorescent agent similarly as MTL with the already

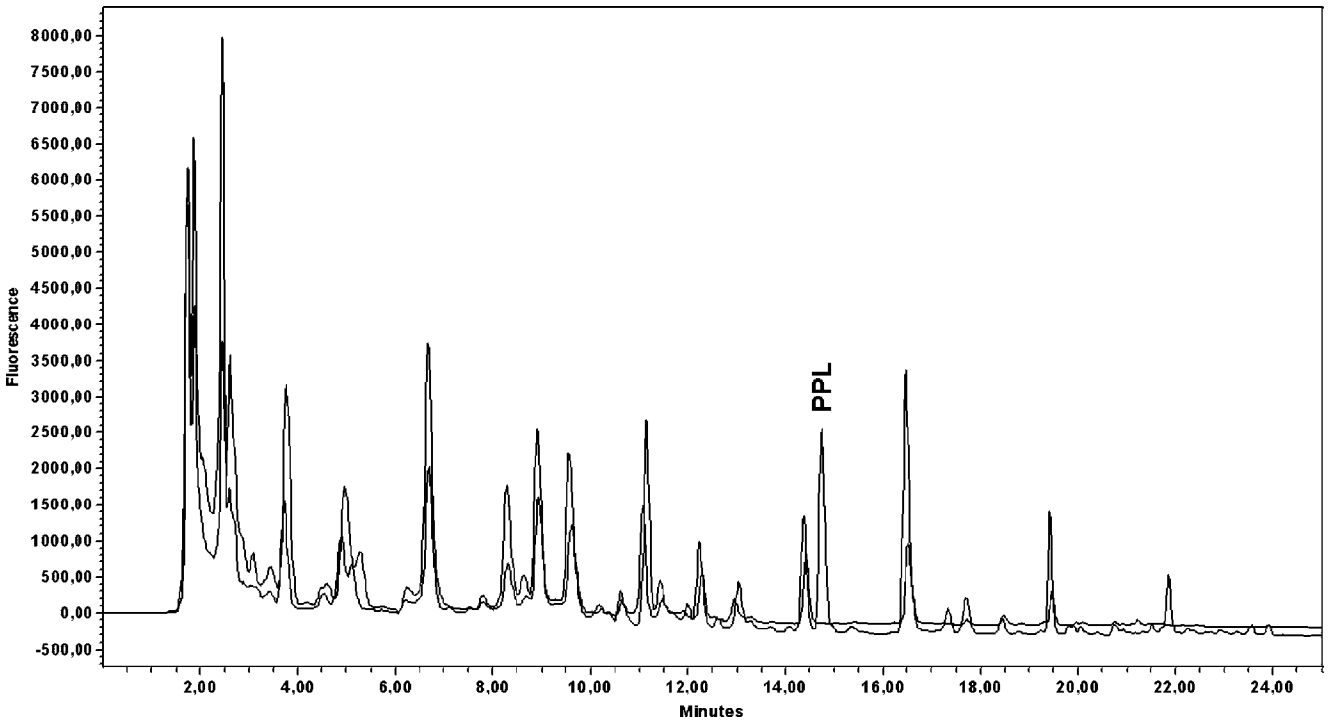

Fig. 2 Chromatogram overlay. *PPL* (50 μg ml^{-1}) spiked to the matrix (fermentation broth of PPL nonproducing B2 mutant strain) and the matrix itself. HPLC: Luna C_{18} column (250×4.6 mm i.d.; particle size, 5 μm; Phenomenex); mobile phase: (A) 20 mM ammonium formate pH 4.7, acetonitrile 10:1 *v/v* and (B) ACN, flow rate, 1.25 ml min^{-1}; injection volume, 25 μl; linear gradient elution (min/%B): 0/30; 1/30; 13/65; 15/100, washing step (100% B) 4 min, equilibration step (30% B) 7 min. Fluorescence detection: λ_{ex}=240 nm; λ_{em}=417 nm, gain switch from 10 to 100 at sixth minute of analysis

existing method [8]. Moreover, application of this method for NDL analysis enables simultaneous determination of both lincomycin precursors.

Three parameters of derivatization reaction, reaction temperature, time, and volume of a sample added to the reaction, were optimized to obtain the best conditions for both MTL and NDL derivatizations.

Reaction temperature The temperature was tested in the range from 55°C to 80°C in 5°C steps. While the derivatization of NDL was temperature independent, the derivatization of MTL yielded better results with increasing temperature up to 75°C (data not shown), which was therefore chosen for MTL and NDL simultaneous derivatization.

Reaction time and volume of a sample added to the reaction The reaction time was tested for 1–5 h with 1-h steps; the volume of the sample added was tested from 200 to 400 μl in 50-μl steps. Four hours and 200 μl were determined as optimal parameters for both analytes (data not shown).

Chromatographic procedure Aside from potassium phosphate buffer [8], ammonium formate and sodium borate were examined. Among them, ammonium formate proved the best efficiency in the PPL separation. Since separation of the MTL and NDL derivatives was pH independent in the range from 2.75 to 4.75, the same mobile phase as for PPL (pH 4.7) was chosen because the analytes in question are usually determined in all tested samples. The elution gradient was further modified to separate analytes from the matrix as well as to enable simultaneous analysis of both compounds of interest. The considerably more complex gradient (see "Experimental") than in the case of analysis by Yurek et al. [8] had to be developed in order to accomplish this aim.

Method validation

Selectivity The selectivity was performed to determine the optimal conditions for quantification of PPL, MTL, and NDL in fermentation broth of *S. lincolnensis*. Under chromatographic conditions described in this study, all three analytes were well separated, and no significant interfering components from the sample matrix were detected by fluorescence detection with the designated chromatographic parameters. For details, see Figs. 2 and 3.

Calibration curve and lower limit of quantification The calibration curves were prepared at six concentration levels, 3.75, 7.50, 15.0, 30.0, 50.0, and 100; 2.50, 5.00, 7.50, 15.0, 20.00, and 40.0; and 3.75, 5.00, 7.50, 15.0, 20.0, and

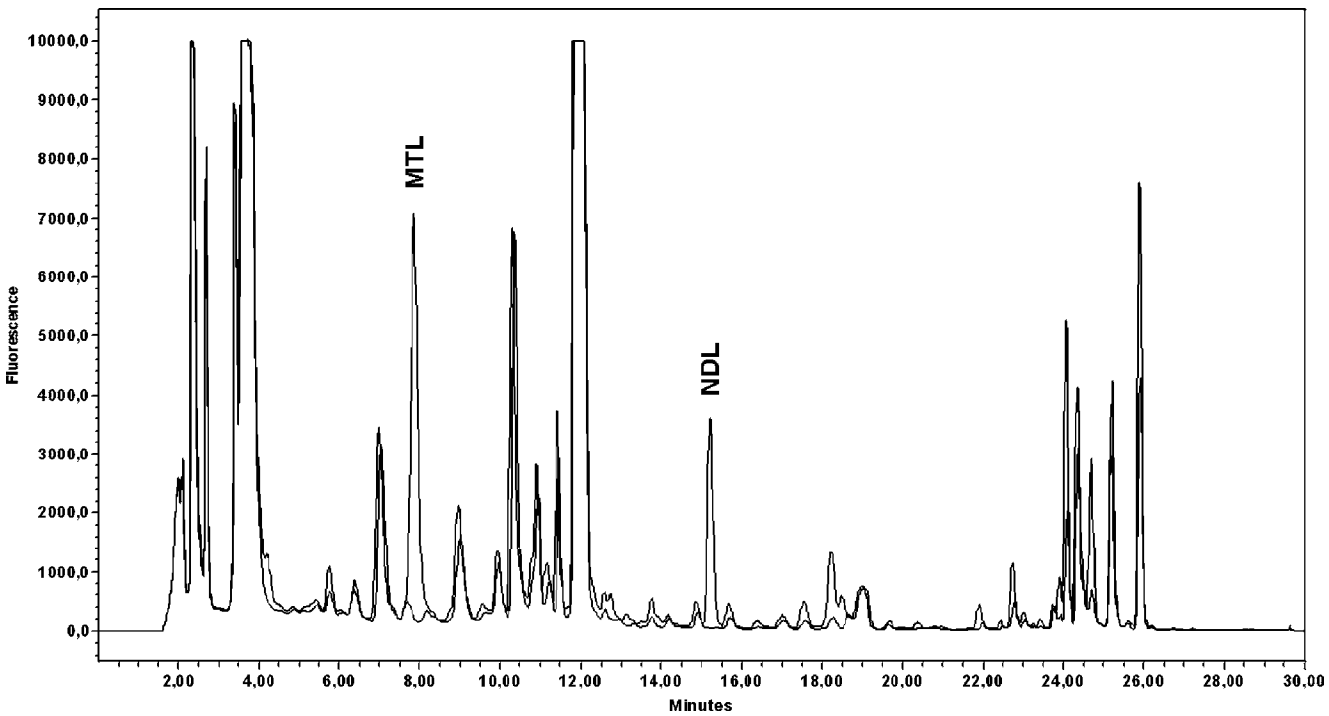

Fig. 3 Chromatogram overlay. *MTL* and *NDL* (30 μg ml^{-1}) spiked to the matrix (fermentation broth of MTL and NDL nonproducing N mutant strain) and the matrix itself. HPLC: Luna C_{18} column (250× 4.6 mm i.d.; particle size, 5 μm; Phenomenex); mobile phase: (A) 20 mM ammonium formate pH 4.7, ACN (10:1 *v*/*v*) and (B) ACN, flow rate, 1 ml min^{-1}; injection volume, 10 μl; linear gradient elution (min/% B): 0/15; 5/33; 15/44; 16/100, washing step (100% B) 4 min, equilibration step (15% B) 7 min, equilibration step (30% B) 7.0 min. Fluorescence detection: λ_{ex}=420 nm; λ_{em}=525 nm, gain 100

Table 1 Parameters of calibration curves including LLOQ values for PPL, MTL, and NDL

Analyte	Retention time (min)	Regression equation	Correlation coefficient	LLOQ (μg ml^{-1})
PPL	14.7	A=2.19 c . 10^5–4.38 . 10^4	0.9995	3.75
MTL	7.4	A=2.38 c . 10^6–1.55 . 10^6	0.9999	2.50
NDL	14.9	A=9.14 c . 10^5–2.64 . 10^6	0.9998	3.75

A area under the chromatographic peak, c concentration (μg ml^{-1})

40.0 μg ml^{-1} for PPL, MTL, and NDL, respectively. The characteristics of the calibration curves including LLOQ values are shown in Table 1.

Accuracy and precision The accuracy and precision of the assays are summarized in Table 2. The accuracy for PPL ranged from 98.4% to 112% with the precision (relative standard deviation) from 2.6% to 6.5%, for MTL from 98.0% to 109% with the precision (RSD) from 2.4% to 8.8%, and for NDL ranged from 99.0% to 111% with the precision (RSD) from 1.9% to 4.9%.

The results indicate that both methods are accurate, precise, and reproducible.

Stability The stability of the oxidized PPL, oxidized PPL derivative, MTL derivative, and NDL derivative during sample storage and processing was evaluated with results given in Table 3. Oxidized PPL is stable for 6 h at RT and 21 days at −20°C. Oxidized PPL derivative was unstable, and therefore, the HPLC analysis must be performed immediately after the derivatization (a delay up to 2.5 min can be tolerated). Routine analysis of PPL may be carried out using pre-column derivatization reactor where PPL can be derivatized automatically prior to the analysis. MTL derivative is stable at RT for 16 h. Longer storage than 16 h, even at −20°C, causes a considerable degradation of the derivative. NDL derivative is stable for 24 h at RT and 21 days at −20°C. Determined values of stability allow carrying out the analysis of PPL, MTL, as well as NDL under conditions described in this paper.

Method application

The above described methods enable to determine three key intermediates of the lincomycin biosynthetic pathway. Therefore, they represent a useful tool for analysis of lincomycin nonproducing mutants blocked in different steps of the pathway. Identification of the lincomycin precursors in strains inactivated in selected cluster genes will help to assign a particular gene either biosynthetic function in one of the respective branches or a regulatory one operating at the cluster level.

PPL has been determined in several selected samples in the concentration range from 10 to 50 μg ml^{-1}. PPL determination in the fermentation broth of mutant Q strain is illustrated in the Fig. 4.

MTL was found at very low concentrations slightly passing the LLOQ of the method, while NDL was not found in the presented samples. However, the both analytes were easily detectable being spiked to the fermentation broths at concentrations which were expected in the real samples. The absence of NDL was in accord with the predicted functions of the tested genes. The low levels of MTL might be due to either its fast depletion by competing catabolic pathways or yet unknown regulation of the MTL synthesis, e.g., feedback inhibition of a foregoing reaction step.

Table 2 Intra-day and inter-day repeatability of methods for PPL, MTL, and NDL analyses in fermentation broth of *S. lincolnesis* (n=6)

Analyte	Nominal concentration (μg ml^{-1})	Intra-day assay		Inter-day assay	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
PPL	3.75 (LLOQ)	98.4	6.53	88.7	9.24
	40.0	111	2.56	103	5.38
	100	112	3.68	101	4.47
MTL	2.50 (LLOQ)	103	8.82	87.6	7.92
	15.0	109	2.40	91.8	8.09
	40.0	98.0	4.69	91.9	6.86
NDL	3.75 (LLOQ)	105	4.32	102	8.93
	15.0	99.0	1.94	107	5.36
	40.0	111	4.87	106	5.08

Table 3 Stability of PPL, MTL, and NDL products in fermentation broth of *S. lincolnesis* (n=3)

Analyte	Nominal concentration (μg ml^{-1})	Autosampler tray at RT		Storage at −20°C	
		Stability period	Accuracy[a] (%)	Stability period (days)	Accuracy[a] (%)
Oxidized PPL	3.75	8 h	95.2	21	103
	100	8 h	96.3	28	105
Oxidized PPL derivative	3.75	2.5 min	92.0	Unstable	–
	100	2.5 min	93.8	Unstable	–
MTL derivative	2.50	16 h	93.6	Unstable	–
	40	16 h	104	Unstable	–
NDL derivative	3.75	24 h	95.7	21	91.2
	40	24 h	106	21	98.8

[a] Accuracy at the end of stability period

Conclusion

Two assays for three lincomycin precursors, PPL, MTL, and NDL, were developed. MTL and NDL can be analyzed simultaneously. Sensitivity and selectivity of the proposed methods allow their application in the lincomycin biosynthetic pathway studies, for which they were primarily developed. The major advantages of the developed methods are their high efficiency and their relative simplicity due to the lack of the fermentation broth purification step.

Methods for determination of PPL, MTL, and NDL in fermentation broth of *S. lincolnensis* are important tools for investigating a set of nonproducing mutants specifically blocked in the particular steps of the lincomycin biosynthesis. This study will substantially contribute to clarifying of biosynthetic pathway and will break new ground for targeted genetic manipulations yielding new lincomycin derivatives.

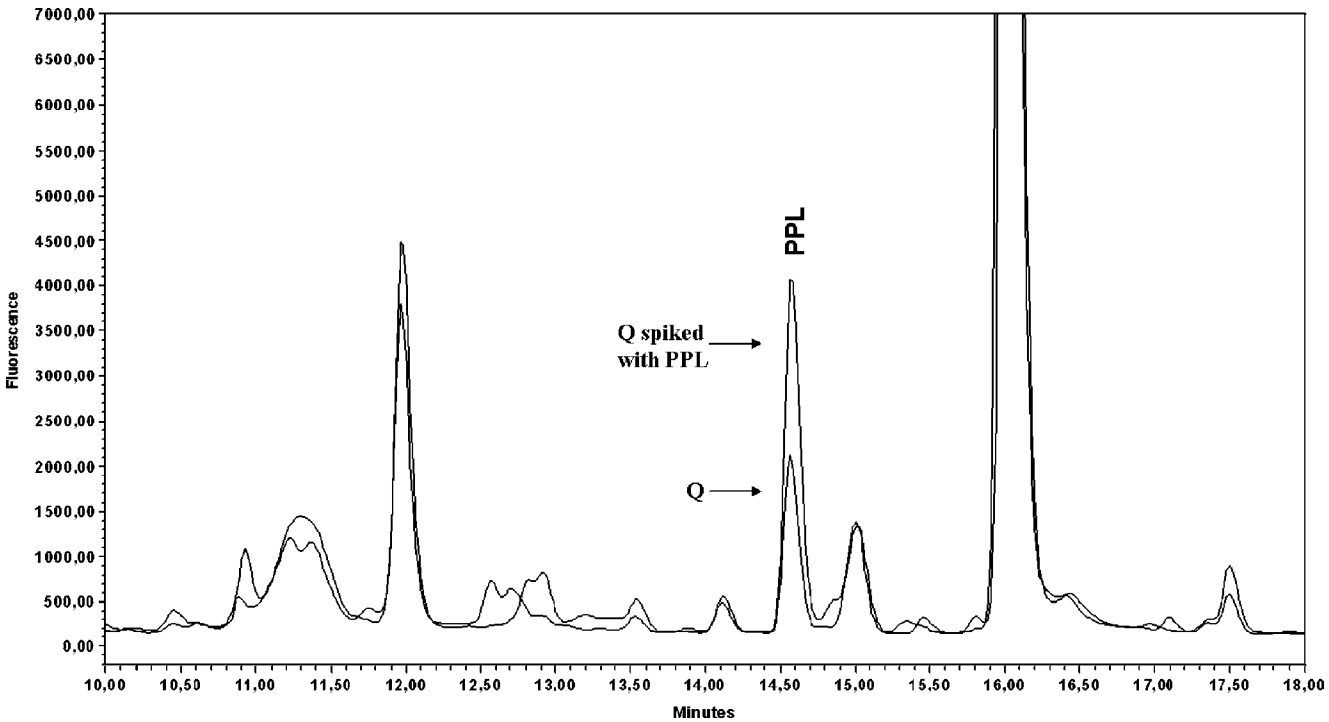

Fig. 4 Detail of a chromatogram overlay. *PPL* (50 μg ml^{-1}) spiked in sample Q (fermentation broth of Q mutant strain) and sample Q. HPLC: Luna C_{18} column (250×4.6 mm i.d.; particle size, 5 μm; Phenomenex); mobile phase: (A) 20 mM ammonium formate pH 4.7, ACN (10:1 *v*/*v*) and (B) ACN, flow rate, 1.25 ml min^{-1}; injection volume, 25 μl; linear gradient elution (min/%B): 0/30; 1/30; 13/65; 15/100, washing step (100% B) 4 min, equilibration step (30% B) 7.0 min. Fluorescence detection: λ_{ex}=240 nm; λ_{em}=417 nm, gain switch from 10 to 100 at sixth minute of analysis

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Mutasynthesis of Lincomycin Derivatives with Activity against Drug-Resistant Staphylococci▽†

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The lincomycin biosynthetic gene *lmbX* was deleted in *Streptomyces lincolnensis* ATCC 25466, and deletion of this gene led to abolition of lincomycin production. The results of complementation experiments proved the blockage in the biosynthesis of lincomycin precursor 4-propyl-L-proline. Feeding this mutant strain with precursor derivatives resulted in production of 4′-butyl-4′-depropyllincomycin and 4′-pentyl-4′-depropyllincomycin in high titers and without lincomycin contamination. Moreover, 4′-pentyl-4′-depropyllincomycin was found to be more active than lincomycin against clinical *Staphylococcus* isolates with genes determining low-level lincosamide resistance.

Lincosamides form a small yet clinically important group of antibiotics. One of the naturally occurring members of this group, lincomycin A (LIN), is active against many Gram-positive bacteria, such as staphylococci and streptococci. Its semisynthetic derivative clindamycin (CLI) is prescribed for the treatment of some infections caused by anaerobic bacteria and is also applied against the causative agent of malaria, *Plasmodium falciparum* (9, 22).

Biosynthesis of lincomycin proceeds via two separate branches from tyrosine and D-glucose to the aglycone 4-propyl-L-proline (PPL) and methylthiolincosamide (MTL), respectively. Condensation of these two precursors via an amide bond by a multimeric synthetase yields *N*-demethyllincomycin (NDL), which is subsequently methylated to form LIN (3) (Fig. 1A). Various LIN derivatives have been prepared chemically, and in particular, the 4′-alkyl-4′-depropyllincomycin set has been determined to be more active and have a broader antimicrobial spectrum than LIN has. Of this set, 4′-butyl, pentyl, and hexyl analogs have been shown to be particularly effective (12, 13). Moreover, demethylated and chlorinated 4′-alkyl-1′-demethyl-4′-depropylclindamycins had higher activities against *Plasmodium* spp. than clindamycin did (14). These more potent 4′-alkyl derivatives of lincomycin can be prepared by a multistep and costly chemical synthesis (13). Alternatively, feeding or genetic modifications of the natural biosynthetic pathway could be used. For example, the addition of PPL derivatives with extended alkyl residues to fermentation broths of a producer strain in a process termed precursor-directed biosynthesis has been described previously (25). This resulted in a mixture of both LIN and its more biologically active derivative being produced, which is not desirable because of the need to separate and purify the product of interest.

In this study we present a new mutasynthetic approach to the preparation of the two known 4′-alkyl-4′-depropyllincomycins (Fig. 1B) by feeding a mutant strain defective in PPL biosynthesis with PPL derivatives as a practical alternative to total chemical synthesis. We tested the biological activity of these LIN derivatives on a collection of *Staphylococcus* strains with defined resistance profiles that have been described previously (15–17).

The early reactions involved in biosynthesis of proline derivatives are the same for lincomycin and several pyrrolobenzodiazepine antibiotics (5, 10, 11). Presumably, the genes shared by lincomycin and benzodiazepine gene clusters could code for enzymes of PPL biosynthesis. To confirm the hypothesis and obtain a mutant defective in PPL production, we deleted one of the shared genes, *lmbX*, in the LIN-producing *Streptomyces lincolnensis* ATCC 25466 type strain. The inactivation of *lmbX* was achieved by the Redirect targeting system (4) using the LK6 cosmid, which bears the whole LIN biosynthetic gene cluster (7), inactivation primers Xf (5′-CGCGCCCATCCTGCACAGCGCACCGGAGGAAGCATGATCATTCCGGGGATCCGTCGACC-3′) and Xr (5′-GAGAAAAGAGCCGCTGACGCAAGGGGCCCTCGGCGACTATGTAGGCTGGAGCTGCTTC-3′) (nucleotide extensions with sequence identity to regions upstream and downstream of *lmbX*, respectively, are underlined) and checking primers ch*X*f (5′-CCGGCATCAACGACT-3′) and ch*X*r (5′-CCAGATGGAACGAATTCA-3′). The *lmbX* deletion strain is hereafter referred to as the Δ*lmbX* strain. For detection of LIN in fermentation broth, we cultivated the type and mutant strains and performed ultra-performance liquid chromatography (UPLC) analysis of the respective broths by the method of Olsovska et al. (18). We revealed that LIN production was under the limit of detection in the Δ*lmbX* strain (Fig. 2). The minor peak that eluted at 2.95 min had a UV spectrum different from that of LIN.

Next, we tested the ability of the LIN precursors PPL and MTL to complement the mutation in feeding experiments, in which the Δ*lmbX* strain was cultivated on GYM agar plugs

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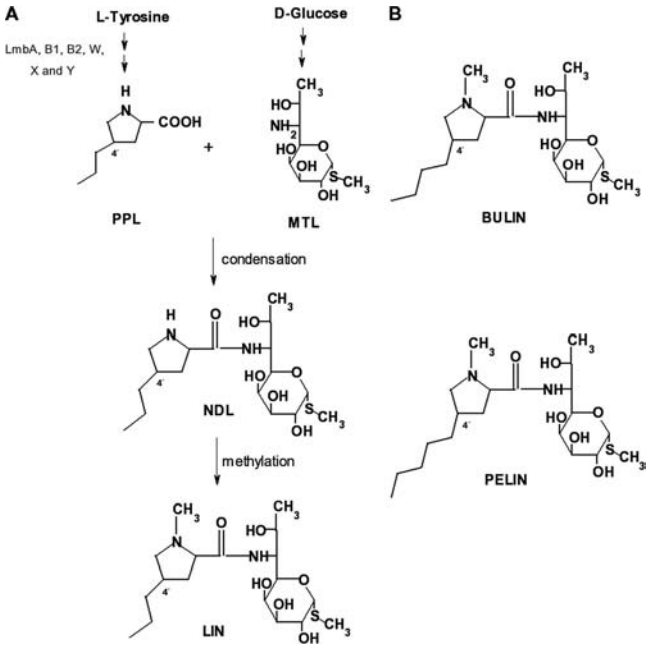

FIG. 1. (A) Biosynthesis of lincomycin. The genes involved in biosynthesis of PPL are *lmbA*, *lmbB1*, *lmbB2*, *lmbW*, *lmbX*, and *lmbY*. PPL, 4-propyl-L-proline; MTL, methylthiolincosamide; NDL, *N*-demethyllincomycin; LIN, lincomycin. (B) 4′-Alkyl derivatives of lincomycin. BULIN, 4′-butyl-4′-depropyllincomycin, PELIN, 4′-pentyl-4′-depropyllincomycin.

(glucose [4 g/liter], yeast extract [4 g/liter], malt extract [10 g/liter], $CaCO_3$ [2 g/liter], agar [12 g/liter] [pH 7.2]) with PPL or MTL (6) added at a final concentration of 200 mg/liter for 10 days at 28°C. Agar plugs were subsequently placed on B1 agar (beef extract [10 g/liter], peptone [10 g/liter], NaCl [5 g/liter], agar [20 g/liter] [pH 7.2]) overlaid with the indicator strain *Kocuria rhizophila* CCM 552. Growth inhibition zones, indicating the production of antimicrobial compound, were detected after 24 h of incubation at 30°C. The production of the compound was detected only in the case of feeding with the precursor PPL. UPLC analysis of the fermentation broth confirmed the antimicrobial compound to be LIN (based on a comparison of retention times and UV spectra with those of the LIN standard) (Fig. 2). The restoration of LIN production after PPL addition proved the participation of LmbX in the PPL biosynthetic branch. Nevertheless, the precise enzymatic role of LmbX remains unknown and is the aim of further studies.

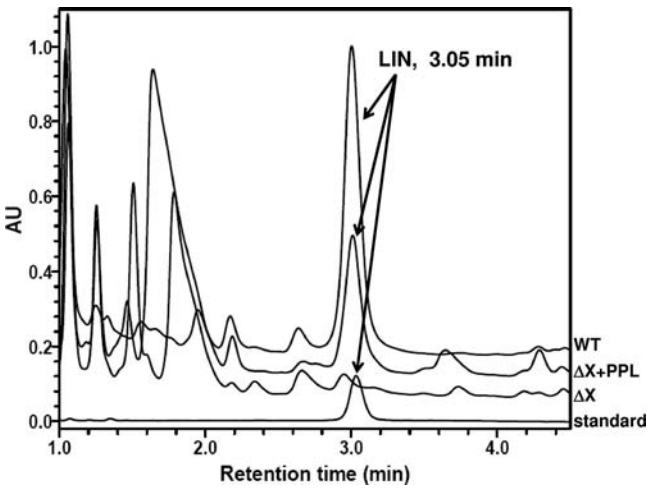

FIG. 2. Chromatographic analysis (UPLC) of fermentation broth. Detail of a chromatogram overlay is shown. The *y* axis shows fluorescence in arbitrary units (AU). Four samples, *S. lincolnensis* ATCC 25466 (wild type [WT]), Δ*lmbX* mutant strain (ΔX), Δ*lmbX* strain fed with PPL (ΔX+PPL), and LIN standard (62.5 mg/liter), were examined. The UPLC conditions follow: bridged ethyl hybrid (BEH) C_{18} column (50 by 2.1 mm [inner diameter]; particle size, 1.7 μm); mobile phase, solvent A (1 mM ammonium formate [pH 9.0]) and solvent B (acetonitrile); linear gradient elution − solvent A: solvent B (vol/vol): 78.0:22.0% at 0 min, 78.0:22.0% at 3 min, 47.5:52.5% at 10 min; flow rate, 0.4 ml min^{-1}; column temperature, 35°C; and injection volume, 5 μl.

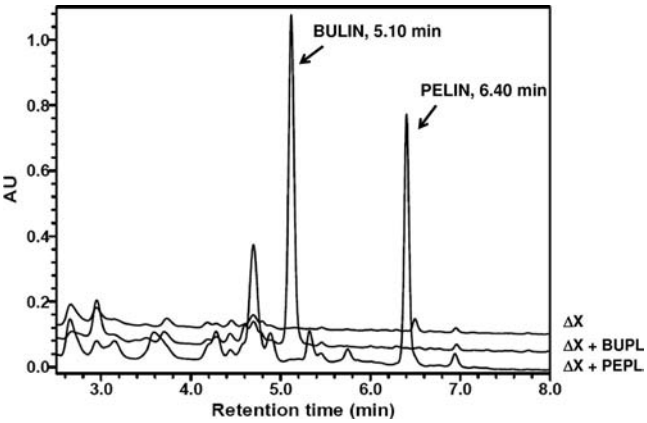

FIG. 3. Chromatographic analysis (UPLC) of fermentation broth. Detail of a chromatogram overlay is shown. The *y* axis shows fluorescence in arbitrary units (AU). Three samples, Δ*lmbX* mutant strain (ΔX), Δ*lmbX* mutant strain fed with BUPL (ΔX+BUPL), and Δ*lmbX* mutant strain fed with PEPL (ΔX+PEPL), were examined. The UPLC conditions follow: bridged ethyl hybrid (BEH) C_{18} column (50 by 2.1 mm [inner diameter]; particle size, 1.7 μm); mobile phase, solvent A (1 mM ammonium formate [pH 9.0]) and solvent B (acetonitrile); linear gradient elution − solvent A: solvent B (vol/vol): 78.0:22.0% at 0 min, 78.0:22.0% at 3 min, 47.5:52.5% at 10 min; flow rate, 0.4 ml min^{-1}; column temperature, 35°C; and injection volume, 5 μl.

In order to feed the Δ*lmbX* strain with PPL derivatives, we prepared 4-butyl-L-proline and 4-pentyl-L-proline (BUPL and PEPL, respectively) based on the aldol condensation of protected L-pyroglutamic acid with corresponding aldehyde. The resulting aldols were dehydrated using MsCl-Et_3N to yield 4-alkylidenepyroglutamates, which gave *cis*-4-substituted pyroglutamates after hydrogenation of the double bond. Inversion of the configuration at C-4 by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) followed by a two-step reduction of the resulting amides led, after deprotection, to BUPL and PEPL. For details of synthetic procedures and analytical data, see supplemental material.

In mutasynthetic experiments, BUPL and PEPL were added at the start of fermentation in AVM medium (18) to a final concentration of 100 mg/liter. We found that addition of either BUPL or PEPL to cultivation broth of the Δ*lmbX* mutant defective in PPL production led to the formation of new compounds (Fig. 3). The compounds were isolated by UPLC or high-performance liquid chromatography (HPLC) in order to perform mass spectrometry and nuclear magnetic resonance

TABLE 1. Summary of characteristics of the selected staphylococcal isolates and comparison of MICs of PELIN, CLI, and LIN

Strain[a]	Species[b]	Resistance genotype	Resistance phenotype[c]	MIC (μg/ml)		
				LIN	CLI	PELIN
134PL	STHA	*ermC*	ELC	32	>16	32
44KR	STEP	*ermC*	ELC	64	>16	64
31UL	STHA	*ermC*	ELiCi	1	0.5	0.5
32PL	STEP	*ermC*	ELiCi	1	0.125	0.25
29OL	STHA	$vgaA_{LC}$	LC	64	16	32
123PL	STHA	*lnuA*	L	128	2	8
102OL	STEP	*lnuA*	L	64	1	2
202BB	STHA	*msrA*	E	1	0.125	0.25
129OV	STEP	*msrA*	E	1	0.25	0.25
65OL	STHA	*ermC* $vgaA_{LC}$	ELC	128	4	128
1036UL	STEP	*ermC* $vgaA_{LC}$	ELC	>256	>16	64
113OL	STHA	*msrA lnuA*	EL	128	1	8
32OL	STEP	*msrA lnuA* $vgaA_{LC}$	ELC	>256	16	64
CIP 107907	STAU	*vgaA*		4	0.25	0.5
ATCC 29213	STAU			1	0.125	0.25

[a] Strain designations are described in references 15 and 17. CIP 107907 is *Staphylococcus aureus* CIP 107907, and ATCC 29213 is the lincosamide-sensitive strain *Staphylococcus aureus* ATCC 29213.

[b] STHA, *Staphylococcus haemolyticus*; STEP, *Staphylococcus epidermidis*; STAU, *Staphylococcus aureus*.

[c] E, erythromycin resistance; L, lincomycin resistance; C, clindamycin resistance; Li, inducible lincomycin resistance; Ci, inducible clindamycin resistance.

experiments (for details, see supplemental material and Fig. S2 and S3 in the supplemental material). The results of these experiments confirmed synthesis of either 4′-butyl-4′-depropyllincomycin (BULIN) or 4′-pentyl-4′-depropyllincomycin (PELIN) and, simultaneously, indicated the broad substrate flexibility of the LmbC enzyme, which is responsible for the recognition and activation of PPL, prior to the synthesis of NDL (S. Kadlčík, unpublished data). This phenomenon has been observed for various enzymes involved in the biosyntheses of secondary metabolites (8, 19, 21) and enables a great structural diversity of products.

The antimicrobial activities of LIN and CLI derivatives with extended alkyl chains against a collection of *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Salmonella schottmuelleri* strains have been determined previously (12). We used a set of 13 clinical macrolide- and/or lincosamide-resistant *Staphylococcus* isolates (15–17) for a series of experiments evaluating the antimicrobial activity of BULIN and PELIN, depending on the presence of certain resistance genes. *S. aureus* ATCC 29213 and *S. aureus* CIP 107907 were used as a lincosamide-sensitive indicator strain and a strain carrying the *vgaA* gene, respectively. The agar diffusion method (24) showed PELIN to be more biologically active than BULIN; therefore, further experiments were conducted only with PELIN. We determined the MIC of PELIN by the agar dilution method in microtitration plates. A 2-μl inoculum of a 0.5 McFarland suspension was spotted onto 100-μl Mueller-Hinton agar plugs with the following antibiotics and concentrations: PELIN, 0.125 to 128 μg/ml; LIN, 0.125 to 256 μg/ml; and CLI, 0.125 to 16 μg/ml. Microtitration plates were incubated at 37°C for 24 h. As summarized in Table 1, strains expressing the constitutive *ermC* gene, coding for rRNA adenine *N*-6-methyltransferase and determining macrolide-lincosamide-streptogramin B (MLS_B) resistance (23), were resistant to PELIN to the same level as CLI and LIN. Furthermore, high susceptibility of strains with an inducible *ermC* gene showed that PELIN, just as LIN and CLI, did not induce resistance. The recently described $vgaA_{LC}$ gene, coding for an ABC transporter, confers resistance not only to streptogramins, as its evolutional variant *vgaA* does (2), but also to lincosamides (16), including PELIN (indicated by the *LC* subscript). Strains carrying the *msrA* gene, coding for another ABC transporter and conferring resistance only to macrolide antibiotics (20) were, as expected, susceptible to all lincosamides tested. Altogether, PELIN, LIN, and CLI had the similar antimicrobial activity against strains with *ermC*, $vgaA_{LC}$, and *msrA* genes (Table 1). In contrast, PELIN was found to have almost the same activity as CLI against isolates with a resistance given by *lnuA* gene, which codes for a lincosamide nucleotidyltransferase (1). Strains carrying this gene, as well as a strain with both *lnuA* and *msrA*, showed higher susceptibility to CLI and PELIN than to LIN. On the other hand, the combination of *lnuA* and *msrA* genes with $vgaA_{LC}$ mimicked the MLS resistance phenotype (Table 1).

In conclusion, we have reported the first example of highly effective mutasynthesis of LIN derivatives. Our more feasible way of synthesis could be used for developing chlorinated alternatives, which have a greater antimalarial activity than currently used clindamycin (14). Nevertheless, we should take into consideration the need for chemical synthesis of the precursors, which is less but still costly and time-consuming. Therefore, preparation of a strain producing hybrid antibiotics on lincosamide without or with minimal chemical modifications is more favorable and is the aim of our future studies.

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ORIGINAL

Comparison of LC Columns Packed with 2.6 μm Core-Shell and Sub-2 μm Porous Particles for Gradient Separation of Antibiotics

Tereza Tylová · Zdeněk Kameník · Miroslav Flieger · Jana Olšovská

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Abstract The recently introduced Kinetex C18 column packed with core-shell 2.6 μm particles is declared to provide similar efficiency and short analysis as Acquity BEH C18 column with 1.7 μm porous particles. Unlike Acquity BEH C18 column, Kinetex C18 column exhibited lower column backpressure making this column compatible to conventional LC systems. The performance of Kinetex C18 column (2.1 × 50 mm) and Acquity BEH C18 column (2.1 × 50 mm) for gradient separation of tetracyclines under acidic conditions (oxytetracycline, tetracycline, chlortetracycline, and doxycycline) and macrolides under alkaline conditions (tylosin, clarithromycin, roxithromycin, and carbomycin) was studied. The columns were compared by evaluation of their experimental peak capacity and its dependence on linear velocity and gradient slope. The maximal experimental peak capacities for analysis of tetracyclines were 51.8 (Acquity BEH C18 column) and 48.4 (Kinetex C18 column). This indicated that Kinetex C18 was a suitable alternative to Acquity BEH C18 column for the analysis of tetracyclines under acidic conditions. On the contrary, the maximal experimental peak capacities for analysis of macrolides on Acquity BEH C18 column was higher (46.7) than that on Kinetex C18 column (36.9). Moreover, application of Kinetex C18 column for the analysis of macrolides under alkaline conditions was limited with respect to its decreasing performance with growing number of injections on the column.

Keywords Column liquid chromatography · Sub-2 μm particles · Acquity BEH C18 column · Core-shell particles · Kinetex C18 column · Macrolides · Tetracyclines

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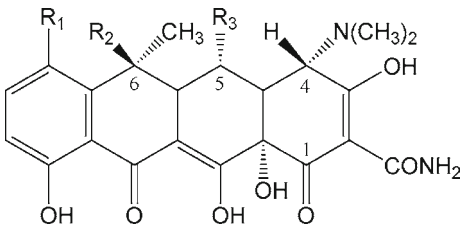
Introduction

High performance liquid chromatography represents nowadays the most widespread separation technique applied commonly in pharmacology, toxicology, clinical analysis as well as various research fields [1–4].

The efficiency of chromatographic separation can be described by van Deemter equation. The comparison of van Deemter curves for column particles of different size reveals that the smaller particles are used the more effective separation is obtained. This statement led to the development of sub-2 μm particles [5] giving birth to ultra high-performance liquid chromatography (UHPLC). Acquity BEH C18 column packed with 1.7 μm particles is one of the most often used UHPLC sub-2 μm particles columns. The particles based on BEH technology (bridged ethylene hybrid) provide excellent mechanical robustness, chemical stability in wide pH range, high separation efficiency, and short analysis time. On the other hand, particles of this size are responsible for significantly higher back pressure. Therefore, UHPLC columns are not compatible with conventional HPLC systems and their use is thus unavoidably connected with the employment of special instrumentation capable of dealing with the pressure up to 15,000 psi [6]. A great deal of attempts was done in order to overcome this disadvantage and develop separation columns with similar efficiency and short analysis time



	R_1	R_2	R_3
TC	H	OH	H
OTC	H	OH	OH
CTC	Cl	OH	H
DOX	H	H	OH

Fig. 1 Structures of analyzed tetracyclines

compared with UHPLC, but compatible with HPLC systems working usually up to 6,000 psi (400 bar).

These requirements are accomplished in recently introduced technologies fused-core particles [7, 8] and analogous core-shell particles [9, 10]. The particles of Kinetex C18 column (core-shell technology) are composed of a solid core (1.9 μm) surrounded by a thin porous silica layer (0.35 μm). This technology enables that the analyte can diffuse only into the pores of the thin porous layer. The particle size together with porous silica layer provides separation efficiency and analyses time similar to UHPLC, but with lower column backpressure making these columns compatible to conventional LC systems [9, 10].

The first comparative study [11] of fused-core silica and sub-2 μm particles for fast separations in pharmaceutical process development revealed that fused-core packed columns have the same or slightly better separation parameters at a much lower column backpressure. Recently, Gritti et al. [12] have compared Kinetex C18 column and the sub-2 μm particles Acquity BEH C18 column for analysis of large molecule mixtures and have obtained similar peak capacities for both columns. Even though, several publications have investigated Kinetex C18 performance [13–15], there is a lack of studies comparing this column with conventional sub-2 μm particles columns for specific applications in gradient mode.

The aim of this study was to compare the performance of Kinetex C18 column and Acquity BEH C18 column for analysis of low-molecular antibiotics of different chemical properties on UHPLC system.

Both tested columns are declared to be stable in a wide pH range: pH 1–12 for Acquity BEH C18 and pH 1.5–10 for Kinetex. Therefore, the columns were tested under acidic as well as alkaline conditions.

The testing compounds represent antibiotics frequently used in human and veterinary medicine, which currently belong to potential residual environmental pollutants [16]. Tetracyclines (see Fig. 1), namely oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DOX), were separated under acidic conditions, whereas macrolides (see Fig. 2), namely tylosin (TYL), clarithromycin (CLA), roxithromycin (ROX), and carbomycin (CAM) were analyzed under alkaline conditions.

Experimental

Chemicals, Standards

TC was purchased from Spofa (Prague, Czech Republic), OTC from VUAB (Roztoky u Prahy, Czech Republic), TYL from Fluka (Steinheim, Germany), DOX from Calbiochem (San Diego, USA) and CTC, CLA, ROX, and CAM from Sigma–Aldrich (Steinheim, Germany). Acetonitrile (ACN), methanol, and trifluoracetic acid (99.95%; TFA) were of LC/MS grade and were obtained from Biosolve (Netherlands), and formic acid (98–100%) was purchased from Merck (Germany). Ammonium hydroxide A.C.S. reagent (29% aqueous NH_4OH solution) was purchased from Sigma–Aldrich (Steinheim, Germany). Ammonium formate was prepared of formic acid of the respective molarity and ammonium hydroxide was added until the required pH value was obtained.

Standard Stock Solution Preparation

Standard stock solutions were prepared with methanol at a concentration of 1 mg mL^{-1}. Tetracycline standard mixture was prepared by mixing equal volumes of TC, OTC, CTC, and DOX standard stock solutions resulting in a final concentration of individual compounds of 100.0 μg mL^{-1}. Macrolide standard mixture consisted of CLA, ROX, TYL, and CAM methanolic solutions spiked into 50% methanol to the concentrations of 500, 300, 50 and 50 μg mL^{-1}, respectively.

Chromatographic System

Chromatographic analyses were carried out on the Acquity UPLC™ system equipped with 2996 Photo Diode Array (PDA) Detector (cell volume, 500 nL, optical path length, 10 mm). The dwell volume of the UHPLC system was 0.17 mL and was measured as described in [17]. The capillary connecting chromatographic column and PDA detector was 24 cm long with internal diameter of 177.8 μm. MassLynx V4.1 software was used for data processing. Samples were separated on Acquity BEH C18

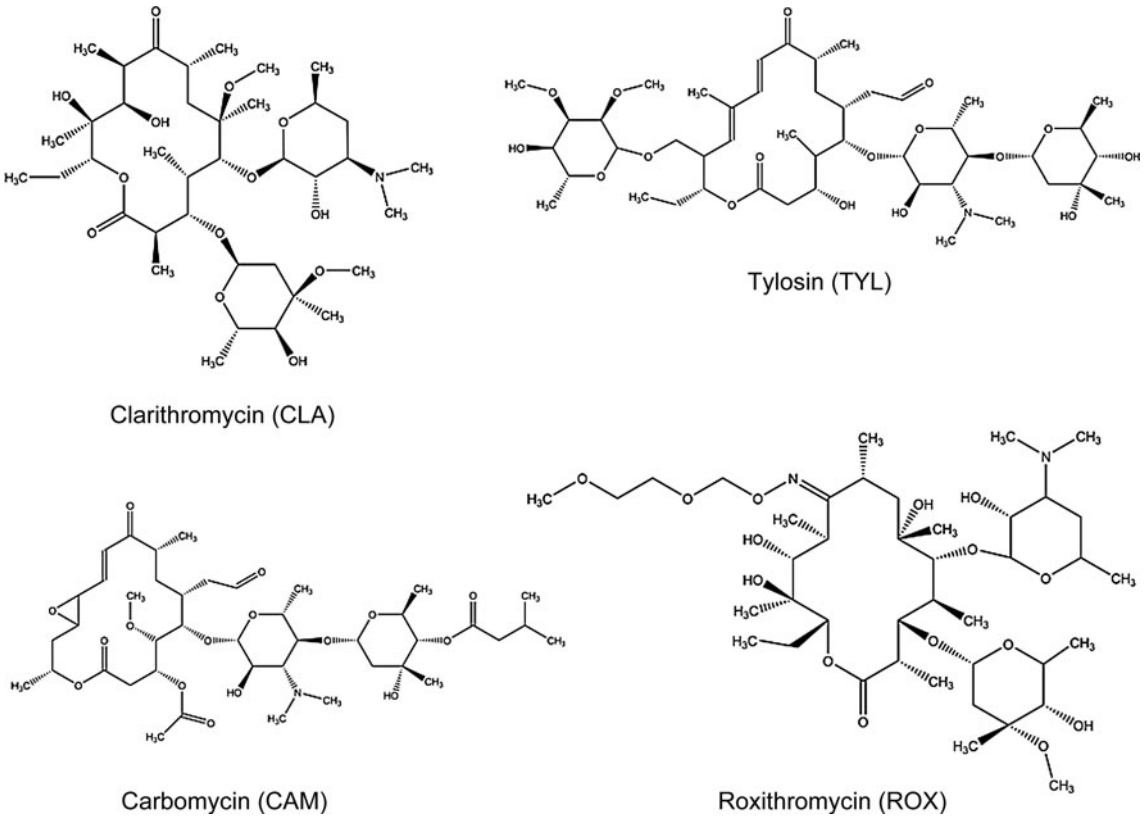


Fig. 2 Structures of analyzed macrolides

column (50 × 2.1 mm i.d., particle size; 1.7 μm, Waters) and Kinetex C18 column (50 × 2.1 mm i.d., particle size; 2.6 μm, Phenomenex). One microliter of sample was injected by Acquity Sample Manager in partial loop with needle overfill mode (the loop volume was 10 μL).

Chromatographic Conditions

Tetracyclines

Tetracyclines were separated on both columns at 40 °C using the mobile phase consisted of solvent A, TFA-water (0.05:99.95, *v/v*) and solvent B, ACN, injection volume was 1 μL. The gradient started at solvent composition A:B 95:5 (*v/v*) and changed to A:B 70:30 (*v/v*) ($\Delta\varphi = 0.25$) during the respective time of gradient t_g. The PDA detector was operating in the range from 200 to 400 nm with the sampling rate of 10 spectra per second and the chromatograms were extracted at 350 nm.

Macrolides

Macrolides were separated on both columns at 40 °C using the mobile phase consisted of solvent A, 1 mM ammonium formate pH 9 and solvent B, ACN, injection volume was 1 μL. The gradient started at solvent composition A:B 80:20 (*v/v*) and changed to A:B 35:65 (*v/v*) ($\Delta\varphi = 0.45$). The PDA detector was operating in the range from 194 to 400 nm with the sampling rate of 10 spectra per second. The chromatograms for further processing were extracted at wavelengths of 194, 194, 240, and 286 nm for ROX, CLA, CAM, and TYL, respectively.

Comparison of Column Performance under Gradient Conditions

The chromatographic column performance under gradient conditions was characterized by experimental peak capacity (P), which was calculated as follows:

$$P = 1 + \frac{t_N - t_1}{(1/N)\sum_{i=1}^{i=N} \omega_i} \quad (1)$$

where t_N and t_1 represent the gradient elution times of the most and less retained peak, respectively, N is the number of peaks selected for the calculation ($N = 4$ for tetracycline and 2 for macrolide analyses), and ω_i is the baseline peak width of the i^{th} peak measured as follows:

$$\omega_i = \frac{2\left(t_{1/2,r,i} - t_{1/2,f,i}\right)}{\sqrt{1.38629}} \quad (2)$$

where $t_{1/2,r,i}$ and $t_{1/2,f,i}$ are the experimental elution times of the rear and front parts of the peak measured at half of its height [12].

In order to make an appropriate comparison of columns performances the linear gradient was applied with constant intrinsic gradient steepness, G, defined as [18, 19]:

$$G = S\beta t_0 \quad (3)$$

where S is the slope of the relationship between the logarithm of the retention factor measured under isocratic conditions (k) and the organic solvent concentration (φ) in the case of linear solvent strength retention model (LSSM), β represents gradient slope, and t_0 is the column hold-up time. The LSSM describes the retention behavior and the dependence of the retention factor of the compound on the mobile phase composition under gradient elution conditions [18, 20, 21].

According to the equation:

$$S = \Delta log k / \Delta\varphi, \quad (4)$$

the values of S for given compound, column and separation conditions were obtained from two isocratic measurements with φ varied for tetracyclines and macrolides, respectively, on both tested columns [18].

Since the total column porosity ε_T of the tested columns is different ($\varepsilon_T = 0.654$ and 0.542 for Acquity BEH C18 and Kinetex C18, respectively [12]), the volumetric flow rate F for both columns was adjusted with respect to their ε_T:

$$F = u_0 \pi r^2 \varepsilon_T \quad (5)$$

where r is the internal column radius, u_0 is the linear velocity dependent on the column length (L), and t_0:

$$u_0 = \frac{L}{t_0}. \quad (6)$$

The change of solvent composition during the gradient, $\Delta\varphi$, was deliberately kept constant at 0.25 for tetracycline and 0.45 for macrolide analyses on both columns. P was measured for 16 values of u_0 ranging from 0.037 to 0.589 cm s^{-1} with 0.037 cm s^{-1} steps. These measurements were performed for three different $\beta/u_0 = 0.047$, 0.142, and 0.425 m^{-1}, where β depends on time of gradient t_g as follows:

$$\beta = \frac{\Delta\varphi}{t_g}. \quad (7)$$

Table 1 summarizes linear velocities u_0 with corresponding volumetric flow rates F at the two columns and times of gradient t_g for the three tested values of β/u_0.

Table 1 Summary of parameters employed during the Acquity BEH C18 and Kinetex C18 columns comparison

	Acquity BEH C18	Kinetex C18	β/u_0 (m^{-1})		
			0.047	0.142	0.425
u_0 (cm s^{-1})	F (mL min^{-1})		t_g (min)		
0.037	0.050	0.041	240.00	80.00	26.67
0.074	0.100	0.083	120.00	40.00	13.33
0.110	0.150	0.124	80.00	26.67	8.89
0.147	0.200	0.166	60.00	20.00	6.67
0.184	0.250	0.207	48.00	16.00	5.33
0.221	0.300	0.249	40.00	13.33	4.44
0.258	0.350	0.290	34.29	11.43	3.81
0.294	0.400	0.331	30.00	10.00	3.33
0.331	0.450	0.373	26.67	8.89	2.96
0.368	0.500	0.414	24.00	8.00	2.67
0.405	0.550	0.456	21.82	7.27	2.42
0.441	0.600	0.497	20.00	6.67	2.22
0.478	0.650	0.539	18.46	6.15	2.05
0.515	0.700	0.580	17.14	5.71	1.90
0.552	0.750	0.622	16.00	5.33	1.78
0.589	0.800	0.663	15.00	5.00	1.67

Result and Discussion

Development of UHPLC Methods

Tetracyclines

During the UHPLC method development, three mobile phases differing in the strength of organic acids (acetic acid, trifluoroacetic acid, formic acid in water) were tested. In all the cases, the ACN was used as an organic modifier. The best separation of all analytes on both columns was achieved with formic acid–water (0.05:99.95, *v*/*v*) as solvent A, and ACN as solvent B of the mobile phase.

The significant effect of the column temperature on separation of tetracyclines was observed and studied in the range from 30 to 60 °C with 5 °C steps. The higher the temperature was the lower retention times of analytes were achieved. At lower temperatures (30, 35 °C), the poor resolution of early-eluting peaks (OTC, TC) was obtained, while at higher temperatures (from 45 to 60 °C), the later-eluting analytes (CTC, DOX) were only partially separated. This phenomenon was observed on both Acquity BEH C18 and Kinetex C18 columns. Therefore, the temperature of 40 °C was chosen as a compromise parameter for both columns.

Macrolides

Strongly acidic (0.1% trifluoracetic acid), acidic (1 mM ammonium formate, pH 4.75) and alkaline (1 mM ammonium formate, pH 9) aqueous parts of mobile phases were

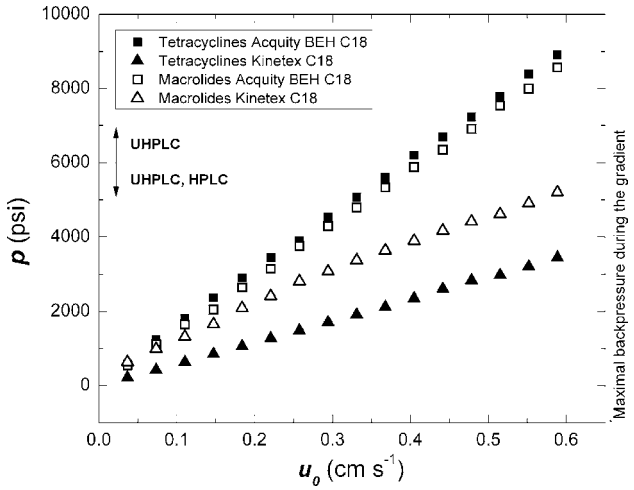


Fig. 3 The dependence of maximal column backpressure p on linear velocity u_0 for Acquity BEH C18 and Kinetex C18 columns

tested for macrolides separation. The alkaline conditions were the most convenient, which corresponds with previously published data [22]. ACN as an organic modifier was chosen with respect to low cut off, so it enables sensitive detection of CLA and ROX, which absorb at 194 nm. The effect of column temperature on macrolides analysis was tested in the range from 30 to 60 °C with 5 °C steps. The increasing temperature improved significantly the separation of the analytes and peaks symmetry in the whole tested range. However, considering the alkaline conditions (pH 9) employed for the separation, the temperature of 40 °C was applied with respect to column life-time.

Comparison of the Column Performances on UHPLC System

The dependence of maximal column backpressure achieved during the gradient on u_0 in the range from 0.037 to 0.589 cm s^{-1} is shown in Fig. 3. The maximal backpressure on Acquity BEH C18 column achieved at $u_0 = 0.589$ cm s^{-1} was 8,565 psi (macrolides) and 8,910 psi (tetracyclines), while on Kinetex C18 column, it reached 5,205 psi (macrolides) and 3,450 psi (tetracyclines) at the same u_0. In the whole studied range of u_0, the Kinetex C18 column backpressure was lower than 6,000 psi (400 bar), which enables to use this column in HPLC mode.

Further, the performance of the two tested columns was compared with UHPLC system by evaluation of their experimental P. As described earlier, the intrinsic gradient steepness G has to be kept constant for a fair comparison of the columns performance. The crucial parameter determining G is parameter S (see Eq. 4). It was revealed that the values of S are slightly higher for Kinetex C18 than for Acquity BEH C18 column for all analytes with maximal difference of 13.8%. However, this difference affects the values of G only negligibly as shown in Table 2. Since this variation influences the experimental peak capacity insignificantly [12], the parameter S was considered to be comparable for both columns and therefore did not need to be taken into account for the columns performance comparison. Three curves describing the dependence of experimental P on u_0 at three different gradient slopes β/u_0 constructed for both tetracyclines and macrolides on the two chromatographic columns are depicted in Fig. 4. The curves revealed that experimental P considerably decreased with increasing u_0. Even though P is according to the literature independent on u_0, this phenomenon is in accordance with previously performed experiments [12]. Additionally, experimental P is apparently strongly dependent on gradient slope β/u_0 so that higher P is obtained when lower gradient slope β/u_0 is applied. Nevertheless, low gradient slope β/u_0 is in principle connected with long analysis time, which is inconsistent with desired high-throughput

Table 2 Parameters S and G for tetracyclines and macrolides on Acquity BEH C18 and Kinetex C18 columns

	Acquity BEH C18				Kinetex C18			
		β/u_0 (m^{-1})				β/u_0 (m^{-1})		
	S_i	0.047	0.142	0.425	S_i	0.047	0.142	0.425
		$G = S\beta t_0$				$G = S\beta t_0$		
Tetracyclines								
OTC	2.609				2.628			
TC	2.127	0.0039	0.0116	0.0347	2.196	0.0040	0.0121	0.0364
CTC	1.030				1.141			
DOX	0.778				0.884			
Macrolides								
TYL	1.822				1.825			
CLA	0.819	0.0039	0.0118	0.0354	0.880	0.0041	0.0124	0.0372
ROX	0.759				0.846			
CAM	0.305				0.347			

G values were obtained from the arithmetic mean of S_i values for tetracyclines or macrolides

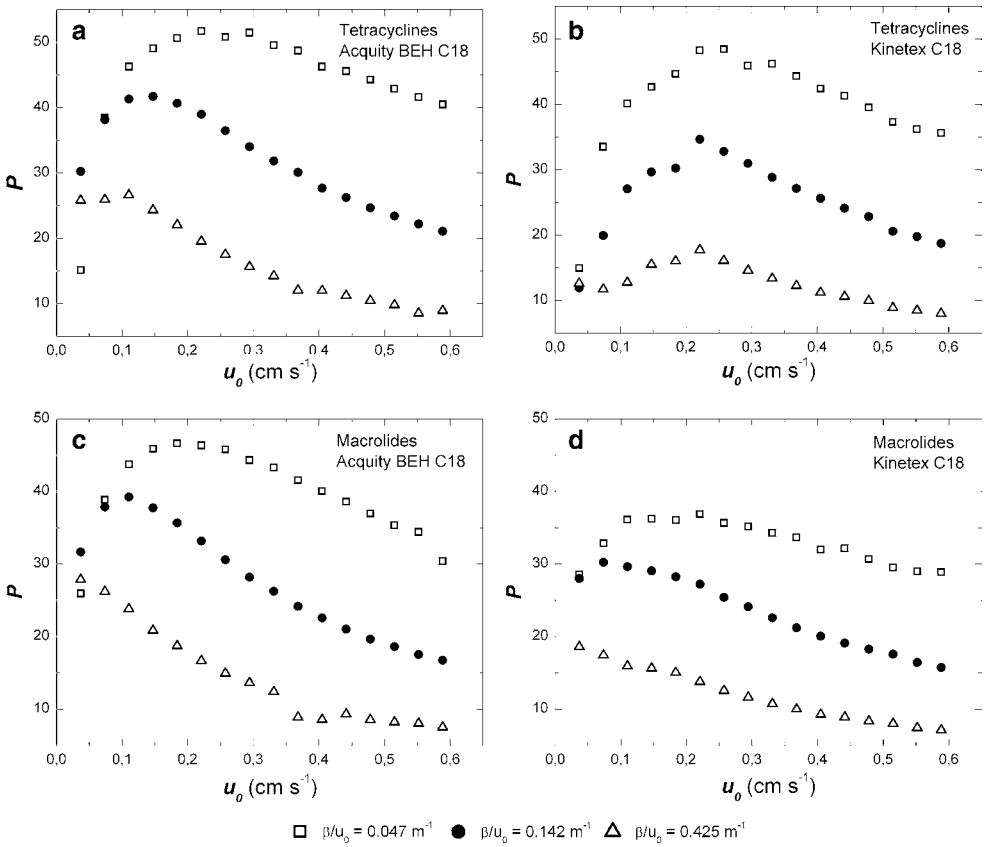


Fig. 4 Experimental peak capacity as a function of linear velocity u_0 in gradient separation. **a** Tetracyclines on Acquity C18 column; **b** tetracyclines on Kinetex C18 column; **c** macrolides on Acquity C18 column; **d** macrolides on Kinetex C18 column

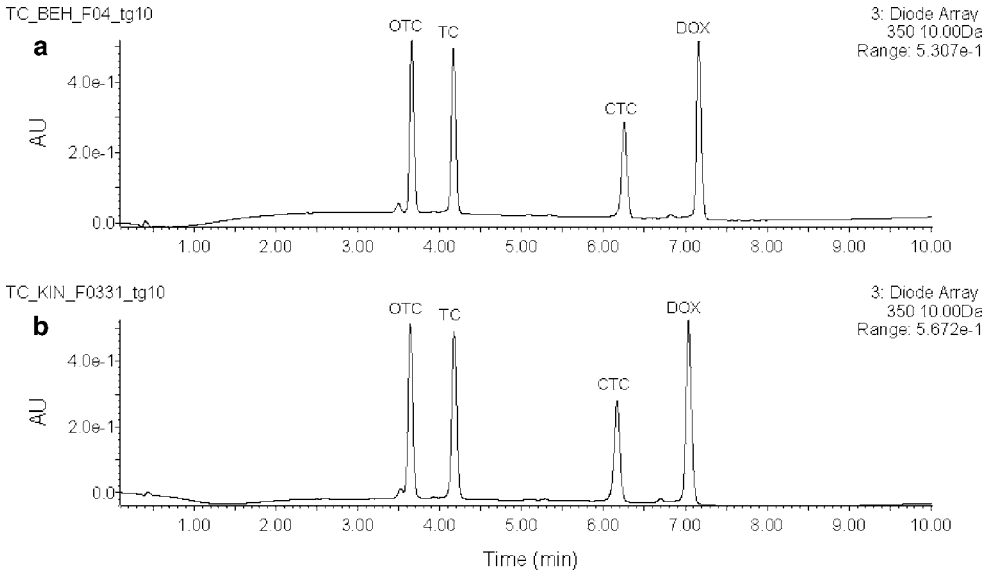


Fig. 5 UHPLC chromatogram of gradient separation of tetracyclines. **a** Acquity BEH C18 column; **b** Kinetex C18 column. Chromatographic conditions: mobile phase: 0.05% trifluoracetic acid and acetonitrile; column temperature 40 °C; linear velocity $u_0 = 0.294$ cm s^{-1}; gradient time $t_g = 8.89$ min; change of solvent composition during the gradient time $\Delta\varphi = 0.25$; gradient slope $\beta/u_0 = 0.142$ m^{-1}; injection volume 1 μL; UV: 350 nm

analyses. Therefore, the compromise between P and gradient slope β/u_0 represents the crucial task.

Tetracyclines

The analyses of tetracyclines on Acquity BEH C18 and Kinetex C18 columns employing $u_0 = 0.294$ cm s^{-1} and $\beta/u_0 = 0.142$ m^{-1} as a compromise between experimental P and analysis time are shown in Fig. 5.

For all applied conditions, all four analytes were baseline separated with good peak symmetry. The only exception with poor peak symmetry was found on both columns at $u_0 = 0.037$ cm s^{-1} and gradient slope $\beta/u_0 = 0.047$ m^{-1} for CTC and DOX, which also

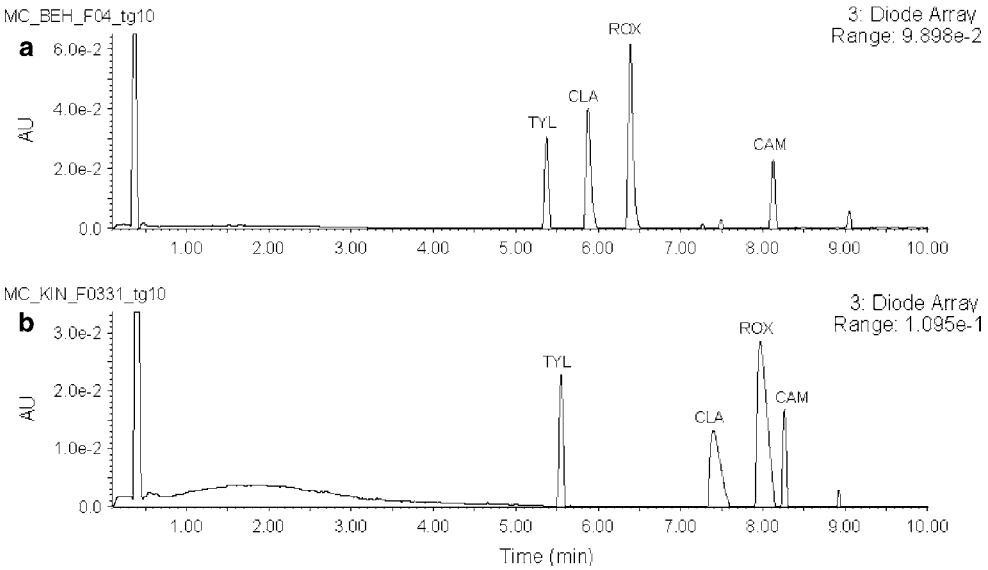


Fig. 6 UHPLC chromatogram of gradient separation of macrolides. **a** Acquity BEH C18 column; **b** Kinetex C18 column. Chromatographic conditions: mobile phase: 1 mM ammonium formate pH 9 and acetonitrile; column temperature 40 °C; linear velocity $u_0 = 0.294$ cm s^{-1}; gradient time $t_g = 8.89$ min; change of solvent composition during the gradient time $\Delta\varphi = 0.45$; gradient slope $\beta/u_0 = 0.142$ m^{-1}; injection volume 1 µL; UV: extracted at maximal wavelength of the analytes-maxplot

resulted in a very low peak capacity at this u_0 and β/u_0.

The maximal experimental P of Acquity BEH C18 column was 51.8 (at $u_0 = 0.221$ cm s^{-1} and $\beta/u_0 = 0.047$ m^{-1}) and for Kinetex C18 was 48.4 (at $u_0 = 0.221$ cm s^{-1} and $\beta/u_0 = 0.047$ m^{-1}). In general, the experimental P of Acquity BEH C18 column was for tetracycline analysis found to be slightly higher than that of Kinetex C18 column. On the contrary, Gritti et al. [12] recently published results revealing better performance of Kinetex C18 column compared with Acquity BEH C18 column under gradient conditions. However, proteins–analytes of different properties were used in their study. Therefore, the discrepancy can be possibly explained by faster mass transfer in Kinetex C18 column that affects positively the analysis of large molecules, but the analysis of the low-molecular weight tetracyclines appears to be influenced by this parameter less significantly.

Interestingly, the maximal experimental P for Kinetex C18 column was for all the three β/u_0 achieved at same value of $u_0 = 0.221$ cm s^{-1}. On the other hand, the maximal experimental P for Acquity BEH C18 column was at higher β/u_0 ratios shifted to lower u_0: $u_0 = 0.110$ cm s^{-1} for $\beta/u_0 = 0.425$ m^{-1}, $u_0 = 0.147$ cm s^{-1} for β/u_0 0.142 m^{-1}, and $u_0 = 0.221$ cm s^{-1} for $\beta/u_0 = 0.047$ m^{-1} (see Fig. 4a, b). The steepness of the experimental P decrease with increasing u_0 was for both columns very similar.

Macrolides

The chromatograms of macrolides analyses on both columns at $u_0 = 0.294$ cm s^{-1} and $\beta/u_0 = 0.142$ m^{-1} representing a reasonable compromise between experimental P and time of gradient t_g are shown in Fig. 6.

Under all u_0 and gradient slopes β/u_0 applied, macrolides were well separated on Acquity BEH C18 column with good peak symmetry rates for all peaks. Kinetex C18 column also separated macrolides sufficiently; however, the peak symmetry rates of CLA and ROX were not optimal. Unlike TYL and CAM, retention times of CLA and ROX on both columns differed significantly. Moreover, worsening performance of Kinetex C18 for CLA and ROX from one injection to another was observed. Figure 7 depicts analysis of macrolides on Kinetex C18 column ($u_0 = 0.221$ cm s^{-1} and $\beta/u_0 = 0.047$ m^{-1}) and the same analysis on the same column after 70 injections. The Kinetex C18 column was after 70 injections incapable of separating CLA and ROX; however, analysis of TYL and CAM including their retention times did not differ considerably. This phenomenon observed on two newly employed Kinetex C18 columns is apparently connected with alkaline pH of the mobile phase and with the two specific analytes—CLA and ROX. The cause of this phenomenon and whether it has a more general relevance remains unclear and requires further investigation. With respect to this fact, CLA and ROX were excluded from the evaluation of the columns performance by experimental P and only TYL and CAM were in case of both columns considered.

The maximal experimental P of Acquity BEH C18 column was 46.7 (at $u_0 = 0.184$ cm s^{-1} and $\beta/u_0 = 0.047$ m^{-1}) and for Kinetex C18 was 36.9 (at $u_0 = 0.221$ cm s^{-1} and $\beta/u_0 = 0.047$ m^{-1}). The experimental P of Acquity BEH C18 column was up to $u_0 = 0.294$ cm s^{-1} for all gradient slopes β/u_0 substantially higher than that for Kinetex C18 column. However, the differences between experimental P at higher u_0 were negligible (see Fig. 4c, d). In other words, the steepness of

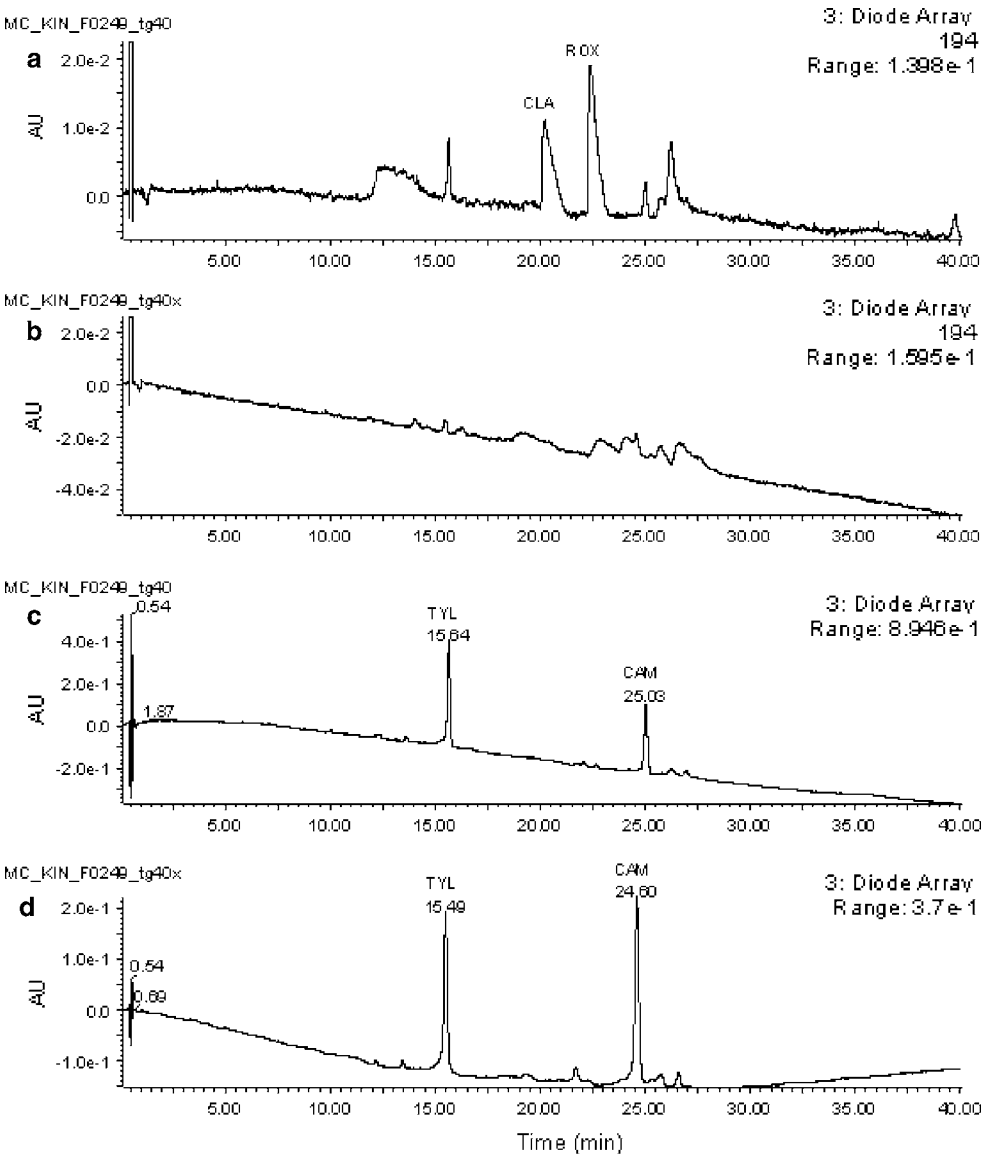


Fig. 7 UHPLC chromatogram of gradient separation of macrolides on Kinetex C18 column. **a**, **c** analysis of initial injection; **b**, **d** analysis after 70 injections. Chromatographic conditions: Mobile phase: 1 mM ammonium formate pH 9 and acetonitrile; column temperature 40 °C; linear velocity $u_0 = 0.221$ cm s^{-1}; gradient time $t_g = 40$ min; change of solvent composition during the gradient time $\Delta\varphi = 0.45$; gradient slope $\beta/u_0 = 0.047$ m^{-1}; injection volume 1 μL; UV: **a**, **b** 194 nm; **c**, **d** extracted from 240 to 286 nm

experimental P decrease with increasing u_0 is much flatter in case of Kinetex C18 column.

Conclusion

The chromatographic performance of Kinetex C18 and Acquity BEH C18 columns in gradient separation of low-molecular tetracycline and macrolide antibiotics on UHPLC system was tested by evaluation of experimental peak capacity P and its dependence on linear velocity u_0 for three gradient slopes β/u_0. Under all conditions, higher P was achieved on Acquity BEH C18 column for both antibiotic groups; however, the difference was dependent on specific parameters and was more pronounced for macrolides. The markedly lower column backpressure generated on Kinetex C18 column during the gradient elution confirmed its compatibility with conventional HPLC system. On the other hand, unlike Acquity BEH C18 column, Kinetex C18 column exhibited dramatically decreasing performance with growing number of injections for analysis of two macrolides (CLA and ROX). This phenomenon is connected to alkaline conditions (pH 9) but was not observed neither for the other macrolides (TYL and CAM) nor tetracyclines analyzed under acidic conditions. In conclusion, Kinetex C18 column represents a convenient alternative to Acquity BEH C18 column for analysis of tetracyclines under acidic conditions, but exhibited substantial limitations for analysis of macrolides under alkaline conditions.

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Short communication

Hyphenated ultra high-performance liquid chromatography–Nano Quantity Analyte Detector technique for determination of compounds with low UV absorption

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ABSTRACT

A novel universal aerosol-based detector Nano Quantity Analyte Detector – NQAD™, connected with an ultra-performance liquid chromatography system is described. The detector was employed for detection of selected antibiotic compounds – macrolides (oleandomycin, erythromycin, troleandomycin, clarithromycin and roxithromycin) that are hard to detect using classical UV detectors due to the lack of chromophores. The determined lowest detection limits under isocratic conditions for these compounds ranged from 3.0 to 5.4 μg/mL. The suitability of the detector connected with ultra high-performance liquid chromatography in the gradient mode was tested on a more complex mixture containing 12 antibiotics. The detector exhibited full compatibility under both the elution modes when UHPLC separations were achieved in relatively short run times.

1. Introduction

UV detection is often considered to be one of the most widespread detection techniques combined with liquid chromatography. However, the universality of UV detection is limited because it requires the presence of a chromophore in the analyte molecule. UV detectors are not sensitive enough for a great many analytes especially those with a lack of π electrons. This is especially important when using ultra high-performance chromatography (UHPLC) where low injection volumes are usually employed (up to 5 μL) and where the volume of the UV detector flow cells can be decreased. Therefore, a novel hyphenated method is introduced in this paper that combines UHPLC with a novel commercially available aerosol-based detector (Nano Quantity Analyte Detector – NQAD™).

UHPLC is a modern separation technique providing considerable high-throughput analysis compared with HPLC. Hardware adjustments allow UHPLC systems to work at extreme pressures, up to 100 MPa [1]. This is necessary for operation with sub-2 micron particles in the chromatographic columns for UHPLC applications and cannot be achieved using conventional liquid chromatographic systems [2]. UHPLC generally yields significantly higher separation efficiencies and shorter run times compared to ordinary HPLC columns. According to the Van Deemter equation, when the particle size of the chromatographic sorbent is decreased, the efficiency of the separation process increases and the efficiency does not diminish at higher flow rates or linear velocities [3].

NQAD™ is a novel aerosol-based detector for HPLC that is also termed in the literature as the condensation nucleation light-scattering detector (CNLSD) [4]. NQAD™ uses condensation nucleation technology. The principle of the technique is based on nebulization and evaporation of the mobile phase at elevated temperature and consequent analyte condensation with supersaturated auxiliary water vapor. This leads to creation of relatively large droplets that are later detected using scattered light with a laser-photodetector system set-up at perpendicular arrangement. This increase in particle size tremendously increases the light-scattering signal and dramatically increases the sensitivity in comparison to ELSD [5]. Only particles above a critical size can act as condensation nucleation sites that increase the signal–noise ratio due to discrimination of small droplets from the mobile phase. The producer states that the detector allows measuring of compounds in low nanogram on column ranges and the NQAD™ dynamic range should span from below 1 ng to over 10 μg on the column [6]. However, even lower LODs were published with a laboratory built CNLSD for various compounds [4,7]. The technique is also suitable for compounds that lack π electrons and that are thus hard to detect using UV methods. Such a universal detection technique should have extensive applications in liquid chromatography including UHPLC as it

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was already published with the laboratory built CNLSD connected to several other separation techniques including HPLC [4,7,8], capillary electrophoresis [9] and capillary electrochromatography [10].

Some representatives of macrocyclic antibiotics, macrolides, can cause difficulties in UV detection. These compounds represent a large group of very similar, naturally occurring antibiotics produced mostly by *Streptomyces* sp. They consist of a macrocyclic lactone ring to which typically two sugars, one of which is an amino sugar, are attached. They are amongst medicines that have recently been widely used to treat a broad spectrum of infectious diseases. It is assumed they can also appear in the environment through misuse [11]. This implies the necessity of suitable, reliable and high-throughput methods for their determination.

Up to the present time, in addition to GC [12], mostly HPLC with various detectors has been used for macrolide analysis. Several methods with UV detection were developed in the past [13]; however, many macrolides lack π-electrons and are thus hard to detect in the UV range even at low wavelengths. Therefore, various techniques have been investigated for macrolide detection. Fluorescence was used by e.g. Pakinaz and Khashaba [14]; however, sample pre-treatment is required for the detection. Electrochemical detection [15–18] may offer a much better choice because of the presence of electro-active groups in their molecules and is undoubtedly the most frequently used technique today. Most recently, mass spectrometry, the most universal detector by far, has also been applied to macrolide detection [11,19,20]. All the above techniques for analysis of macrolides were reviewed by e.g. Marzo and Dal Bo [21], Kanfer et al. [22] and Danaher et al. [23].

This work was concerned with testing a novel hyphenated UHPLC-NQAD™ technique employing the first commercially available detector using condensation nucleation light-scattering principle for detection of five selected macrolides (Mixture I) and 12 other antibiotics with various structures and chromatographic properties (Mixture II) and to compare this method with UV detection.

2. Experimental

2.1. Chemicals, standard solutions

The solvents used in UHPLC were of gradient grade. Acetonitrile 99.95% (ACN) Biosolve and Methanol 99.95% (Chromapur GG) were purchased from Chromservis (Czech Republic).

The standard stock solutions were prepared with methanol–water (50:50, v/v) at a concentration level of 1 mg/mL. Standard solutions with the required concentration were obtained by dilution of the stock solutions with methanol–water (50:50, v/v).

Mixture I contained the following macrolides: oleandomycin (OLE), erythromycin (ERY), troleandomycin (TRO), clarithromycin (CLA) and roxithromycin (ROX) at a concentration of 50 μg/mL.

Mixture II contained following antibiotics: metronidazole (MET), vancomycin (VAN), chloramphenicol (CHL), cycloheximide (CYC), lincomycin (LIN), griseofulvin (GRI), clindamycin (CLI), clarithromycin (CLA), roxithromycin (ROX), rapamycin (RAP), streptovitacin A (STV) and carbomycin (CAM) at a concentration of 62.5 μg/mL.

MET, VAN, CHL, CYC, LIN, GRI, CLI, ERY, CLA, ROX and RAP were obtained from Sigma–Aldrich, Germany and were of UV grade (>95%). STV, OLE, TRO and CAM were purer than 90% and were kindly provided by Prof. Jaroslav Spížek, Institute of Microbiology of the Academy of Sciences of the Czech Republic, v.v.i., Czech Republic.

2.2. UHPLC

Samples were analyzed with the Acquity UHPLC system (Waters, Milford, MA, USA) using Waters BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm), column temperature, 50 °C; data sample rate, 20 Hz; filter constant, 0.5; injection volume, 5 μL; flow rate, 0.4 mL/min. Mobile phases consisted of water (A) and acetonitrile (B), both containing 0.01% or 0.04% NH_4OH for NQAD™ and UV detection, respectively. The stock solution of aqueous NH_4OH solution had a concentration of 24%.

2.2.1. Isocratic mode

Mixture I was separated under isocratic conditions A:B 55:45 (v/v). The separated compounds were detected by both the NQAD™ and UV techniques.

2.2.2. Gradient mode

Mixture II was separated under gradient conditions (min/%A) 0/5; 7/27; 12/33; 17/88; 18/100; 20/100. Each analysis was followed by a column equilibration step (2 min). The separated compounds were detected by both the NQAD™ and UV techniques.

2.3. NQAD™ detection

The Quant NQAD™ (Quant Technologies, LLC; Blaine, Minnesota, USA) was used for detection of antibiotics under

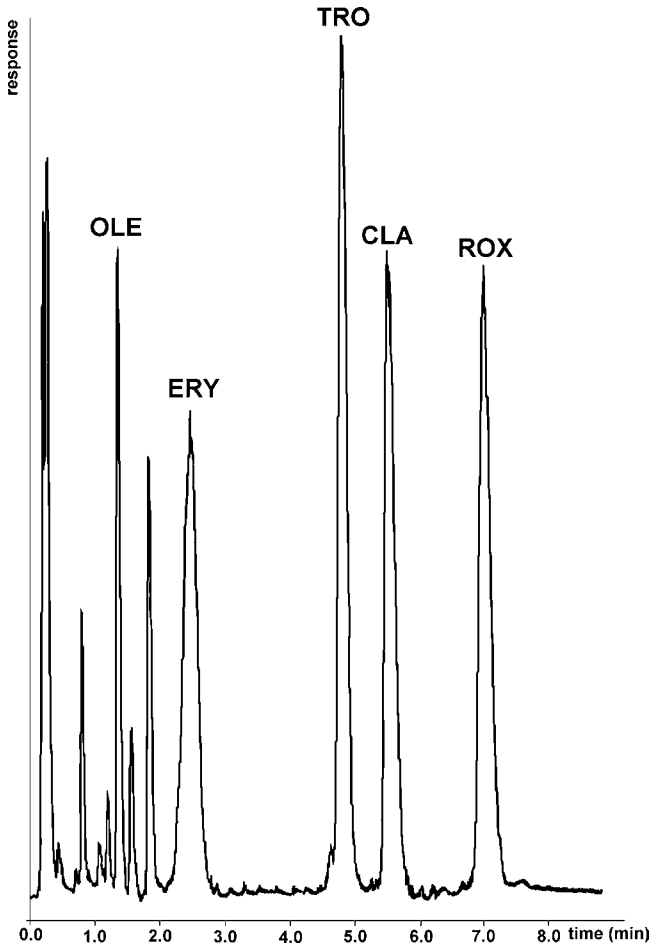

Fig. 1. Isocratic separation of Mixture I with NQAD™ detection. OLE, ERY, TRO, CLA, ROX at a concentration of 50 μg/mL, BEH C18 column (50 × 2.1 mm I.D., 1.7 μm), column temperature, 50 °C; injection volume, 5 μL; flow rate, 0.4 mL/min, water (A) and acetonitrile (B), both containing 0.01% NH_4OH (55:45, v/v), NQAD™ detection: 35 °C, gain 10×.

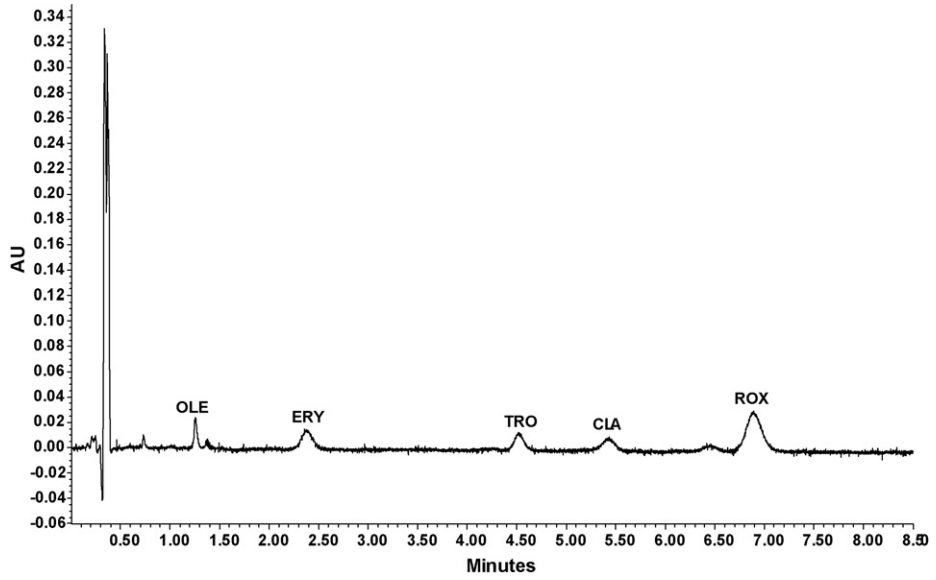

Fig. 2. Isocratic separation of Mixture I with UV detection. OLE, ERY, TRO, CLA, ROX at a concentration of 50 μg/mL, UV detection 194 nm, for chromatographic conditions, see Fig. 1.

UHPLC conditions. The evaporation temperature of the nebulizer-condensation part was set at 35 °C, the scan rate was 50 s^{-1} and the gain was 10×. The data were collected and processed with an integrator LCI-100 (PerkinElmer, Waltham, MA, USA).

2.4. UV detection

2996 photodiode array detector (Waters) operating in the range from 194 to 690 nm was used for antibiotic detection under UHPLC conditions. The macrolides were quantified at extracted wavelength 194 nm where they exhibited their maxima in the used range. The other antibiotics analyzed in Mixture II were monitored at their UV maxima – MaxPlot (MET 318 nm, VAN 206 nm, CHL 278 nm, CYC 203 nm, GRI 295 nm, RAP 278 nm, STV 203 nm, CAM 240 nm). Data were processed with Empower 2 software (Waters).

2.5. Calibration curve, LOD, LOQ

Calibration curves over the linear range from 3.125 to 100 μg/mL for OLE, ERY, TRO, CLA and ROX were determined

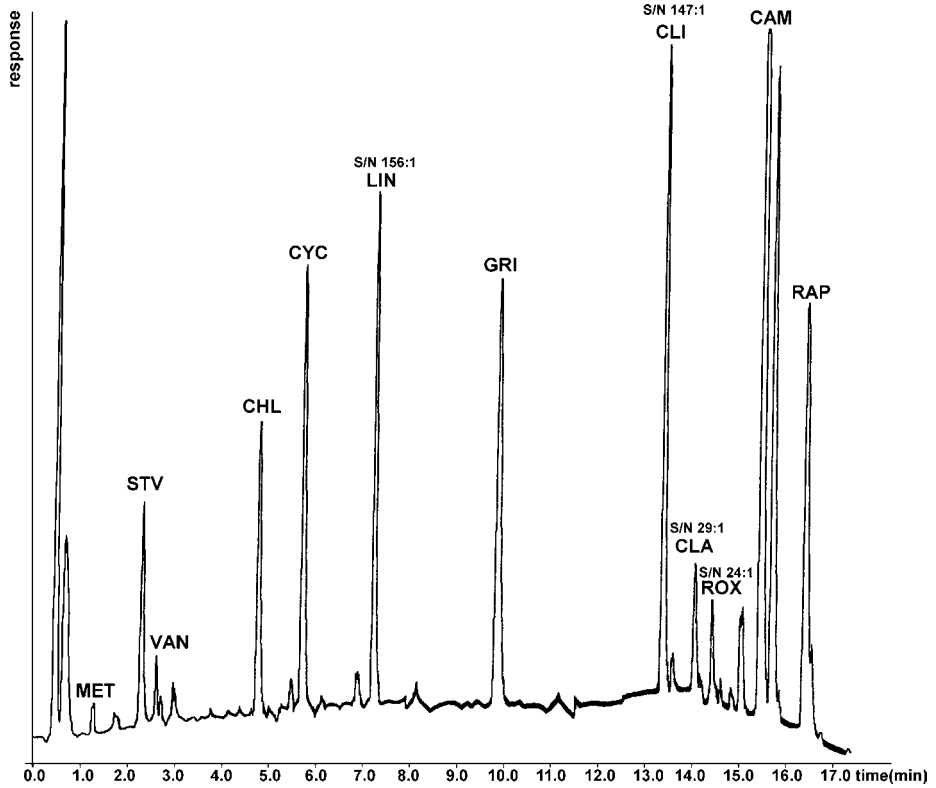

Fig. 3. Gradient separation of Mixture II with NQAD™ detection. MET, VAN, CHL, CYC, LIN, GRI, CLI, ERY, CLA, ROX, RAP, STV, OLE, TRO and CAM at a concentration of 62.5 μg/mL, gradient (min/%A) 0/5; 7/27; 12/33; 17/88; 18/100; 20/100, for other conditions, see Fig. 1, NQAD™ detection.

Table 1
LOD and LOQ parameters for UV and NQAD™ detection and respective calibration curves.

Analyte	LOD (μg/mL)		LOQ (μg/mL)		R^2		Calibration curves	
	NQAD™	UV	NQAD™	UV	NQAD™	UV	NQAD™	UV
OLE	3.0	10.3	10.0	33.9	0.999	0.998	$y = 60110x - 873$	$y = 1060x + 1180$
ERY	5.4	18.2	18.0	60.1	0.998	0.994	$y = 188794x - 710649$	$y = 2480x - 1280$
TRO	4.6	12.5	15.0	41.4	0.999	0.997	$y = 143931x + 327974$	$y = 1790x + 13800$
CLA	5.4	10.9	17.6	36.0	0.998	0.998	$y = 163431x - 550962$	$y = 2010x + 1980$
ROX	5.1	9.6	16.7	31.6	0.998	0.999	$y = 169400x - 260384$	$y = 6950x - 4210$

at concentration levels of 100.0, 50.0, 25.0, 12.5, 6.3 and 3.1 μg/mL.

LOQ and LOD values were obtained from the calibration curves. The LOD and LOQ were calculated as follows: LOD = $3.3\sigma/S$ and LOQ = $10\sigma/S$, where S is slope of the calibration curve and σ is the standard error. The calibration parameters were obtained from an average of three independent curves.

3. Result and discussion

The separation of the target compounds was optimized under basic pH conditions. There were tested several buffers including ammonium formate and ammonium acetate at the range pH 9.0–10.0. Ammonium hydroxide was the only additive that allowed complete baseline separation of the compounds. This was important especially in the case of the last eluting peaks. Simultaneously, the other buffers caused substantially higher noise of the baseline that did not enable any detection of the analytes. Furthermore, a significant influence of the ammonium hydroxide concentration in the mobile phase was observed for the NQAD™ detection. While both the tested concentrations of ammonium hydroxide had no effect on the UV mode, the higher concentration (0.04%) caused an elevated baseline noise in the NQAD™ mode. After decreasing the ammonium hydroxide concentration to 0.01%, the noise value dropped and the separation was still adequate. As a result, the mobile phase composition (buffer type, concentration, additives, etc.) probably represents the limiting parameters in the NQAD™ universal detection.

Another parameter of the separation as temperature in the range 25–50 °C was tested. The optimal separation was achieved at 50 °C (data not shown).

The parameters of the NQAD™ and UV detectors were compared using two model mixtures. Mixture I represents a mix of five macrolides with poor UV response. The baseline separation of all five compounds of Mixture I was achieved under isocratic conditions. Fig. 1 depicts the separation of Mixture I at a concentration level of 50.0 μg/mL with NQAD™ detection. Fig. 2 depicts the same separation with UV detection. The resolutions of the neighboring peaks were greater than 1.5 (data not shown). Some other unknown peaks appeared in the chromatograms (see Figs. 1 and 3). Probably they come from impurities in the used solvents and standards. However, in every case these unknown peaks were sufficiently separated from the target compounds and therefore they do not interfered with them. It is evident from Figs. 1 and 2, on the basis of visual comparison of the two chromatograms alone, that NQAD™ detection is significantly more sensitive than UV detection for all the macrolides.

The LODs and LOQs of the studied macrolides obtained by NQAD™ and the UV mode are compared in Table 1. The LOD values in the NQAD™ mode range from 3.0 to 5.4 μg/mL. The obtained values are in good agreement with the parameters mentioned on the supplier/producer website [6]. The LOD values in the UV mode range from 9.6 to 18.2 μg/mL, i.e. they are approximately three times higher than when NQAD™ was used. Several articles can be found where authors determined the LOD of macrolides with other detectors. De la Huebra and coauthors published the LOD of ROX, OLE detected by a coulometric detector in the range 2.2–6.2 μg/mL [16]. Another macrolide, medacamine, was detected by voltametric detection down to an LOD 1.0 μg/mL [15]. Amperometric detection of CLA and ROX achieved 10 μg/mL [18] and the LOD was improved with an increased injection sample volume to 2.5 μg/mL. Fluorescence detection after derivatization of the sam-

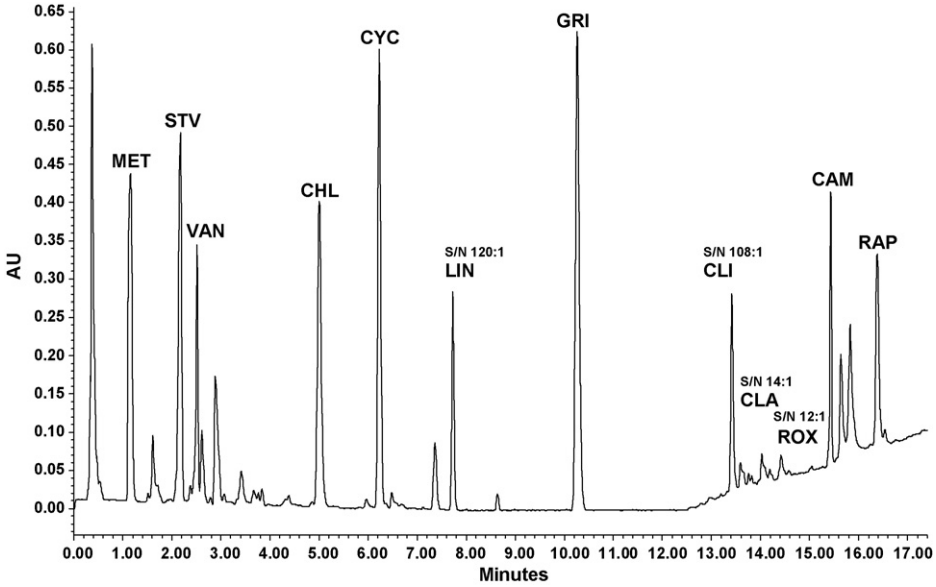

Fig. 4. Gradient separation of Mixture II with UV detection. Concentration of individual components (MET, VAN, CHL, CYC, LIN, GRI, CLI, ERY, CLA, ROX, RAP, STV, OLE, TRO and CAM), 62.5 μg/mL, UV detection displayed at their individual UV maxima (MaxPlot, see Section 2), for chromatographic conditions, see Fig. 3.

ples was also shown to be suitable for macrolide analysis with an LOD 9.8–17.5 ng/mL for ERY, CLA, ROX [14]. To the best of our knowledge, there is no publication describing macrolide detection using light-scattering detectors. Amongst other antibiotics, mainly aminoglycosides have been detected by this technique, with an LOD in the range 1.7–2.5 μg/mL [24,25]. Mass spectrometry was published as the most sensitive method (LOD 0.5 ng/mL; ERY, CLA, ROX) [11]; however, operational and instrumental costs can be excessive. Therefore NQAD™ can be a suitable compromise as universal and sensitive detector for compounds with poor limits of UV detection [3].

The suitability of NQAD™ connected with LC in the gradient mode was tested on more complex Mixture II containing 12 bioactive compounds with various structures. The chromatograms of both detection techniques are presented in Figs. 3 and 4. Even though the baseline of NQAD™ increased slightly with increasing retention time, no other difficulties were observed with NQAD™ detection under a linear acetonitrile gradient (see Fig. 3). Finally, a drop in baseline level was observed at the end of gradient runs, caused probably by lower ratio of water in the mobile phase and by related changes of surface tension in the mobile phase. The comparison of single peak heights for both detection techniques is variable. Some of the analytes, e.g. CLI, LIN, CLA, ROX, achieved a better signal-to-noise ratio with NQAD™ than with the UV detector (see Figs. 3 and 4). Especially these compounds can cause difficulties in UV detection due to low UV detector response. However, VAN, STV and MET exhibited lower sensitivity by NQAD™ compared with the UV detector. The other compounds included in Mixture II showed similar detection response. The different response of the detector toward the analyzed compounds can be caused by several factors including different refractive index, density of the analytes and molecular weight that influence signal also in the case of classical ELSD technique. Moreover, in the case of CNLSD method the response can be influenced by surface tension and polarity of the analyte. That can effect condensation nucleation process which involves the growths of droplets by condensation of an externally introduced vapor with the analytes [5].

4. Conclusions

Several macrolides that are poorly detectable with UV detectors, because of the lack of UV absorbing chromophores, were detected with a novel aerosol-based NQAD™ detector combined with UHPLC separation. The determined LOD were from 3.0–5.4 μg/mL, which is in the same range as the LOD of electrochemical methods and approximately 3 times higher than the LOD of the tested UV detector. Adequate UHPLC separation of these compounds was achieved within 8 min. In addition to macrolides, the NQAD™ detector was also suitable for detection of other antibiotics of various structures, such as lincosamides, piperidines, glycopeptides, chloramphenicol, etc. Moreover, the detector was shown to be fully compatible with UHPLC in the isocratic as well as the gradient mode. The method could be applied to the environmental analysis of residual antibiotics in matrices including wastewaters, e.g. manures and soil exposed to such materials.

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Ultra-high-performance liquid chromatography fingerprinting method for chemical screening of metabolites in cultivation broth

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ABSTRACT

A fingerprinting method for chemical screening of microbial metabolites, potential antibiotics, in spent cultivation broths is described. The method is based on high-throughput ultra-high-performance liquid chromatography (UHPLC) separation with UV detection (photodiode array detector). Thirteen antibiotic standards and four cultivation broths were used for the method development. The comparison of ten liquid–liquid and solid phase extraction protocols for sample clean-up and pre-concentration revealed that Oasis HLB C18 sorbent gives the best recoveries. The Acquity BEH C18 chromatographic column was chosen for the samples separation with respect to its universality, selectivity, efficiency and robustness. The method is presented by two 3D fingerprints for every sample that was obtained under different, acidic and alkaline, UHPLC conditions. The acidic mobile phase consisted of 0.5% phosphoric acid with methanol and the alkaline mobile phase of 1 mM ammonium formate, pH 9 with acetonitrile. Each pair of 3D fingerprints includes the following physico-chemical information: polarity (retention time), presence and characterization of chromophores (UV spectra), compound concentration (detector response), and acid–base properties (influence of different pH of the aqueous parts of mobile phases on retention times). The sample extraction and method validation were assessed with relative standard deviation (RSD) of 0.5, 5.0 and 20.0% for retention times, peak areas and minor compound peak areas, respectively.

1. Introduction

Bacterial secondary metabolites represent one of the most important sources of bioactive compounds. Almost one half of described antibiotics originate from actinomycetes [1]. Recently, the rate of new antibiotics discovery has declined dramatically and this trend is continuing [2]. In many instances, known compounds are rediscovered [3–5], although Watve et al. estimated that only about 3% of the existing compounds have been described so far [6]. Chemical fingerprinting based on chemical analysis of antibiotics and other secondary metabolites excreted to cultivation broth represents one possible discovery tool exploring a "chemical picture" of the produced set of metabolites without their individual isolation [7]. The applied method that includes both sample preparation and analysis must be universal enough to detect the maximal number of analytes present in the sample. In addition, the fingerprints are to provide physico-chemical information for all unknown compounds (*e.g.* to allow a tentative identification or classification to a specific compound class). Finally, the method should be high-throughput to facilitate screening of a large number of samples. The fingerprints then not only can be used *per se*, but also combined with genetic screening, *e.g.* detection of secondary metabolic genes, operons or clusters. Also, in combination with taxonomical identification the fingerprints may predict horizontal gene transfer among related strains as indication of usefulness for antibiotic production [8].

Chemical fingerprinting always has been based on a chromatographic technique [9,10], and most recently chromatographic techniques hyphenated with various means of detection have been applied: LC–UV [11,12], LC–ELSD [13,14], LC–MS [11,12,14], LC–NMR [11]. Since none of the detectors is fully universal, their advantages and disadvantages must be considered [15–17]. In principle, the information gained by any of the detectors varies in terms of sensitivity and selectivity, but is applicable for obtaining fingerprints. UHPLC represents a current state-of-art liquid chromatographic technique. It approximately enables six times faster analyses than HPLC [18] adding to its suitability for application

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in the screening of a large number of samples. Also, selectivity and sensitivity are considerably higher than with standard HPLC columns [19,20]. To date, no UHPLC fingerprinting protocols that specifically is optimized for compounds present in cultivation broth has been introduced and only one publication focusing on UHPLC analysis of cultivation broth is available [18]. Here, we describe a fingerprinting method with UHPLC and photodiode array detection (DAD) that was developed for bacterial secondary metabolites produced in spent cultivation broths. Broths that were obtained from culturing four actinomycetes and a set of 13 antibiotic standards were used to develop a suitable extraction approach and the most efficient UHPLC conditions. The major classes of antibiotics (β-lactames, coumarins, glycopeptides, lincosamides, macrolides, piperidines, polyenes, quinolones, tetracyclines, *etc.*) that cover the existing range of physico-chemical properties (chemical structure, polarity, spectral and acid–base properties) were included into the set of antibiotic standards; Fig. S1 in Supplementary data illustrates their structures.

2. Experimental

2.1. Chemicals

The solvents used as UHPLC mobile phase were of the gradient grade. Acetonitrile (ACN; 99.95%, Biosolve, Netherlands), methanol (MeOH; 99.95%, Chromapur GG) and dichloromethane (DC; min. 99%, Chromapur G) were purchased from Chromservis (Czech Republic). Trifluoroacetic acid (TFA; 99.95%, ULC/MS) was obtained from Biosolve (Netherlands) and formic acid (HCOOH; 99%) from Merck (Germany). *Ortho*-phosphoric acid (H_3PO_4; 99%), ammonium hydroxide (NH_4OH; 29%, A.C.S. reagent) and acetic acid (glacial, min. 99%) were purchased from Sigma–Aldrich (Germany). Diethylether (EE; p.a.) and ethyl acetate (EA; p.a., 99.7%) were obtained from Lach-Ner (Czech Republic). HPLC grade water was prepared by Milli-Q reverse osmosis, Millipore (USA). Amberlite XAD-4 (Amb) was purchased from Supelco (USA) and was used to make a 500 mg-Amberlite-cartridge. Oasis HLB 3cc (50 mg) cartridges (Hlb) were obtained from Waters (USA).

The standard stock solutions of antibiotics were prepared with MeOH or water at a concentration of 1 mg mL^{-1}. Two mixtures A1 and A2 containing six and seven antibiotic standards, respectively, were used for the development of extraction and UHPLC methods.

Mixture A1 contained the following antibiotic standards: cephalosporin C (CEC), penicillin G (PEG) dissolved in water; griseofulvin (GRI), tylosin (TYL), lincomycin A (LIN) dissolved in MeOH; and streptovitacin A (STV) dissolved in 50% MeOH. Standard stock solutions of these antibiotics were mixed and diluted with 50% MeOH so that the final concentration of each compound was 100 μg mL^{-1}.

Mixture A2 contained the following antibiotic standards: novobiocin (NOV), ristocetin (RIS), ofloxacin (OFL) dissolved in water; roxithromycin (ROX), natamycin (NAT), chlortetracycline (CTE) and chloramphenicol (CHL) dissolved in MeOH. Standard stock solutions of these antibiotics were mixed and diluted with 50% MeOH so that the final concentration of each compound was 100 μg mL^{-1}.

CEC, ROX, CHL, LIN, GRI, PEG, TYL, NOV, RIS, OFL, NAT, CTE were obtained from Sigma–Aldrich (Germany) and were of UV grade (>90%). STV was kindly provided by Jaroslav Spížek, Institute of Microbiology of the Academy of Sciences of the Czech Republic, v.v.i. (Czech Republic).

2.2. Cultivation

Four actinomycete strains (E1, E2, E3, and E4) were cultivated. Spores were inoculated in GYM broth (50 mL) (glucose 4 g L^{-1}, yeast extract 4 g L^{-1}, malt extract 10 g L^{-1}, $CaCO_3$ 2 g L^{-1}, pH 7.2) and cultivated in a rotary shaker for 24–48 h at 28 °C. Then, fresh GYM broth (50 mL) was inoculated with 5% of the pre-culture and cultivation continued for 10 days at 28 °C. Cells were centrifuged for 10 min at 4000 rpm and 4 °C. The spent cultivation broth (supernatant) was used for extraction (extracts E1, E2, E3, and E4).

2.3. Fingerprinting method development

2.3.1. Extraction of cultivation broth

Liquid–liquid extraction (*LLE*). The standard mixtures A1 and A2 were diluted 10-fold with water to the concentration of 10 μg mL^{-1} for each antibiotic. Two milliliters of diluted A1 or A2 mixture were mixed with 2 mL of various organic solvents (EE, DC, EA, or EA acidified: EA–acetic acid 95:5, v/v), the emulsion was shaken for 10 min and the organic phase was removed. The procedure was repeated twice and all the three organic fractions were put together, evaporated and reconstituted in 200 μL 50% MeOH so that the theoretical concentration (100 μg mL^{-1}) corresponds with the concentration of the original mixture A1 or A2. This sample was measured by UHPLC under acidic conditions (see Section 2.4.2) and peak areas of the antibiotics were compared with peak areas of antibiotics in the original A1 or A2 mixtures. The recovery was then calculated as the ratio of the respective peak areas.

Solid phase extraction (*SPE*). Hlb and Amb cartridges were conditioned with 3 mL MeOH and equilibrated with 3 mL water. Two milliliters 10-fold diluted A1 or A2 solution was added. The column was then washed with 3 mL water and the absorbed antibiotics were eluted with 1 mL MeOH (fraction F100) or with 1 mL of 40% MeOH (fraction F40) and subsequently with 1 mL 90% MeOH (fraction F90/40). The eluent was evaporated and dissolved in 200 μL 50% MeOH so that the theoretical concentration (100 μg mL^{-1}) corresponded with the original mixture A1 or A2. This sample was analyzed by UHPLC under acidic conditions (see Section 2.4.2). The recovery was assessed as described above for LLE.

The extraction procedures giving the best recoveries were subsequently tested with cultivation broths E1–E4.

2.3.2. UHPLC conditions

The following five UHPLC columns were tested:

- Vision HT C18 column (50 mm × 2.0 mm I.D., particle size 1.5 μm), Grace (HT C18);
- Acquity UPLC BEH Shield RP18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm), Waters (BEH Shield);
- Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm), Waters (BEH C18);
- Acquity UPLC BEH C18 column (100 mm × 2.1 mm I.D., particle size 1.7 μm), Waters (BEH C18 10 cm);
- Acquity UPLC BEH Phenyl column (50 mm × 2.1 mm I.D., particle size 1.7 μm), Waters (BEH Phenyl).

The gradient programs g10, g15, g25, g30, g40 and g60 were used. Isocratic elution of 5% B (organic modifier) was set for 1.5 min (g10 and g15) or 2.0 min (g20 to g60) before the gradient elution. Then, the ratio of solvent B linearly increased from 5% to 100% in 10, 15, 20, 25, 30, 40 and 60 min for gradient programs g10, g15, g25, g30, g40 and g60, respectively. The column was then washed with 100% B for 1.5 min and equilibrated for 1.0 min (5% B).

Section S1 in Supplementary data summarizes the specific UHPLC conditions used during the study.

2.4. Final fingerprinting method

2.4.1. SPE

Hlb cartridge was conditioned with 3 mL MeOH, equilibrated with 3 mL water and then 3 mL cultivation broth was loaded. After that, the cartridge was washed with 3 mL water and absorbed substances were eluted with 1 mL MeOH. The eluent was evaporated to dryness, reconstituted in 200 μL 40% MeOH and centrifuged (13,000 rpm). This sample represented the extract for UHPLC analysis.

2.4.2. UHPLC

The UHPLC analyses were performed on Acquity UPLC system, equipped with the 2996 PDA detection system operating from 194 to 600 nm (Waters). Data were processed with Empower 2 (Waters). The chromatographic conditions were: Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm, Waters); flow rate, 0.4 mL min^{-1}, data sample rate, 20 pts s^{-1}; filter constant, 0.5; injection volume, 5 μL. Every sample was analyzed under acidic and alkaline conditions.

Acidic conditions. Mobile phase consisted of solvent A, 0.5% H_3PO_4 in water, and solvent B, MeOH. Samples were eluted by a linear gradient program (min/%B): 0/5; 1.5/5; 16.5/100 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B). Total analysis time was 19.0 min. The column oven was set to 55 °C. The data were recorded from 210 to 600 nm.

Alkaline conditions. Mobile phase consisted of solvent A, 1 mM ammonium formate pH 9.0, and solvent B, ACN. Samples were eluted by a linear gradient program (min/%B): 0/5; 2.0/5.0; 18.0/65.8 with subsequent column clean-up for 1.0 min (100% B) and equilibration for 1.0 min (5% B). Total analysis time was 20.0 min. The column oven was set to 30 °C. The data were recorded from 194 to 600 nm.

2.5. Data processing

Peak symmetry, selectivity, area, height, and retention time were calculated by Empower 2 (Waters). Symmetry $S = 0.5\,W/F$, where W means peak width at 5% of peak height and F means time from width start point at 5% of peak height to retention time; resolution $R = 1.18\ (t_{R2} - t_{R1})/(W_1 + W_2)$, where t_{R1} and t_{R2} mean retention times of the two assessed peaks, W_1 and W_2 mean peak widths at 50% peak height; peak quality $Q = h/A$, where h represents peak height and A means peak area. S and Q were used for evaluation of the peak shape, whereas R and Q were used to estimate the separation efficiency and selectivity. All antibiotic standards in mixtures A1 and A2 and following unknown compounds in the extracts E1–E4 were assessed: E1a, E1b and E1c (extract E1); E2d and E2e (extract E2); E3f and E3g (extract E3); E4h, E4i and E4j (extract E4). All the unknown compounds as well as antibiotic standards showed characteristic UV spectra that facilitated monitoring of individual compounds under different UHPLC conditions. The UV spectra of the analytes did not differ significantly under acidic and alkaline conditions. Compared compounds in the extracts E1–E4 were selected so that different polarity (retention time) and signal response (minor and major peaks) were always involved.

2.6. Validation and column robustness

SPE extraction and UHPLC analysis of cultivation broths E1–E4 was performed in six replicates using the final conditions. Retention times and peak areas of selected unknown analytes that were detected in the extracts E1–E4 (see Section 2.5) were compared within the six replicates in order to evaluate the fingerprinting method repeatability as defined in the validation guidelines [21].

Five hundred actinomycete spent cultivation broths were extracted and analyzed by UHPLC under final conditions using two different BEH C18 columns, one for the acidic and the other for alkaline conditions. The testing extract E2 was repeatedly injected and analyzed at the beginning and after every 20 samples, *i.e.* 26 injections of E2. The column robustness was assessed by comparison of the 26 chromatograms of E2 and was expressed as repeatability of retention times and areas of selected peaks.

3. Results and discussion

3.1. Development of extraction protocol

Actinomycetes secrete many of the secondary metabolites to their cultivation broth in relatively low concentrations. The cultivation broth is a complex matrix that contains not only bacterial metabolites, but also essential nutrients. Therefore, the sample preconcentration and clean-up prior to the analysis is mandatory.

SPE is generally more universal and repeatable than LLE [22]. The former method also complies with requirements for high-throughput. However, LLE previously has been used widely for natural products extraction [12,23]; therefore both SPE and LLE were evaluated.

LLE was examined with four water non-miscible solvents of different polarity (EE, DC, EA, and acidic EA) and the results summarized in Table 1 indicated that DC and EA yielded the best recovery results though only in case of half of the antibiotic standards more than 50% actually were recovered. In particular, a low recovery was achieved for more polar antibiotics (CEC, RIS, LIN, STV, and OFL).

The SPE method was tested with two sorbents, Amb and Hlb. Amb has been used widely to recover natural products from biomatrices including cultivation broth [23–26]. Hlb was used to extract, for example, LIN from cultivation broth [18] and various antibiotics from wastewater [27]. The nature of Hlb provides many advantages compared to the classical silica-based SPE cartridges, such as low elution volume, higher recovery of polar metabolites, proprietary cleaning process, and minimal drying effect, all factors that contribute to excellent repeatability.

Table 1 summarizes recovery rates of antibiotic standards extracted with Amb and Hlb. Hlb (fraction 100) is capable of extracting all antibiotics except CEC. The recoveries exceeded 50% except for RIS (26.3%) and CTE (46.2%). Amb recovered more antibiotics than any solvent in LLE, however, the recovery rates are much lower than those of Hlb. The extraction of broths E1–E4 also confirmed that Hlb is more efficient than Amb. It mostly provided UHPLC fingerprints that showed peaks of the same compounds but with significantly higher response indicating more efficient recovery (see Fig. 1).

The solvent composition for dissolving of the Hlb extract was optimized. MeOH–water and MeOH–1% acetic acid in ratios 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, and 30:70 (v/v) were compared. It was found that water and 1% acetic acid yielded the same results whilst the concentration of MeOH much more influenced the recovery of specific compounds. Generally, pure MeOH yielded higher recovery rates of less polar compounds. If the ratio of MeOH was reduced to 40%, the recovery of less polar compounds was the same or slightly reduced, but that of the more polar compounds improved considerably. Therefore, 40% MeOH was used for the reconstitution of the Hlb extract.

3.2. Development of UHPLC conditions

The antibiotic mixtures A1 and A2 and cultivation broth extracts E1–E4 were used for the UHPLC method development. Actinomycete metabolites that are produced in low amounts may be

Table 1
Recovery rates of antibiotics extracted by different liquid–liquid and solid phase extraction techniques.

Antibiotic	CEC	RIS	LIN	STV	OFL	CHL	CTE	PEG	GRI	TYL	NAT	ROX	NOV
Polarity of antibiotic	More polar ↔ Less polar												
Wavelength (nm)	260	199	197	201	295	278	368	197	295	287	304	199	324
LLE													
Diethylether	0.0	0.0	0.0	0.0	2.4	64.2	4.2	0.0	71.3	1.0	1.9	33.5	34.0
Dichloromethane	0.0	0.0	0.0	4.6	39.1	30.9	27.3	0.0	65.3	49.8	0.5	49.9	51.4
Ethylacetate	0.0	0.0	0.0	14.6	2.7	98.6	66.7	53.9	71.4	8.03	0.4	4.7	87.0
Ethylacetate acidified	0.0	0.0	0.0	9.3	0.4	79.1	5.7	37.2	78.9	1.6	1.1	0.0	63.3
SPE													
Amberlite Fraction F100	0.0	0.0	35.3	5.4	12.5	37.9	2.6	0.0	20.3	25.6	19.3	26.5	24.2
Amberlite Fraction F40	0.5	0.0	0.0	0.0	5.1	2.9	4.5	0.0	4.6	5.0	2.0	0.0	3.2
Amberlite Fraction F90/40	0.0	0.0	9.3	2.2	3.6	17.6	0.2	0.0	7.5	7.4	6.0	9.5	12.8
HLB Fraction F100	**0.0**	**26.3**	**90.1**	**84.3**	**90.5**	**95.6**	**46.2**	**60.4**	**95.4**	**87.3**	**91.3**	**91.4**	**94.3**
HLB Fraction F40	0.0	57.2	72.7	76.0	2.0	0.0	7.9	60.1	5.5	1.2	0.0	0.0	0.0
HLB Fraction F90/40	0.0	0.0	18.7	2.7	74.3	86.0	44.8	8.6	86.5	83.5	84.3	76.2	84.0

CEC – cephalosporin C, RIS – ristocetin A, LIN – lincomycin A, STV – streptovitacin A, OFL – ofloxacin, CHL – chloramphenicol, CTE – chlortetracycline, PEG – penicillin G, GRI – griseofulvin, TYL – tylosin, NAT – natamycin, ROX – roxithromycin, NOV – novobiocin; LLE – liquid-liquid extraction, SPE – solid phase extraction.
The bold values refer to the extraction protocol which provided best results and were therefore chosen for the final method (or at least considered for this in case of 0.25% TFA).

easily missed by inappropriate conditions. Therefore, the goal was to develop a method that facilitates detection of the maximal number of analytes in the broth by well-separated peaks with reasonable shape. The conditions chosen for the UHPLC method development reflect those used in published HPLC methods for analysis of natural products, secondary metabolites and antibiotics in crude extracts, cultivation broths, *etc.* [12,18,23,28–30] and emerging trends in separation techniques (*e.g.* sub-2 μm-particle chromatographic columns and alkaline mobile phase).

3.2.1. Linear gradient

The optimal linear gradient elution was developed under UHPLC conditions described in Supplementary data, S1.1. Specifically, the method is aimed at efficient fingerprinting of unknown compounds. Thus, the elution gradient has to run from a very low ratio of organic modifier to its maximum. The only crucial parameter of the gradient is its duration, pronouncing its slope and determining the peak capacity of the analysis. The longer the duration of the gradient, the better the separation is obtained. To comply with high-throughput requirements, however, the analysis time should not be any longer than necessary. Thereby, the gradient duration of g10, g15, g25, g30, g40, and g60 (see Section 2.3.2) were tested. The shorter the gradient time was, the higher the response and *Q* of particular analytes were obtained in the extracts E1–E4. However, the longer the gradient, the better the separation (higher *R* values) was observed. For instance, *Q* of peaks of E1b and E1c compounds (see Fig. 2) were as follows: 0.14 (g60), 0.20 (g40), 0.25 (g30), 0.29 (g25), 0.34 (g20), 0.26 (g15) and 0.33 (g10), whilst *R* for E1b and E1c was following: 1.93 (g60), 1.37 (g40), 1.15 (g30), 1.02 (g25), 0.76 (g20), 0.62 (g15) and 0.42 (g10). The same trend was visible for most compounds in E1–E4. As a compromise, g25 was chosen with respect to *Q* and *R* values as well as to the analysis time that is compatible with high-throughput requirements. Since all compounds present in E1–E4 and all antibiotics in A1 and A2 were eluted within 16 min, the analysis time was shortened without alternation of the gradient slope.

Further modification of the gradient program by replacing ACN as organic modifier by MeOH is described in Section 3.2.3. To sum up, the following gradients were found to be the most suitable: g25 (min/%B): 0/5; 2.0/5.0; 18.0/65.8 when ACN was used and g15 (min/%B): 0/5; 1.5/5; 16.5/100 for MeOH.

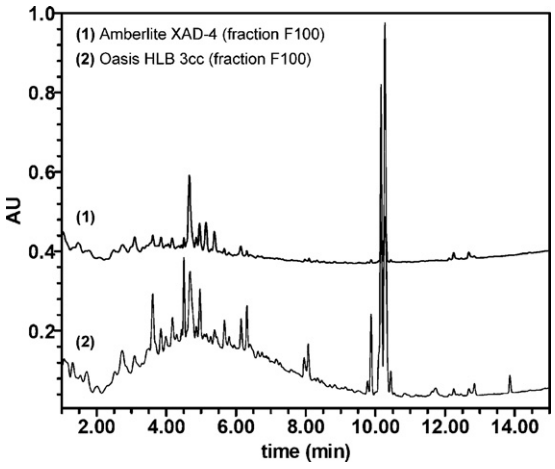

Fig. 1. The comparison of Amberlite XAD-4 and Oasis HLB 3cc for extraction of cultivation broth E1. UHPLC conditions (see also Section 2.4): Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm), mobile phase: solvent A: 0.5% H_3PO_4 in water, and solvent B, MeOH; linear gradient mode (min/%B): 0/5; 1.5/5; 16.5/100 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B); flow rate, 0.4 mL min^{-1}; column temperature, 55 °C; injection volume, 5 μL; UV detection: extracted at maximal wavelengths in the range from 210 to 600 nm (max plot).

3.2.2. Aqueous part of the mobile phase

Various acidic and alkaline modifiers and buffers under UHPLC conditions that are described in Supplementary data, S1.2 were explored: TFA (0.25%, 0.1%, 0.05%, and 0.005%), H_3PO_4 (0.5%, 0.1%, 0.05%, and 0.005%), HCOOH (0.1%), 1 and 5 mM ammonium formate of different pH, water, and ammonium hydroxide (1.2×10^{-2}%). Table 2 summarizes the *Q* values for antibiotic standards that were analyzed with different aqueous parts of the mobile phase.

Pure water as aqueous part of the mobile phase led to an insufficient elution of many antibiotic standards, namely RIS, LIN, PEG, TYL, ROX, and NOV that were not detected in the chromatogram at all. Moreover, most compounds of the extracts E2 and E4 were not separated sufficiently. An addition of acidic additives improved the separation and detection of the most antibiotics compared to pure water. The data in Table 2 show that 0.25% TFA yielded the best *Q* values. However, signals intensity of STV, LIN, PEG and ROX were suppressed significantly, which cannot be deduced from the *Q* value alone. The signal suppression together with increased noise is a crucial disadvantage of TFA. Therefore, 0.5% H_3PO_4 was chosen as a compromise between optimal *Q* and *S* values and peak height. The most convenient solvent turned out to be 0.5% H_3PO_4, also for analyses of the extracts E1–E4. As an example, Fig. 3 compares different aqueous parts of the mobile phase in UHPLC analyses of the extract E2.

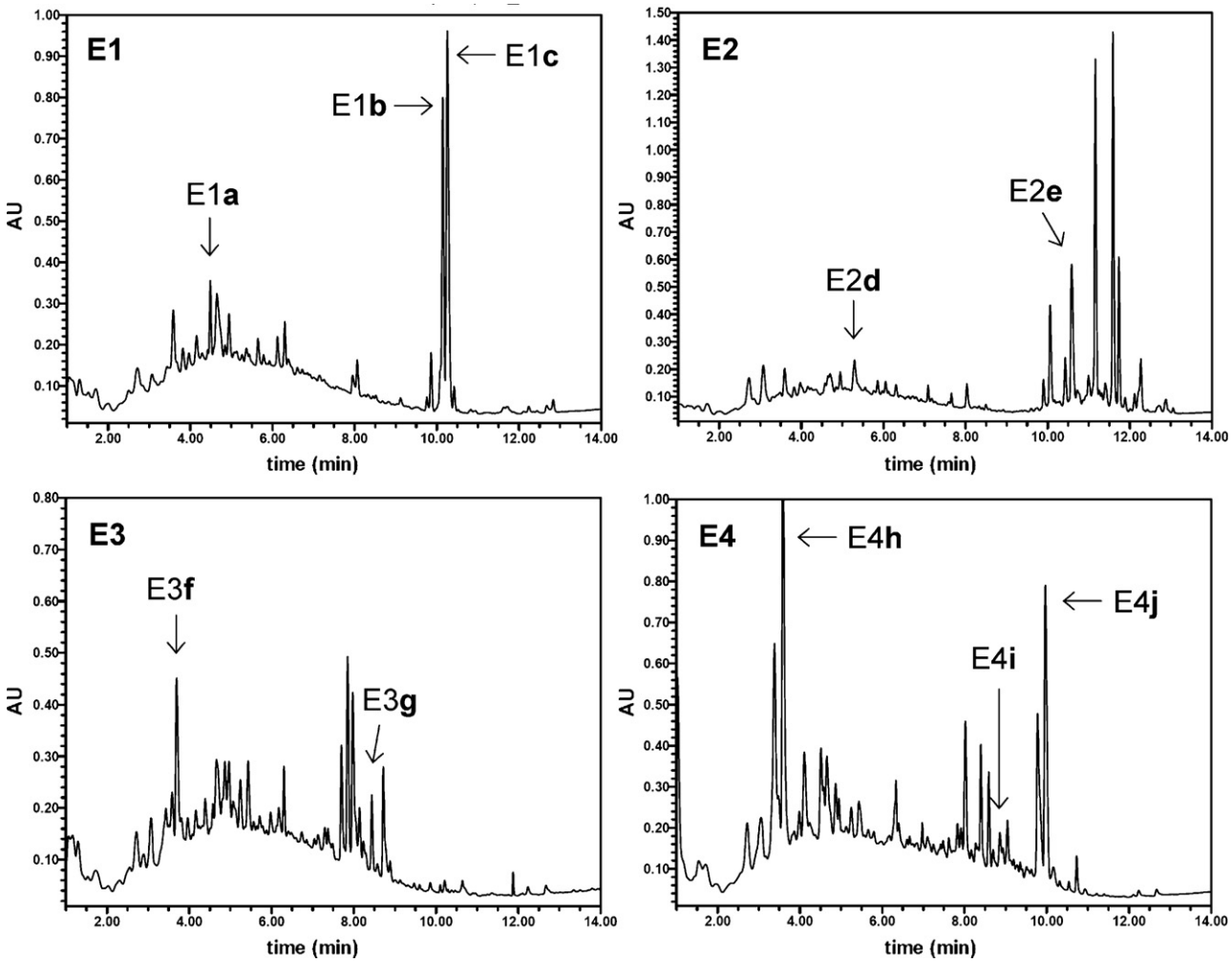

Fig. 2. UHPLC 2D fingerprints of cultivation broth E1, E2, E3 and E4 under acidic conditions. UHPLC conditions (see also Section 2.4): Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm); mobile phase: solvent A, 0.5% H_3PO_4 in water, and solvent B, MeOH; linear gradient mode (min/%B): 0/5; 1.5/5; 16.5/100 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B); flow rate, 0.4 mL min^{-1}; column temperature, 55 °C; injection volume, 5 μL; UV detection: extracted at maximal wavelengths in the range from 210 to 600 nm (max plot). The labels E1a–E4j represent different unknown compounds present in the cultivation broths E1–E4 (see also Section 2.5).

Alkaline conditions generally lowered the separation quality for many antibiotics. Nevertheless, the *Q* values for RIS, LIN, TYL, and ROX were higher with alkaline solvents compared to most acidic solvents tested (see Table 2). Fluctuations in pH may have caused the asymmetric peak shape of some antibiotic standards (STV, PEG, TYL peaks tail or front) and the poorly resolved peaks that were detected in the extracts E3 and E4 when ammonium hydroxide (1.2×10^{-2}%) was used as aqueous part of the mobile phase. In this context, better results were obtained with ammonium formate, which was studied in the pH range from 8.0 to 10.0 with increasing 0.5 steps and identified pH 9.0 as optimum. One mM and 5 mM ammonium formate (pH 9.0) yielded similar *Q* and *S* values; therefore, 1 mM ammonium formate was chosen with respect to extended column life-time.

To sum up, 0.5% H_3PO_4 and 1 mM ammonium formate pH 9.0 were chosen for further development of two parallel UHPLC methods, one under acidic and the other under alkaline conditions. The merit of the latter is the provision of information about analyte acid–base properties.

Apart from UV (DAD), MS detectors are very frequently applied for fingerprinting or profiling since they provide more specific characterization of unknown compounds. Therefore, the applicability of this method for MS detectors was considered. The crucial parameter represents mobile phase composition–H_3PO_4 cannot be used for MS. However, 0.1% TFA and 0.1% HCOOH, that only yielded slightly less optimal results than 0.5% H_3PO_4, offers itself an alternative. One mM ammonium formate (alkaline conditions) as well as 0.1% TFA and 0.1% HCOOH (acidic conditions) are fully compatible with MS [31–33].

3.2.3. Organic part of the mobile phase

For acidic and alkaline aqueous parts of the mobile phase, the organic modifier was studied under UHPLC conditions that are described in Supplementary data, S1.3 and S1.4.

The contribution of the organic content of the mobile phases MeOH–ACN 100:0, 95:5, 50:50, 5:95, and 0:100 (v/v), were only compared under acidic conditions. MeOH yielded much better resolution of several compounds in the extracts E1–E4 compared to ACN and MeOH–ACN mixtures. For example, the *R* value of compounds E1b and E1c was 1.49 compared to 0.86 with ACN. Compared to ACN, however, MeOH increased the analysis time in the same gradient resulting in broader peaks and thus worse *Q* values. Therefore, sharper gradients (g10, g15 and g25) with MeOH as organic modifier were applied. Gradient g15 showed the best results with respect to *Q* and *R* parameters. A higher MeOH absorption cut-off (205 nm), compared to ACN (194 nm), discriminates absorption maxima under 205 nm (*e.g.* LIN, PEG, ROX). However, this is compensated by the simultane-

Table 2
Parameter *Q* for antibiotic standards analyzed by UHPLC with different aqueous parts of mobile phase.

Aqueous part of mobile phase	1.2×10^{-2}% ammonium hydroxide	**1 mM ammonium formate**	5 mM ammonium formate	Water	1 mM ammonium formate	5 mM ammonium formate	0.005% H_3PO_4	0.05% H_3PO_4	0.1% H_3PO_4	**0.5% H_3PO_4**	0.1% HCOOH	0.005% TFA	0.05% TFA	0.1% TFA	**0.25% TFA**
pH	10.5	9.0	9.0	7.3	4.7	4.7	3.1	2.2	2.0	1.6	2.3	3.2	2.3	1.9	1.6
CEC	0.79	**0.34**	0.44	0.30	0.47	0.22	0.23	0.17	0.17	**0.15**	0.19	0.27	0.14	0.19	**0.34**
RIS	0.40	**0.36**	0.29	n/a	0.12	0.09	0.13	0.11	0.11	**0.11**	0.11	0.10	0.29	0.30	**0.40**
LIN	0.38	**0.36**	0.35	n/a	0.13	n/a	0.08	0.15	0.17	**0.19**	0.11	n/s	0.25	0.28	**0.37**
STV	0.20	**0.24**	0.24	0.25	0.24	0.21	0.25	0.25	0.25	**0.24**	0.23	0.25	0.23	0.22	**0.22**
OFL	0.21	**0.38**	0.32	0.06	0.22	0.29	0.22	0.34	0.35	**0.35**	0.27	0.25	0.25	0.35	**0.42**
CHL	0.27	**0.27**	0.26	0.26	0.26	0.25	0.29	0.29	0.29	**0.29**	0.26	0.28	0.25	0.24	**0.25**
CTE	0.12	**0.12**	0.15	0.19	0.30	0.26	0.15	0.21	0.23	**0.27**	0.16	0.21	0.22	0.24	**0.31**
PEG	0.09	**0.17**	0.10	n/a	0.16	0.18	0.30	0.30	0.31	**0.31**	0.24	0.26	0.28	0.27	**0.33**
GRI	0.31	**0.30**	0.29	0.28	0.28	0.28	0.32	0.32	0.32	**0.31**	0.28	0.28	0.27	0.26	**0.31**
TYL	0.11	**0.30**	0.30	n/a	0.16	0.22	0.11	0.15	0.18	**0.26**	0.12	0.11	0.22	0.25	**0.33**
NAT	0.13	**0.28**	0.22	0.28	0.32	0.32	0.11	0.18	0.20	**0.25**	0.14	0.13	0.22	0.23	**0.29**
ROX	0.17	**0.24**	0.21	n/a	0.06	0.10	0.06	0.10	0.12	**0.16**	0.08	0.08	0.16	0.19	**0.27**
NOV	0.17	**0.25**	0.16	n/a	0.16	0.19	0.31	0.31	0.31	**0.33**	0.28	0.28	0.27	0.27	**0.30**

n/a – not present in the chromatogram. For UHPLC conditions see Supplementary data S1.2.
The bold values refer to the mobile phase which provided best results and were therefore chosen for the final method (or at least considered for this in case of 0.25% TFA).

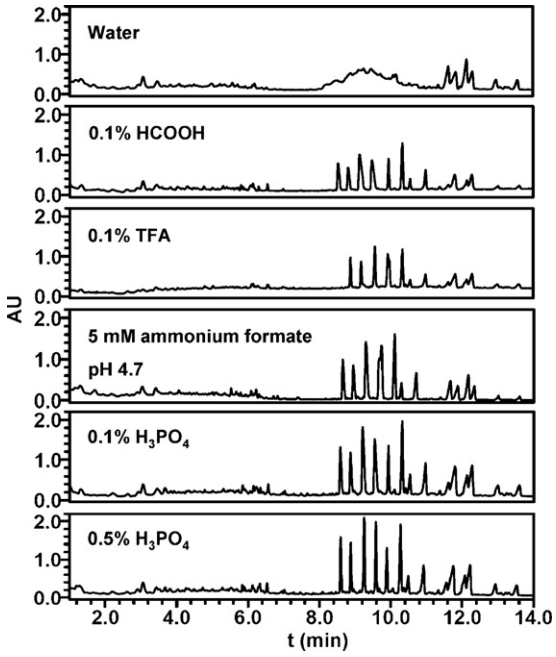

Fig. 3. The comparison of different aqueous parts of the mobile phase for UHPLC analysis of extract E2. UHPLC conditions (see also Supplementary data, S1.2): Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm); mobile phase: solvent B, ACN; linear gradient mode (min/%B): 0/5; 2.0/5.0; 27.0/100 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B); flow rate, 0.4 mL min^{-1}; column temperature, 30 °C; injection volume, 5 μL; UV detection: extracted at maximal wavelengths in the range from 194 to 600 nm (max plot).

ous analysis under alkaline conditions as described in the ongoing text.

MeOH and ACN as organic modifiers tested with 1 mM ammonium formate, pH 9, yielded comparable results. Therefore, ACN was chosen with respect to lower absorption cut-off, which prevents that compounds with low UV absorption may be missed. As a result, MeOH and gradient g15 was used together with acidic conditions (0.5% H_3PO_4) and ACN and gradient g25 with alkaline conditions (1 mM ammonium formate, pH 9.0).

3.2.4. Chromatographic columns

Five chromatographic columns (see Section 2.3.2) were chosen for the fingerprinting method development and they were tested subsequently under UHPLC acidic and alkaline conditions specified in Supplementary data, S1.5 and S1.6.

Under acidic conditions, HT C18 and BEH Shield did not exhibit better *Q* values for any single antibiotic and the separation of the extracts E1–E4 was less efficient compared to the other columns. BEH Phenyl yielded significantly better results, especially concerning the *Q* parameter for PEG. However, similar or slightly better parameters also were achieved with BEH C18 and BEH C18 10 cm columns. The extracts E1 and E2 were more efficiently separated on BEH Phenyl, but fingerprints of the extracts E3 and E4 showed more fully resolved peaks on BEH C18. As an example, the comparison of different chromatographic columns for UHPLC analysis of the extract E3 is presented in Fig. 4. HT C18 and BEH Shield are not stable in pH 9.0. The separation parameters of BEH C18, BEH C18 10 cm and BEH Phenyl columns were very similar under alkaline conditions. BEH C18 10 cm provided similar separation results as BEH C18. However, it extended the analysis time without significantly improving the analysis. The fact that C18 ligand is generally more universally selective than the C6-phenyl ligand recommended the choice of the BEH C18 column for both acidic and alkaline conditions.

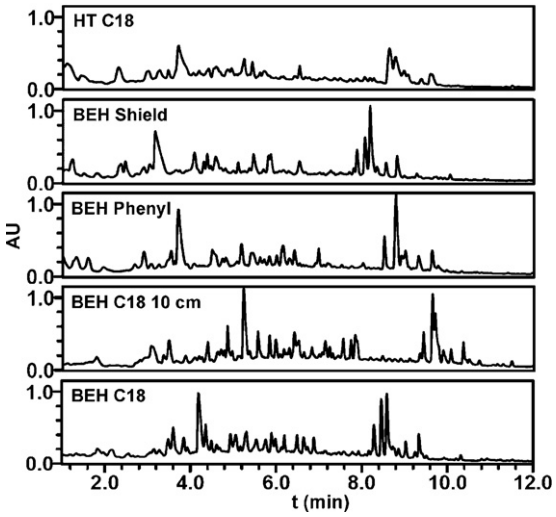

Fig. 4. The comparison of different chromatographic columns for UHPLC analysis of extract E3. UHPLC conditions (see also Supplementary data, S1.5): Mobile phase: solvent A, 0.5% H_3PO_4 and solvent B, MeOH; linear gradient mode (min/%B): 0/5; 1.5/5; 16.5/100 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B); flow rate, 0.4 mL min^{-1}, column temperature, 30 °C; injection volume, 5 μL; UV detection: extracted at maximal wavelengths in the range from 210 to 600 nm (max plot).

3.2.5. Column temperature

The influence of the column temperature on the separations at 30, 40, 50, 55, 60, and 65 °C under UHPLC acidic and alkaline conditions (see Supplementary data S1.7 and S1.8) was tested. The higher the temperature was used, the better the separation was: improved peaks shape and shorter analysis times were obtained. This applied for all extracts and most antibiotics except PEG, which was probably not stable at 60 and 65 °C. To eliminate the risk of losing thermolabile compounds, the temperature of 55 °C was chosen. Under alkaline conditions, increasing temperature did not improve the separation efficiency. Conversely, the peak response of all antibiotics as well as most compounds in E1–E4 was the same or even decreased; therefore, the temperature of 30 °C was applied.

3.3. Validation and column robustness

Repeatability of the fingerprinting method is the only parameter which is necessary to validate with respect to its purpose. The results of the method validation are summarized in Table 3 for acidic UHPLC conditions and in Table 4 for alkaline conditions. In both milieus, retention times are stable with RSD under 0.5% in all cases. As far as the peak area repeatability is concerned, RSD is under 5% with the exception of one minor peak under acidic conditions (E2d, 16.1%) and two minor peaks under alkaline conditions (E1a, 10.6% and E2d, 8.75%). In consideration that the concentration of the minor peaks corresponds to the limits of quantification, the RSD acceptance criteria of 20% are in accordance with the validation guidelines [21].

The BEH C18 column robustness under acidic conditions is sufficient for the analysis of 500 samples as corroborated by the RSD of retention times and areas of selected peaks of the extract E2, which was within the 5% limit for all 26 analyses of the extract E2 (data not shown). However, under alkaline conditions, the column was robust enough only for 180 samples; the RSD values were within 5% for the first 10 analyses of the extract E2. Then, the column separation parameters worsened significantly (data not shown). This may have been caused by precipitation of the sample matrix in high pH. The column robustness under both conditions is sufficient for its purpose, but the sample number limit has to be taken into consideration.

3.4. Fingerprinting method application

This fingerprinting method was designed for the separation of a wide spectrum of unknown compounds focusing on secondary metabolites of bacteria, potential antibiotics. This is why antibiotic standards of various polarity and properties were included in the method development. The developed fingerprinting method is illustrated by two 3D chromatograms (see Figs. 5 and 6). Different appearance of four fingerprints characterizing four different actinomycete strains E1–E4 (see Fig. 2) demonstrates that the method is able to distinguish various bacterial strains on basis of metabolites that they produce and excrete into the cultivation broth. Each

Table 3
Repeatability of the final fingerprinting method (acidic conditions).

Compound[a]	Wavelength (nm)	Retention time (min)	Retention time RSD (%)	Area (mV s)	Area RSD (%)
E1a	220	4.49	0.16	524	4.27
E1b	228	10.1	0.07	2030	1.65
E1c	237	10.3	0.07	3560	1.81
E2d	263	5.29	0.02	1010	16.1
E2e	210	10.6	0.01	2030	2.39
E3f	210	3.69	0.25	1200	3.09
E3g	220	8.44	0.11	476	1.87
E4h	254	3.58	0.09	4500	2.09
E4i	322	8.86	0.02	240	2.12
E4j	284	9.97	0.04	1620	1.49

[a] See Fig. 2.

Table 4
Repeatability of the final fingerprinting method (alkaline conditions).

Compound	Wavelength (nm)	Retention time (min)	Retention time RSD (%)	Area (mV s)	Area RSD (%)
E1a	194	3.85	0.45	670	10.6
E1b + E1c	233	12.2	0.02	6470	1.80
E2d	268	4.32	0.11	100	8.75
E2e	194	8.71	0.08	9220	2.74
E3f	194	4.17	0.04	4030	1.89
E3g	220	9.24	0.02	573	3.63
E4h	238	5.06	0.11	3920	2.27
E4i	322	7.49	0.02	178	3.59
E4j	284	6.73	0.10	813	2.32

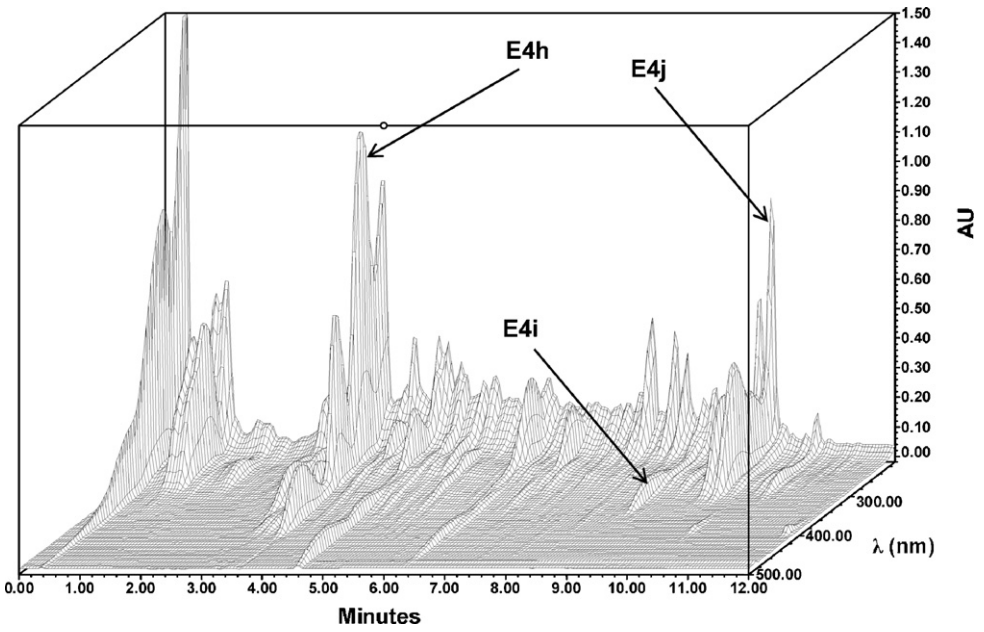

Fig. 5. UHPLC 3D fingerprint of cultivation broth E4 under acidic conditions. UHPLC conditions (see also Section 2.4): Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm); mobile phase: solvent A, 0.5% H_3PO_4 in water, and solvent B, MeOH; linear gradient mode (min/%B): 0/5; 1.5/5; 16.5/100 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B); flow rate, 0.4 mL min^{-1}; column temperature, 55 °C; injection volume, 5 μL; UV detection in the range from 210 to 600 nm.

pair of fingerprints contains physico-chemical information on a set of compounds that is represented by the single peaks in the fingerprint. The detector response reflects the compound concentration and provides the quantitative information whereas the qualitative information consists of retention time, UV spectrum and acid–base properties. The retention time refers to the polarity of the compound and this parameter itself may suggest a tentative classification of the compound into a specific antibiotic group, *e.g.* β-lactames (generally more polar) or macrolides (generally less polar). UV spectra that were obtained for each peak inform about the compound structure in terms of presence and absence of specific chromophores, and together with retention times, they may be used for partial identification or dereplication. The acid–base properties are characterized by the influence of the pH of the mobile phase (aqueous part) on retention times. This is demonstrated by differences in the pair of 3D fingerprints of the extract E4 obtained under both acidic and alkaline conditions (see Figs. 5 and 6, respectively). The fingerprints vary in retention times and even elution order of some compounds indicating their specific acid–base properties. For instance, under acidic conditions, compounds E4 h, E4i and E4j are eluted at 3.6, 8.9, and 10.0 min, whereas, under alkaline conditions, at 6.1, 9.0, and 8.1 min. The retention order of E4i and E4j

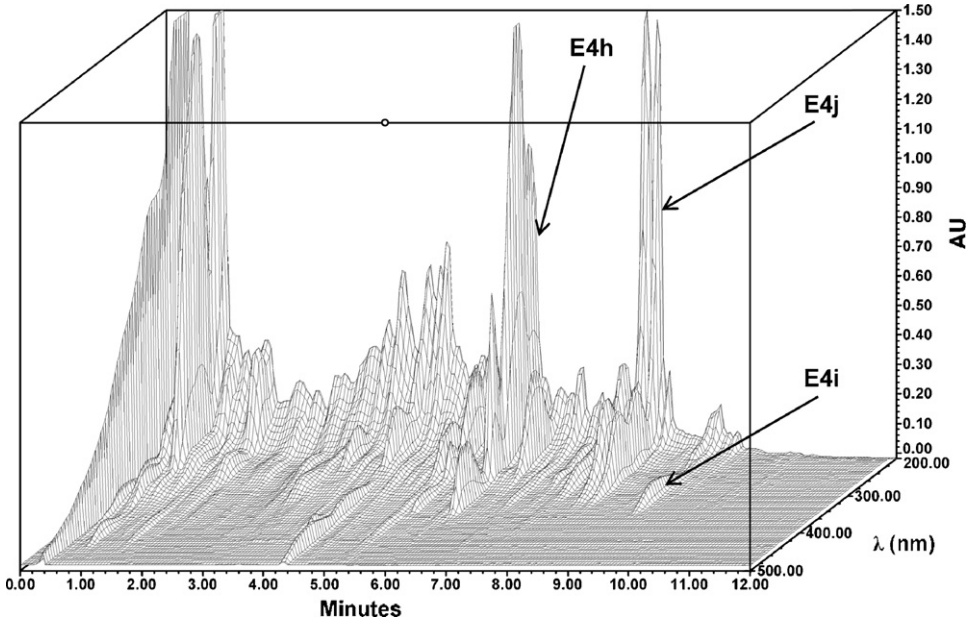

Fig. 6. UHPLC 3D fingerprint of cultivation broth E4 under alkaline conditions. UHPLC conditions (see also Section 2.4): Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm); mobile phase: solvent A, 1 mM ammonium formate pH 9.0, and solvent B, ACN; linear gradient mode (min/%B): 0/5; 2.0/5.0; 18.0/65.8 with subsequent column clean-up for 1.5 min (100% B) and equilibration for 1.0 min (5% B); flow rate, 0.4 mL min^{-1}; column temperature, 30 °C; injection volume, 5 μL; UV detection in the range from 194 to 600 nm.

Table 5
The influence of mobile phase composition on retention times of antibiotics differing in acid–base properties.

Acid–base properties	Antibiotic	pK_a	Retention time[a] (min)	
			Acidic	Alkaline
Acidic	PEG	2.8	7.73	5.27
	CEC	4.8	2.55	0.47
Basic	RIS	7.5	2.70	3.63
	TYL	7.5	8.99	11.43
	LIN	7.8	3.56	7.67
	ROX	9.2	9.96	12.90
	CHL	11.0	4.98	6.21
Amphoteric	OFL	5.7, 7.9	3.92	4.93
	CTE	3.3, 7.6	5.23	5.79
	NOV	4.3, 9.1	12.60	7.57
	NAT	3.8, 7.9	9.20	8.29
Neutral	STV	–	3.86	3.68
	GRI	–	8.62	10.08

PEG – penicillin G, CEC – cephalosporin C, RIS – ristocetin, TYL – tylosin, LIN – lincomycin A, ROX – roxithromycin, CHL – chloramphenicol, OFL – ofloxacin, CTE – chlortetracycline, NOV – novobiocin, NAT – natamycin, STV – streptovitacin A, GRI – griseofulvin.

[a] Retention times were obtained under final acidic and alkaline UHPLC conditions, see Section 2.4.2.

compounds is reversed and the retention time shift of compound E4 h (from 3.6 to 6.1 min) hints its basic properties. More convincing evidence of appearance of the acid–base properties in the fingerprints provide the retention times of the antibiotic standards obtained under acidic and alkaline conditions (see Table 5). The data confirm that all acidic (CEC, PEG) and all basic (RIS, TYL, LIN, ROX, and CHL) antibiotics are more retained on the chromatographic column under their respective corresponding pH conditions. The behavior of amphoteric antibiotics (OFL, CTE, NOV, and NAT) is more complex as it additionally depends on the pK_a values besides of the pH of aqueous part of the mobile phase. Neutral antibiotics (STV and GRI) also show a slight change of retention times, which, in this case, may be affected by other parameters than pH. Generally, the higher the retention times differ, the more probable the prediction is.

Multivariate statistical methods, such as PCA (principal component analysis), may be applied to explore differences and similarities of the fingerprints (after normalization) without consideration of peak identities [34,35]. The other possibility is represented by comparison of particular compounds (peaks) of the fingerprint with data in commercial database (UV spectra) or in-house database of standards measured by the same method (retention times and UV spectra).

The total UHPLC analysis time of 19 and 20 min under acidic and alkaline conditions facilitates a high sample throughput compared to standard HPLC fingerprinting with analysis time usually exceeding 50 min [12,30].

4. Conclusion

The here presented fingerprinting method enables screening of compounds encompassing a broad spectrum of physico-chemical properties including antibiotics of the majority of antibiotic classes. The main prerequisite due to UV detection is the presence of chromophores. Therefore, it is less suitable for aminoglycosides. The major advantage of the presented method is that it facilitates metabolite screening under both acidic and alkaline conditions which provides additional chemical and physical information about the fingerprinted bacterial metabolites: (1) polarity (retention times), (2) structure (presence of chromophores in the range from 194 to 600 nm), (3) concentration (detector response) and (4) acid–base properties (the influence of mobile phase pH on retention times). The fingerprints may be further used for statistical comparison in order to dereplicate already known compounds and strains or to seek correlation between physico-chemical information of the fingerprint and genetic or ecological markers (*e.g.* presence of selected genes responsible for production of antibiotics, locality of the strains origin or their taxonomic identification, *etc.*).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.08.031.

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